Biol Res 30: 149-160 (1997)

Construction of a gene encoding the insect bactericidal protein attacin. Studies on its expression in *Escherichia coli*.

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Attacin, a bactericidal small protein is produced by the giant silk moth ***Hyalophora cecropia.*** *This paper deals with our efforts to clone the attacin cDNA in a bacterial vector to express it in* ***Escherichia coli*** *and produce the protein in sufficient amount for further studies. We chose two inducible expression vector/bacterial cell systems: pPL-lambda/N99cI$^+$ cells which is able to be induced by nalidixic acid, and pET3d/BL21(DE3) cells carrying a T7 RNA polymerase gene which is IPTG-inducible. After cloning in the pPL-lambda system and under no addition of the inducer, isolated transformants carried this plasmid with at least 2 concurrent deletions that drastically affected attacin expression, even though attacin gene seems to be intact as deduced by its PCR amplification. It was concluded that basal attacin expression occurred in this system and bacterial growth was limited. Plasmid deletions may have emerged by selection pressure as a way to avoid bactericidal expression and allow bacteria survival. The second cloning attempt was done in pET3d vector/BL21 cells, that should not express the cloned sequence (they lack T7 RNA polymerase gene). Transformed BL21 cells gave 3 recombinant plasmids, 2 of them presented a C deletion that generated an early stop signal in the attacin coding region. The third clone, pET-ATT18, carrying an intact gene, was transferred to BL21(DE3)-IPTG inducible cells in order to be expressed. Attacin was undetectable in stained gels or by Western blot analysis. However, expression was visualized in grown cells after 30 min of IPTG induction and 5 min of [^{35}S]-methionine labeling, as a 22.5 kDa protein band by using gel electrophoresis and fluorography. This low level of expression drastically affected bacterial growth. Considering that attacin has no lytic activity, these results suggest that this molecule should block bacterial growth directly at the cytoplasm by an unknown mechanism, since no signal peptide coding sequence was incorporated in this gene construction, precluding periplasmic or external destination of this protein.*

Key words: *attacin cDNA, bacterial expression, bactericidal peptide, pET vector*

INTRODUCTION

Attacins are bactericidal proteins found in the hemolymph of the giant silk moth *Hyalophora cecropia* in response to bacterial infection or injury (Boman *et al*, 1985; Sun *et al*, 1991). Antibacterial proteins have also been found in the hemolymph of several

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other lepidopteran and dipteran species (Dickinson *et al*, 1988; Ando *et al*, 1987; Lambert *et al*, 1989; Dimarcq *et al*, 1988).

Attacins constitute one of the main groups of antibacterial proteins in *H. cecropia*. They act bacteriostatically on Gram-negative bacteria (Hultmark *et al*, 1983; Engström *et al*, 1984; Ando & Natori, 1988). Six attacins have been purified, four basic proteins (A, B, C and D) and two acidic/neutral forms (E and F) with molecular weights ranging around 20-23 kDa (Hultmark *et al*, 1983). However, from amino acid and cDNA sequencing data it was found that two genes (one encoding an acidic attacin form and the other, a basic form) were enough to explain the 6 different isoforms (Kockum *et al*, 1984; Engström *et al*, 1984). The six forms resulted from post-translational modifications (Kockum *et al*, 1984). The structure of these two genes (containing 2 introns) and two attacin pseudogenes has been described (Sun *et al*, 1991).

Certain protein domains within the attacin sequence could be of functional importance (Wicker *et al*, 1991) since they are shared with sarcotoxin (Ando *et al*, 1987) and diptericin (Dimarcq *et al*, 1988). Interestingly, the proposed functional domains are coincident with the exons 2 and 3 of the attacin coding region.

