Vectors and P64k gene targeting for tandem affinity purification in Neisseria meningitidis

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Abstract

We present and describe the construction of tagging cassettes and plasmids for tandem affinity purification (TAP) of proteins in Neisseria meningitidis. The tagging cassette is designed for carboxyl-terminal tagging of proteins and it contains only two repeats of IgG-binding units. P64k protein from N. meningitidis was chosen to fuse at these new affinity tags. This protein is well recognized in immunoassays by serum from human convalescent meningococcal disease and it is highly immunogenic in animals. To continue the characterization of this meningococcal antigen, we designed and constructed two vectors for use in TAP purification method. We also carried-out preliminary test to check the correct expression of the protein fused in these vectors. D 2005 Elsevier B.V. All rights reserved.

Keywords: Neisseria meningitidis; P64k; Tandem affinity purification; Vectors

1. Introduction

Tandem affinity purification (TAP) method has been developed for the purification and characterization of protein complexes from yeast (Rigaut et al., 1999). The basic concept of TAP is similar to the epitope tagging strategy but differ on this in the sequential utilization of two tags instead of one. These are the IgG-binding units of Staphylococcus aureus protein A (ProtA) and the calmodulin-binding peptide (CBP).

Between the two affinity tags, the cleavage site for Tobacco Etch Virus (TEV) protease was placed. This enzyme is a site-specific protease that cleaves a seven amino acid recognition sequence and is only found in rare known proteins. After the initial affinity purification step on an IgG matrix, the bound material is released by incubation with TEV protease. A second affinity step is performed by immobilizing the complex with calmodulin coated beads via the CBP tag. Simultaneously, this step removes the TEV protease and further contaminants that may be present. The CBP–calmodulin interaction is calcium dependent and, hence, the removal of calcium ions with chelating agents (e.g., EGTA) can be used to release the material into the solution.

The eluted material can then be resolved by SDS–PAGE and/or directly subjected to tryptic digestion and liquid chromatography-coupled tandem mass spectrometric analysis (LC-MS/MS). The TAP method has proven to be a very useful tool for the detection of interacting partners of a target protein and for deter-mining protein composition of macromolecular complexes with low levels of false positives and false negatives. It has also been used to obtain active complexes for in vitro studies (Veraksa et al., 2005; Dra-kas et al., 2004).

We have adapted the TAP system for use in the fission bacterium, Neisseria meningitidis. The construction of cassettes and vectors for this purpose is reported here. A first vector consists of a TAP tag based in Rigaut et al. but the carboxyl-terminal TAP tag was designed to contain two copies of IgG-binding units of Prot A instead of one and an additional sequence specifically recognized by the CB.Hep1 monoclonal antibody (mAb) was included (Fontirrochi et al., 1993). The CB.Hep1mAb is a mouse made antibody used for affinity purification of a recombinant hepatitis B sur-face antigen virus (r-HBsAg) (Penton et al., 1992). Neisseria spp. are among the bacteria where homologous recombination process is highly efficient, thus to integrate a plasmid into Neisseria spp. genome via homologous recombination, a flanking homologous arms to the bacteria genome should be included into the plasmid in order to induced a single-site crossing over.

Thanks to the high proficient of homologous recombination in yeast and bacteria, the

necessity to construct a plasmid that allows the fusion of the TAP tag to the gene of interest was bypassed. Polymerase chain reaction (PCR) fragments can indeed be used to integrate the TAP tag directly in the genome (Puig et al., 2001). However, the construction of a second vector via standard DNA cloning procedures to intro-duce the C-terminal TAP tag in-frame with the coding region of the P64k protein was achieved. The final recombinant vector could then be stably introduced into the recipient organism by homologous recombination by replacing the endogenous C-terminal region of the wild-type gene. The use of the Kan cassette from pUC4K, allowed the positive selection of N. meningitidis carrying vector.