Regarding attacin mode-of-action as antibacterial protein it seems that the primary target is the outer membrane of the bacterial cell (Carlsson *et al*, 1991). Initially, it has been shown that the permeability barrier function of the outer membrane is affected shortly after addition of attacin to growing cultures of *Escherichia coli* (Engström *et al*, 1984). After prolonged exposure to attacin, physical alterations in the structure of the cell envelope are revealed by electron microscopy as found for diptericin (Ishikawa *et al*, 1992).

It has been shown that attacin caused a specific inhibition of the synthesis of several outer membrane proteins, including *OmpC*, *OmpF*, *OmpA* and LamB. The inhibition is explained by a reduction in the steady-state mRNA levels and, in part, as a result of a block of transcription of the corresponding genes (Carlsson *et al*, 1991). Transcription of OmpR, the activator of ompC and ompF porin genes is also affected by attacin. However, the fine mechanism to explain this inhibition is not well understood.

In order to study in further details the attacin mechanism of action it is necessary to have the purified protein in sufficient amount.

We present here our initial efforts to express cloned acidic attacin gene in *Escherichia coli*, using inducible expression vector systems to allow the production of the protein and, at the same time, minimize the bacterial damage due to its natural antibacterial activity.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli strains DH5α and N99cI$^+$ were obtained from Pharmacia Biotech, Piscataway, NJ, USA. *Escherichia coli* strains BL21 (F^-, *ompT*$^-$, r_B^- m_B^-) and BL21 (DE3), a derivative that carries the T7 RNA polymerase phage gene in the chromosome (Studier *et al*, 1990), were provided by Novagen Inc, Madison, WI, USA. All strains were grown in Luria broth (10 g/l tryptone, 5 g/l NaCl and 10 g/l yeast extract) at 37°C with rotatory shaking (200 rpm). Isolated clones were kept in 50% glycerol at -20°C or frozen at -70°C in 14% glycerol. Plasmid pUC-ATT was a kind gift of *Centro Internacional de la Papa* (CIP), Lima, Perú; pPL-lambda was purchased from Pharmacia Biotech and pET3d and pET11d were from Novagen Inc. Plasmids were isolated essentially according to Birnboim and Doly (1979). For plasmid miniscreening the alkaline lysis method was followed (Sambrook *et al*, 1989).

Cloning and DNA sequencing

Most of DNA manipulations such as 5' end dephosphorylations, kinasing and DNA ligations were carried out according to Sambrook *et al* (1989). Bacterial transformation was done as described by Weston *et al* (1981) and in some cases, using

the protocol proposed by Hanahan (1983). DNA sequencing was carried out as described (Sanger *et al*, 1977) using the Sequenase kit (USB, Cleveland, OH, USA). Double stranded plasmid sequencing was done according to Chen and Seeburg (1985).

DNA agarose gel electrophoresis

Plasmid DNA was visualized in 1-2% submarine agarose gels in TEA buffer (40 mM Tris HCl, 2 mM EDTA and 20 mM sodium acetate adjusted to pH 8 with glacial acetic acid). The gels were stained with 1µg/ml ethidium bromide and photographed under UV light with a Polaroid camera and 667 Polaroid film.

PCR DNA amplifications

PCR assays were done following the Saiki protocol (Saiki, 1989) in 100 µl final volume with 20 mM Tris HCl pH 8.4, 50 mM KCl, 0.2 mM of each dNTP, 50 pmoles of each primer and 2.5 units of Taq polymerase (AmpliTaq, Perkin Elmer, Norwalk, CT, USA). As DNA template, 500 ng of pUC-ATT plasmid containing the acidic attacin cDNA was used. The reaction mix was submitted to 30 cycles in a thermocycler, including the following steps: denaturation at 94°C for 1 min, annealing at 55°C for 2 min and elongation for 3 min at 72°C. Three oligonucleotide primers were designed:

Att-1: 5'AGG AAA CAG ACC ATG GAC GCG CAC GGA GCG CTG ACC CTG AAC

Att-2: 5'TAT ATA TAT AAG CTT ATA TAT ATA GAA TTA AAA TTA

Att-3: 5'AAT AAT AAT CCA TGG AGT AAA TAC ATT TAG

Att-1 is a 42mer corresponding to the 5'end of the gene and it was designed to add a NcoI site (double underlined) which includes an ATG initiation codon previous to the first Asp codon corresponding to the mature protein sequence. Also, four triplets were optimized for expression in E. *coli* (underlined nucleotides). Att-2 is a 36mer derived from complementary sequence to the 3' end of the gene including a HindIII site (double underlined). Att-3 is a 30mer primer which is based on the complementary sequence of the 3' end of the gene covering part of the 3' untranslated region. This primer sequence starts 84 bases beyond the attacin stop codon and also includes a NcoI site (double underlined) to facilitate cloning. PCR products were purified by Magic™ PCR Preps kit (Meize, 1991).

Preparation of attacin peptide polyclonal antibody

A 37 amino acid peptide antigen derived from acidic attacin sequence was chemically synthesized by Bios Chile (Santiago, Chile). The antigen sequence corresponded to the second G peptide domain present in attacin (Hultmark, 1993) and covered from Asp_{123} to Asp_{159}. The antibody was prepared in rabbits according to Harlow and Lane (1988). Two male rabbits were immunized subcutaneously three times with 250 µg of antigen each, in a period of 90 days. The antisera were satisfactory tested at 1:1000 dilution by dot blot assay (Hawkes, 1986) and ELISA (Voller *et al*, 1976) using the purified peptide antigen.

Selective [^{35}S]-methionine labeling of plasmid encoded proteins

The assay was adapted from the protocol described in Current Protocols in Molecular Biology (Tabor, 1990). *E. coli* BL21 (DE3) cells carrying the pET-ATT18 plasmid or the pET vector were grown in 2 ml of Luria media with 80 µg/ml ampicillin until OD_{600} = 0.4. The cells were centrifuged 15 seconds in microfuge and washed 3 times with 1 ml M9 medium (Sambrook *et al*, 1989). The pellets were resuspended in 1 ml M9 medium supplemented with 20µg/ml thiamine and a mixture of 18 amino acids (excepting methionine and cysteine) in 0.005% final concentration. Then, cells were kept growing for 90 min at 37°C with shaking. T7 RNA polymerase was induced by 1 mM IPTG during 30 min incubation. Main bacterial transcription was blocked with 200 µg/ml rifampicin (Sigma-Aldrich R-3501) and incubation was continued for 10 min at 42°C (to maximize rifampicin action) and then 20 min at 37°C. An aliquot

of 0.5 ml was removed for [^{35}S]- labeling with 10 µCi of [^{35}S]-methionine (Sp. act. 1,175 Ci/mmole) for 5 min at 37°C. Cells were pelleted and resuspended in 100 µl of 2X sample buffer (Laemmli, 1970), boiled for 5 min and 20µl were loaded in a 15% polyacrylamide gel and run as described below. The gel was soaked 1 hour in 250 ml of 1 M sodium salicylate used as fluorographic enhancing agent (Chamberlain, 1979), washed with distilled water, dried under vacuum and exposed to X-Omat AR Kodak film for 24 hours.

Protein gel electrophoresis and Western blotting

PAGE-SDS was carried out on 15% or 18% polyacrylamide gels following Laemmli method (Laemmli, 1970). To analyze bacterial lysates or ^{35}S-labeled proteins, 5-10 µl from overnight cultures or 20 µl of labeled cells were mixed with the same volume of 2X sample buffer (0.125 mM Tris HCl pH 8.0, 6% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.05% bromophenol blue) and boiled 10 min before loading a 5 µl aliquot. Minigels (10 x 6 x 0.2 cm) were run at 200 volts for 1 hour. Labeled samples were run in 15% polyacrylamide gels (15 x 12 x 0.75 cm) at 150 volts for 2 hours. Then, the gels were stained 15 min with 0.1% Coomassie blue R250 in 0.1% TCA and 25% isopropanol, or transferred for Western blotting, or prepared for X ray film exposure. Western blots were done according to Towbin *et al* (1979). Nitrocellulose filters were incubated with polyclonal anti-attacin peptide rabbit antiserum diluted 1:100 and revealed with anti-rabbit IgG goat antiserum conjugated to horseradish peroxidase diluted 1:200.