Neisseria meningitidis is the causative agent of meningococcal meningitis. This is a serious health problem throughout the world. The P64k protein from this organism is well recognized in serum from meningococcal disease convalescents. The presence of the protein in more than 80 meningococcal strains has also been verified. It is immunogenic in animal models and the elicited antibody shows bactericidal activity against meningococci. The characterization of this protein may constitute an attractive strategy for vac-cine development against meningococcal disease. P64k seems to be the same protein as the dihydrolipoyl dehydrogenase (E3) protein, a component of pyruvate dehydrogenase complex (Ala Aldeen et al., 1996). Based on this knowledge and to demonstrate the correct expression of these tags, the correct expression of P64k protein was immunodetected via Western blot with the co-expression of the TAP tag. Our results also indicate the potential usefulness of new TAP constructions in prokaryotes, and not only in eukaryotes as it was used until now.

2. Materials and methods

2.1. Bacterial strains and growth conditions Escherichia coli strain XL-1 Blue was used for all cloning. It was grown in Luria–Bertani (LB) broth at 37ºC, supplemented with 100 Ag/mL ampicillin or 50 Ag/mL kanamycin accordingly. The Neisseria menin-gitidis strain CuB385 (B: 4, 7: P1.19, 15) and the subsequent CuB385 transformation described in this work were grown at 37 ºC in brain heart infusion (BHI, Oxoid, UK)

1.5% (w/v) agar plates, in a candle jar, containing an initial OD260 of 0.1 and supplemented with 3 Ag/mL vancomicin, 7.5 Ag/mL colimicin, 12.5 U/mL nistatin and 100 Ag/mL kanamycin as necessary. Culture stocks were prepared in 10% (w/v) skimmed milk and stored at - 70 ºC. The meningococcal protein synthesis was induced after incubation for 1 h at 37ºC by the addition of indolacrylic acid (0.02 mg/ml), allowing the bacteria culture to grow for 11 h.

2.2. Construction of endogenous carboxyl-terminal TAP tagging vectors

The carboxyl-terminal TAP tagging (p TAP-BAC) vector was assembled in p M231 vector in two stages. Initially, a synthetic sequence (SB) was constructed using automatic system Gene Assembler Plus AND Synthetizer (Pharmacia-LKB, Sweden). The SB was designed using Vector NTI software (http://www. invitrogen.com) and it contains a polylinker site (Not I, EcoR I, Xho I, Kpn I, Bgl I) followed by the CBP, TEV sequence and the HVB tag ending with a restric-tion site for BamH I. The SB was cleaved with BamH I enzyme and subcloned into the p M231 vector also cut with BamH I and Puv II enzyme, generating the p TAP vector (Fig. 1A). In the second stage, DNA encoding two IgG domains of the ProtA gene (from p PRIT-2T) was amplified using the primers 7232 ProtA forward and 7233 ProtA reverse (Table 1). The amplified fragments were then purified on agarose gels and cleaved with BamH I and Bgl II enzyme. The cleaved products were ligated into p TAP vector; which were then linearized using BamH I to obtain the final tagging vector, the p TAP-BAC (Fig. 1B). Subsequently, sequencing was carried out to confirm that no changes had been introduced during the vector construction.

2.3. Tap tagging of P64k protein

The 3Vend fragment of the lpdA gene (the gene coding for P64k protein) was TAP tagged by PCR, utilizing p TAP-BAC plasmid as a template for PCR and the two pairs of primers 8131/8132 and 8135/ 8136 respectively (Table 1). The Kan resistant gene was also amplified using the primers 8133/8134, in order to use it as a selection marker. The generated vector was named p TAP-5K3 and it allowed the correct insertion of the

TAP cassette in the N. meningitidis genome by spontaneous homologous recombination event. Consequently, the TAP cassette promoter was the same as that of lpdA gene, followed by it.

2.4. Recombinant DNA techniques, transformation and selection

The standard recombinant DNA techniques were carried out essentially as previously described (Sam-brook et al., 1989). The DNA restriction and modification enzymes were used following the manufacturer's recommendations. For meningococcal transformation, exponentially growing cells were resuspended in BHI supplemented with 10 mM MgCl2 at 0.05 OD260 and statically incubated with plasmid DNA at 10 mg/mL for 1 h at 37 8C. Afterwards, they were plated on BHI-kanamycin plates, and grown within 12 to 24 h. The resistant colonies were purified twice by streaking onto selective plates before preparing stocks for analysis. The double purification was achieved in order to ensure the selection of meningococcal with both diplococcal members effectively transformed. The purification of meningococcal chromosomal DNA and total RNA was carried out following previous published procedures (Guille´n et al., 1993; Shaw and Clewell,1985). The lpdA gene was entirely amplified by PCR using oligonucleotides 1573 and 1206 to confirm the correct insertion and the homologous integration via single-site crossing-over mechanism of the cassette.

2.5. Preparation of whole cell extracts and western blot analysis.

The transformed N. meningitidis expressing the P64k was grown as described in the above procedure for the expression conditions. The bacteria were har-vested by centrifugation at 1500 g for 15 min and washed once with 10 mM Tris, pH 8.0 (lysis buffer). After cell collection, all steps were performed at 0 ±4 oC. The total extracts were prepared using French press and the protein concentration values were determined following the method of Lowry (Stoscheck, 1990), 20 Ag of each sample was loaded per lane and subjected to 10% sodium dodecyl sulfate–polyacrilamide gel electrophoresis (SDS–PAGE) as described (Laemmli, 1970). Western Blots were per-

formed by transferring proteins from acrylamide gels to 0.45 Am pore size nitrocellulose membrane (Tow-bin et al., 1992). The membranes were then blocked with skimmed milk and immunodetection was car-ried out as in (Wedege and Frøholm, 1986); using mAb-114 previously described by (Alvarez et al., 1997) and the CB.Hep1mAb, which recognized the HBV tag sequence specifically included in the TAP cassette.

3. Results and discussion

Proteomic analysis, particularly in the case of methods that use mass spectrometry (ME), needs a reproducible and rapid system for protein purification. In most cases, the general method is based on an affinity purification step that uses tag segment, epi-topes or small protein fused with the target gene in order to have a protein overexpression. Nevertheless, for optimum results, it is preferable to maintain expression of the fusion protein at, or close to, its natural level when we need to study protein–protein interaction. The TAP system gives us this possibility. In order to utilize the TAP system in N. meningitidis, it was necessary to adapt it by adding a novel C-terminal TAP tag to a target gene P64K. C-terminal consisted of an SB that contains not only the TAP system proposed by Rigaut et al. but an additional HVB tag between the TEV sequence and two domains of protA. This sequence is specifically recognized for CB.Hep1mAb allowing both to follow the expression of target protein after homologous recombination in the host cell and to check the correct insertion of the TAP cassette inside the N. meningitidis genome. The SB length was 228 pb. It contains a polylinker site upstream 5Vend, and two sites that are recognized specifically by the EcoR I and BamH I endonuclease downstream 3Vend (Fig. 1A). A C-terminal TAP tag design was chosen instead of an N-terminal design in order to maintain the expression of the target gene under the control of its natural promoter. Moreover, in some expression/host cell systems, the introduction of a C-terminal tag has a smaller perturbing influence on the transcriptional/translational cell machinery than N-terminal located tag sequence. This consideration is particularly relevant if the introduced tag has a high content of the same

type(s) of amino acid residues, which may lead to arrested expression/delayed translational maturation of the target protein.

The multiple cloning sites (MCS) of the p TAP-BAC expression vector allow several cloning strategies, resulting in fusion of the TAP tags with the C-terminal of the gene of interest.

The BS with the BamH I 3Vend site subcloned into the p M231 vector facilitated the introduction of the IgG domain of ProtA, previously amplified via PCR from p PRIT-2T vector and cleaved with BamH I/Bgl II endonuclease enzymes. After the construction of the p TAP-BAC, we inserted the 3Vend of the lpdA gene. To allow the introduction of another segment of the lpdA gene (3Vend fragment that does not codify for the protein) into the vector, further modifications were made to the p TAP-5 vector. Specifically, Kan resistant marker from the p UC4K includes a polylinker site in both ends. The resulting vector, p TAP-5K, not only permitted the clone selection in N. meningitidis but the new insertion of the segment in the BamH I and PsT I restriction sites. Subsequently, the P64k 3Vend fragment (not codified for the protein) was subcloned generating a final vector with all require-ments for the correct fusion of the TAP system in a target protein, P64k.This vector was named p TAP-5K3 (Fig. 2).