RESULTS AND DISCUSSION

Amplification of the cDNA acidic attacin sequence and cloning in the pPL-lambda vector

The pPL-lambda vector is a 5218 bp plasmid expression vector carrying a region of the lambda phage corresponding to the N gene and their regulatory sequences (Ptashne, 1992). The vector contains a single HpaI sequence as a cloning site within the N gene commanded by the P_L strong promoter to express cloned genes (Pharmacia Biotech 1995 catalogue). The P_L promoter is normally blocked by the binding of the cI$^+$ repressor but in the *Escherichia coli* strain N 4830-1, which carries a cI^{857} temperature sensitive mutant repressor, the cloned gene can be induced by a temperature shift over 31°C. However, the P_L promoter may also be induced by 40 µg/ml nalidixic acid in the strain N99 cI$^+$ (Mott *et al*, 1991).

The attacin coding region contained in plasmid pUC-ATT was amplified according to Methods using oligos att-1 and att-2. The product was isolated by 1% agarose gel electrophoresis as a 630 bp band. The gel slice was melted and purified by Magic^{TM} PCR columns (Promega Corporation, Madison, WI, USA) as described by the vendor. This purified band (100 ng), after kinasing the 5' ends with T4 polynucleotide kinase, was blunt-end ligated to the pPL-lambda plasmid previously digested with HpaI and dephosphorylated with calf intestine alkaline phosphatase (Sambrook *et al*, 1989). After ligation, half of the ligation mix was again digested with HpaI to reduce the transformation background due to the vector self-ligation. Transformation assays were done on *E. coli* N99cI$^+$ with half of each ligation mix as described by Sambrook *et al* (1989). The HpaI digested ligation mixture gave 22 Amp^R colonies and the other half, 44 colonies. Screening of these colonies showed that most of them contained plasmids. In order to identify the plasmid carrying the attacin insert, digestion with BamHI and HindIII endonucleases were carried out. From the BamHI digestion, a 1,859 bp fragment containing the attacin insert and a 4,000 bp fragment of vector sequences were expected, and the HindIII digestion should linearize the plasmid. Curiously, none of these clones released the 1,859 bp BamHI fragment. For instance, Figure 1a, lanes 2 to 8, shows an agarose gel with DNA from 7 selected clones after incubation with BamHI. Six of these plasmids migrate as

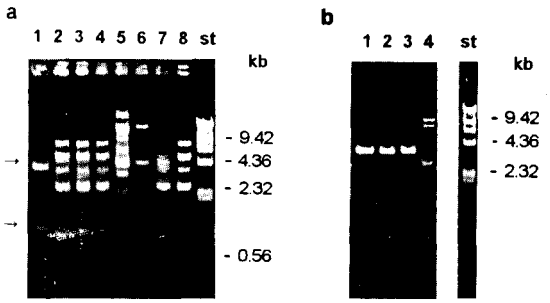

Fig 1. Restriction analysis of some recombinant plasmids after ligation to the pPL-lambda vector and transformation of *E. coli* N99cI+. A 1% agarose gel was loaded with 20 µl plasmid digestions of different pPL-ATT clones. **a:** BamHI digestion on plasmids of clones 2, 4, 5, 12, 13, 17 and 20 in lanes 2, 3, 4, 5, 6, 7 and 8, respectively; lane 1, BamHI/pPL-lambda. Arrows indicate the fragments obtained from the pPL-lambda vector. **b:** HindIII digestion on plasmids from clones 12, 13, 17 and 20 in lanes 1, 2, 3 and 4, respectively. Lane st, HindIII-digested lambda DNA. The number at the sides indicate the size of standard DNA in kilo base pairs.