The N. meningitidis strain CuB385, transformed with the p TAP-5K3, was selected by Kanamicyn. This was possible as the result of the integration of the Kanr cassette into the genome via homologous recombination with P64k gene fragments flanking the homologous arms; thus replacing the wild-type gene with the counterpart-tag. Proof of correct replacement of the wild-type gene was obtained by PCR amplification of the lpdA locus with primers 1573 and 1206 (data not shown). The specific primer pair was designed to amplify the fragment expected from single crossing over integration events in the deferent's trans-formants. A PCR negative control with DNA from untransformed colonies or from bacterial plasmid alone did not yield any of these amplification products. Integration of plasmid DNA into the chromosomes evidently involved single-site crossing over events between homologous regions. In order to achieve a successful homologous recombination process in bacterial, some details should be taken into

account: the use of a plasmid with origin of replication not functionally in Neisseria, the flanking region longer than50 bp, and is critically the use of a competent cell with pili. The previous condition will ensure a successful homologous recombination process.

To determine whether the constructs were functional, the p TAP-5K3 vector was introduced into wild-type N. meningitidis cells to create strain Cu385TAP. Tagging of P64k protein with the TAP cassette was confirmed by immunoprecipitating with IgG sepharose from protein lysates of Cu385TAP. Fig. 3 shows a Western blot using an antibody to detect P64K, specifically 114 mAb, and CB-Hep1-mAb for detecting the binding modules of the TAP tag cassette.

Summarizing, the novel TAP tag cassette could be adapted to other bacteria to allow the purification and identification of additional protein complex. The expression vectors described here have broad application potential for studying protein and protein–protein interactions in bacteria by maintaining the protein normal expression levels. Simultaneously, the several disturbances provoked to the cell by constructions leading to protein overexpression were avoided, specifically when a foreign protein is expressed.

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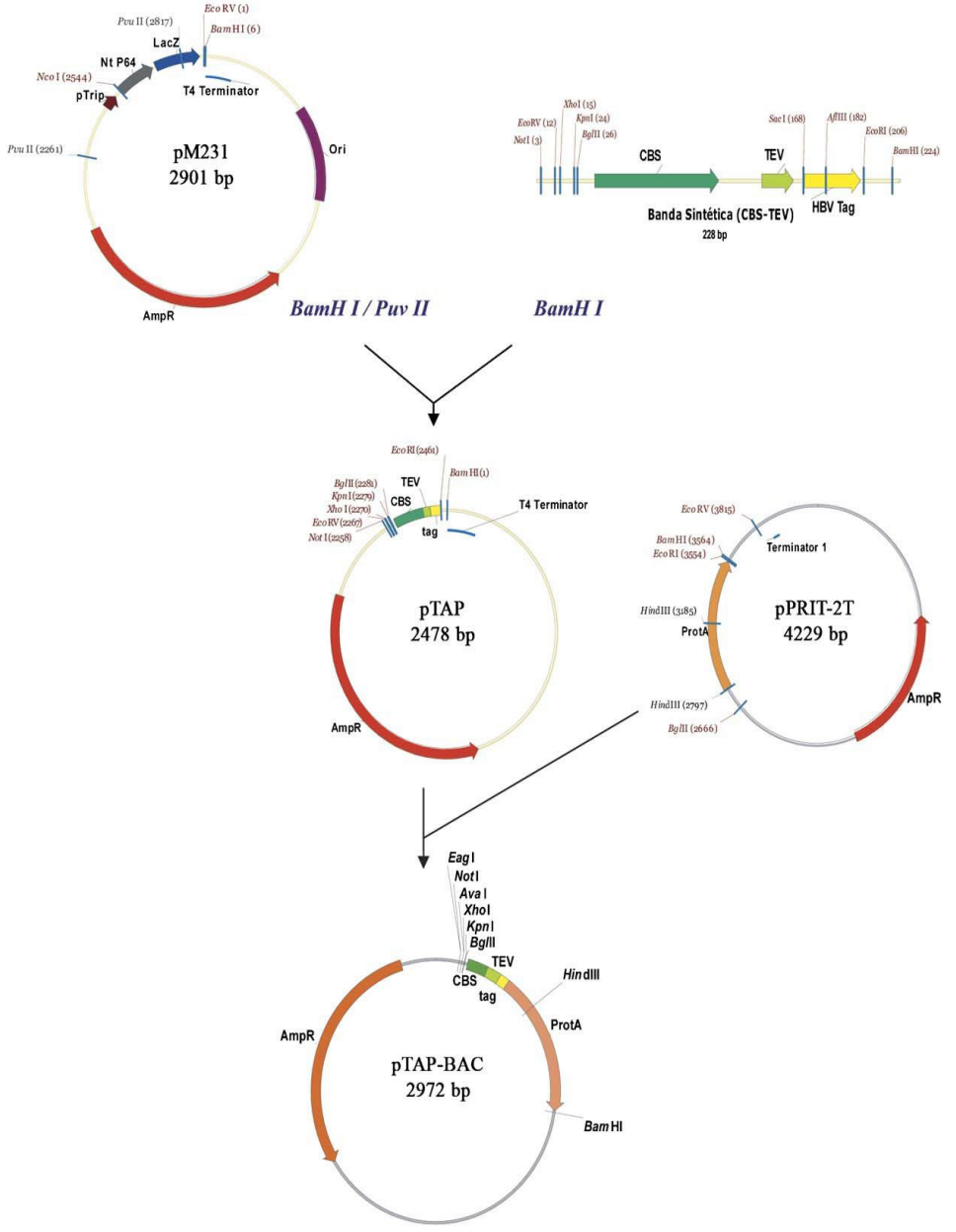
Fig. 1. Vector for TAP targeting in *N. meningitides*. Series of vector for the construction of the p TAP-BAC vector, that allows the fusion of TAP cassette with the target gene.



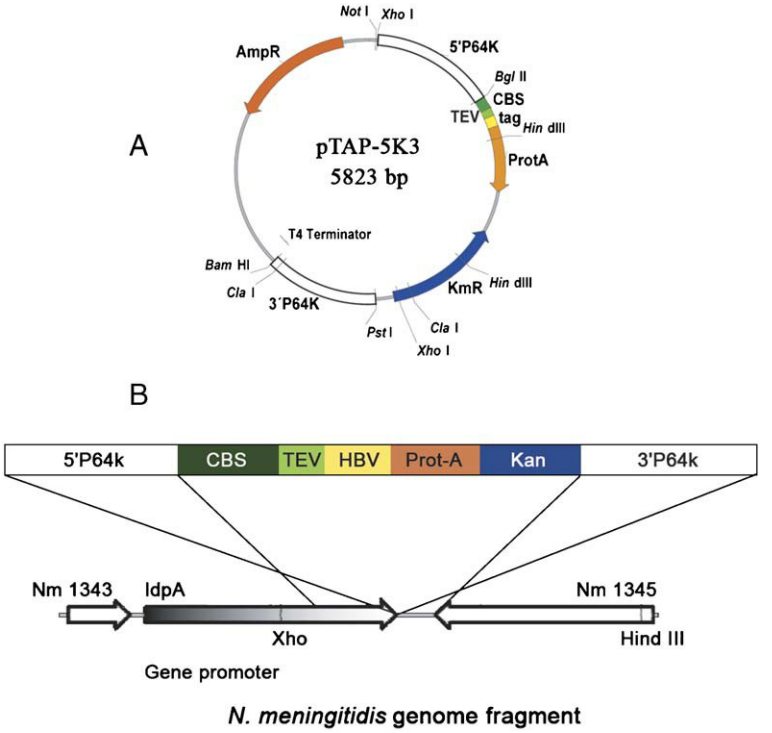


Fig. 2. Schematic diagram of C-terminal tagging strategy. (A) The TAP-5K3 plasmid, which include the tagging cassette and flanking homologous regions to the target gene *lpdA*. (B) Followed by the transformation into N. meningitidis cells. The fragment integration into the genome occurs via homologous recombination by placing the target under the control of the *lpdA* gene promoter.