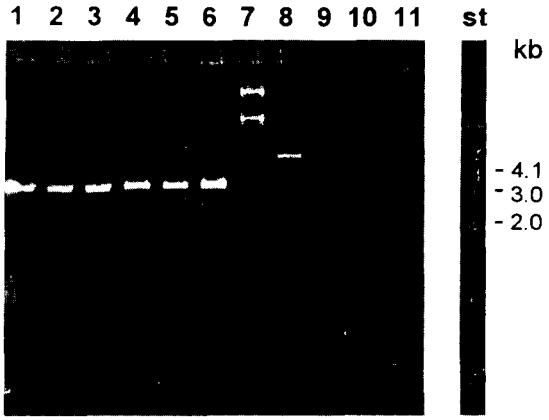

Fig 2. Restriction analysis on recombinant plasmids pPL-ATT12, pPL-ATT13 and pPL-ATT17 isolated from strain N99cI+. A 1% agarose gel was loaded with 20 µl of different digestions. Lane 1, KpnI/pPL-ATT12; lane 2, KpnI/pPL-ATT13; lane 3, KpnI/pPL-ATT17; lane 4, KpnI/EcoRI/pPL-ATT12; lane 5, KpnI/EcoRI/pPL-ATT13; lane 6, KpnI/EcoRI/pPL-ATT17; lane 7, SalI/pPL-ATT12; lane 8, SalI/pPL-lambda; lane 9, KpnI/pPL-lambda; lane 10, HindIII/pPL-lambda; lane 11, pPL-lambda undigested; lane st, 1 kb DNA standard ladder.

undigested DNA and only clone pPL-ATT17 (lane 7) releases a smaller 1.2 kb BamHI fragment as occurs with the pPL-lambda vector (see lower arrow). In addition, HindIII digestion of these clones, which should linearize the plasmids giving a 5,857 bp fragment, produced in some cases a smaller band of about 3,600 bp. Figure 1b shows plasmids from clones pPL-ATT12, 13 and 17 (lanes 1, 2 and 3) after HindIII digestion as linear fragment approximately 3,600 nucleotides long, however clone 20 lacked the HindIII site (lane 4). Since the HindIII site is present only in the attacin amplified fragment (it was incorporated in the att-2 primer) and was not present in the vector (Fig 2, lane 10) this suggests that in spite of the smaller size, the 3,600 bp fragments of clones pPL-ATT12, 13 and 17 may carry part of the attacin cDNA sequence. These results strongly support the idea that these recombinant plasmids have suffered some kind of rearrangement with loss of sequences around both BamHI sites that are flanking the promoter and transcription terminator vector sequences. To evaluate if the attacin gene was still present in them, plasmids from clones pPL-ATT12, 13 and 17 were digested with KpnI (a single site is within the attacin coding region and no sites are in the vector) and double digested with KpnI and EcoRI (which should release a 528 bp fragment containing the promoter region including the 14 initial codons of the gene). Figure 2 showed that a single KpnI site was present in these clones (lanes 1, 2 and 3), however, the size of the recombinant linearized plasmids was around 3,600 bp, indicating that at least a 2.2 kb of the total expected sequence length was missing. In addition, double digestion gave no 528 bp fragment (Fig 2, lanes 4, 5 and 6) suggesting absence of the EcoRI site or that both KpnI and EcoRI sites are indeed very close each other. Actually, the existence of the EcoRI site in these clones was demonstrated by single EcoRI digestion that produced 3,600 linear plasmids from these clones (not shown) as found for KpnI and HindIII digestions. As reference, a single SalI site (lane 8) and no sites for KpnI (lane 9) and HindIII (lane 10) were found in the pPL-lambda vector.

After repeated transformation attempts, colonies containing plasmids with lower molecular weight than the vector were always obtained. Even though the attacin insert was present in these deleted plasmids as confirmed by PCR gene amplification, at least two regions of the vector were deleted since both BamHI sites of the vector that should flank the attacin gene were missing.

One deletion of about 500 bp involved the P_L promoter region and extends from the EcoRI site to some region before the HpaI cloning site. The other deletion would include approximately 1 kb starting just past the HpaI site to reach some point beyond the terminus of the non-functional tet gene present in the vector. This is in part sustained by the absence of a BamHI and a SalI site, normally found in the tet gene of the pPL-lambda vector.

As mentioned, the integrity of the attacin gene was confirmed by PCR amplification of the coding region using primers att-1 and att-2. Figure 3 indicates

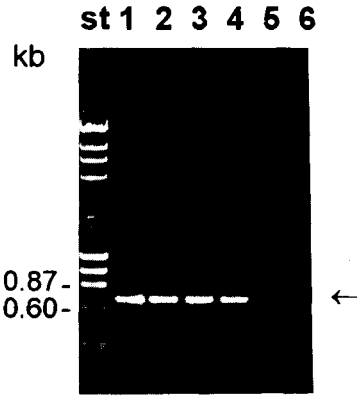

Fig 3. PCR amplification of attacin gene from clones pPL-ATT12, pPL-ATT13, pPL-ATT17 and pPL-ATT20. The assay was done as indicated in Methods. The primers used were att-1 and att-2. Electrophoretic analysis of 10 µl aliquots of amplified products was carried out in a 1% agarose gel. Lane 1, positive control using 500 ng of the pUC-ATT plasmid as a template; lanes 2, 3, 4, 5 were PCR reactions using 100 ng of plasmids pPL-ATT12, pPL-ATT13, pPL-ATT17 and pPL-ATT20, respectively; lane 6, negative PCR control assay without DNA template; lane st, a mixture of 0.5 µg of HindIII-digested lambda DNA and 0.5 µg of HaeIII-digested PhiX174 DNA.

that a 640 bp amplicon was obtained from clones pPL-ATT12, 13 and 17 (lanes 2, 3 and 4), but no fragment was amplified from pPL-ATT20 (lane 5). This last result also agrees with the lack of the HindIII site in the clone pPL-ATT20, as previously displayed in Figure 1b (lane 4).

From these results it was anticipated that clones pPL-ATT12, 13 and 17 will not express the attacin gene after induction with 40µg/ml nalidixic acid and these experiments were not carried out.

Cloning of the attacin gene in the pET3d vector

The pET3d vector was selected to express acidic attacin gene since it carries a T7 promoter for the expression of foreign genes when this vector is induced in the host strain *Escherichia coli* BL21(DE3) (Studier *et al*, 1990). Particularly, this system has been used to clone and express genes that generate toxic products. The host strain, BL21(DE3), contains the T7 phage RNA polymerase gene inserted in the bacterial chromosome. This gene is inducible by IPTG, facilitating a transient expression of a toxic gene until most of the bacteria are killed. The plasmid pET3d was digested with NcoI and the attacin PCR product prepared with att-1 and att-3 primers. After gel isolation, the fragment was cut with the same enzyme and ligated to pET3d as described in Methods. The cloning strategy is summarized in Figure 4. The transformation mixture (approximately 80 ng of DNA) was used in 2 separated transformation events, giving in total 26 Amp^R *E. coli* BL21 transformants. This strain was used to obtain the attacin construction free of deletions or plasmid rearrangements which may be induced in bacteria as a way to survive by inactivating a gene encoding a toxic product, such as attacin. Strain BL21 does not contain the T7 RNA polymerase gene and, at least in theory, the attacin gene should not be expressed. For expression studies the plasmid with the attacin gene was later transferred to the *E. coli* BL21(DE3) strain. Plasmid screening of these 26 colonies allowed us to identify 3 recombinants (pET-ATT11, 18 and 20) in the right orientation respect to the T7 promoter sequence, and 4 in the opposite orientation (pET-ATT12, 14, 17 and 19). As an example, digestions done on clone pET-ATT11 are shown in Figure 5 demonstrating the presence of the NcoI insert with the right orientation of the attacin gene respect to the T7 vector promoter. NcoI digestion released a 671 bp insert as expected (lane 1); KpnI/HindIII double digestion gave a 1,151 bp fragment containing most of the insert and part of the vector sequences (lane 2). If the insert were