Fig. 3. Identification of the correct insertion of the P64k-TAP tag system (in CuB385). Extracts carried out by French press were resolved by 12.5% SDS–PAGE and the gel staining with Coomassie blue dye. Lane 1 correspond to the protein molecular markers, lane 2 and 3 correspond to experiments with and without 2mM DTT. Samples were also analyzed by western blot with CB-Hep1 mAb detecting HVB tag sequence (lane A) and 114 mAb (anti-P64k specifically antibody) (lane B). Using both antibodies the correct insertion of the P64k-TAP tag system was detected. Molecular weights in kiloDaltons are indicated on the left side.

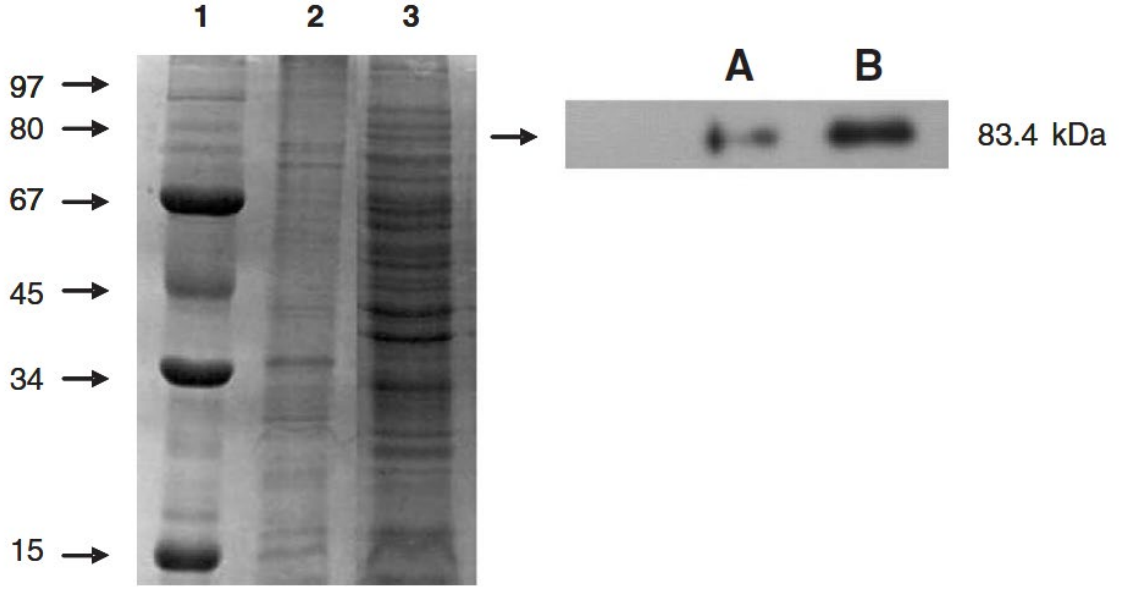


Table 1
Oligonucleotides

Oligonucleotides			Sequence (5′ to 3′)
pTAP-BAC (protA from pPRIT-2T)		7232	GAT GAA TTC GCA CCT AAA GCT GAT AAC AAT
		7469	CTG CAG GTC GAC GGA TCC ACG GAA ATT
pTAP-5K3	lpdA 3′ end	8131	ATT ATC GGC CTC GAG ATG GGT ACG
		8132	TTA AGA TCT CTT TTG CGG AGG CAG GTC
	Kanamicin from pUC19	8133	TGT TGT GTG GAA TTG TGA GC
		8134	CTG AGA TCT GCC TCG TGA AG
	lpdA 5′ end	8135	GTT TAC TGC AGC AAA TGC CGT CTG
		8136	TCA CCA AGG ATC CCG TAT TGG AC
Plasmid fragment		6795	AAC TGC AGG CTT GTA AAC CGT TTT GTG
		6793	AAA GGG AAT AAG GGC GAC ACG
N. meningitidis *LpdA* locus		1573	TTCCATGGTAGATAAAAG
		1206	AAAAAAGAAAACGCCTCC