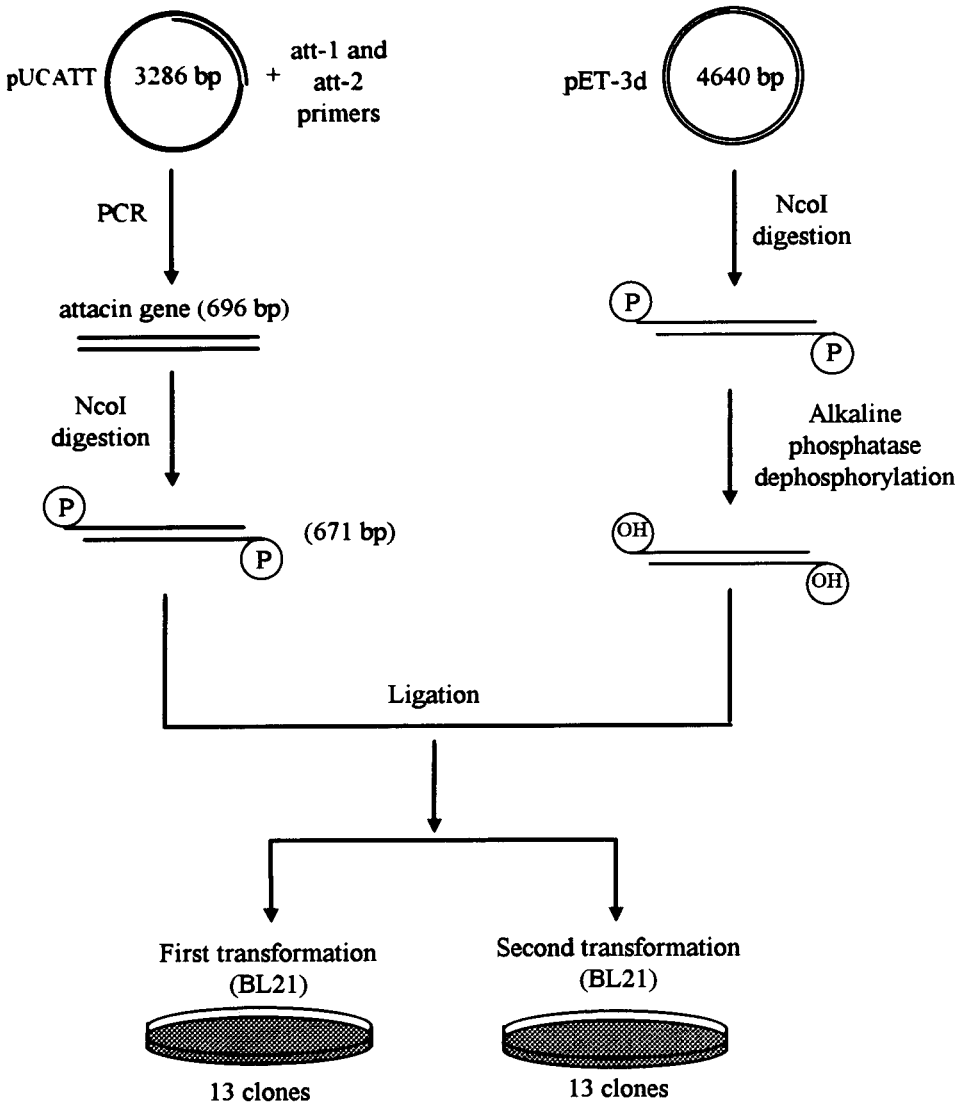

Fig 4. Strategy to obtain the acidic attacin cDNA cloned into the pET3d vector.

in the opposite orientation, this double digestion would have produced a 556 bp fragment. HindIII enzyme only linearized the pET-ATT11 plasmid (lane 3) since a single HindIII site exists in the pET3d plasmid. The remaining transformants were: 15 isolates carrying resealed vectors with no inserts and 4 clones carrying smaller plasmids, with a faster electrophoretic migration than native pET3d plasmid (results not shown).

The plasmids from clones pET-ATT11 and pET-ATT18 were fully sequenced to verify the primary structure of the attacin gene and lack of mutations. Figure 6 displays these sequences. Also, pET-ATT20 was partially sequenced (not shown). It was found that only plasmid pET-ATT18 carried the right attacin sequence. Both pET-ATT11 and pET-ATT20 plasmids shared a C deletion within a run of 3 Cs between codon 8 (Thr=ACC) and codon 9 (Leu=CTG). This deletion generated a translation stop signal (TGA) at codon 9 (Stp boxed in Fig 6). It should be noted that this deletion occurs within the sequence designed as part of primer att-1. The actual native gene sequence does not present a run of 3 Cs at this position. This occurs in pET-ATT18 because codon 8 was optimized

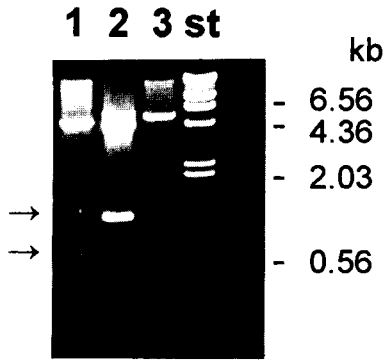

Fig 5. Restriction analysis on pET-ATT11 plasmid containing the attacin amplified gene. Separated digestions were carried out using approximately 0.5 µg of plasmid DNA, incubating 2 hours at 37°C in a final volume of 20 µl and adding 10 units of each enzyme. Lane 1, NcoI digestion; lane 2, HindIII and KpnI double digestion; lane 3, HindIII digestion; lane st, 0.5 µg of HindIII-digested lambda DNA standard.

(ACT→ACC) in the PCR product to enhance the expression in *E. coli*. The plasmids pET-ATT11 and pET-ATT20 were independently isolated from 2 different transformation events, so they do not correspond to a putative originally mutated single clone that could have been amplified during the isolation of transformants due to the tight selection pressure. Nevertheless, it should be noted that all clones derived from a single ligation event. Because of this, and the successful isolation of pET-ATT18, we concluded that this mutation was not present in the att-1 primer itself and emerged later. As we speculate below, probably a basal attacin expression in strain BL21 counter-selected against the cloning of the intact gene sequence and allowed us to isolate attacin mutants.

It is well known the fact that a run of 3 Cs or longer are susceptible to be targets for base deletions (so called "hot spots") as occurring in the histidine operon genes of *Salmonella typhimurium* strains currently used in the Ames test (Levin *et al*, 1982).

Regarding other features found in these clones, we describe here that all changes incorporated in the design of the primers were present, excepting of course, the deleted C reported in pET-ATT11 and pET-ATT20 plasmids. In addition, when the pET-ATT11 and pET-ATT18 sequences were compared to that described for acidic attacin cDNA (Kockum *et al*, 1984), five T→C transitions were identified (asterisks in Fig 6) but these changes are not found when the sequences are compared to the acidic attacin gene (Sun *et al*, 1991), suggesting the existence of sequence polymorphism or errors in the determination of the cDNA sequence.

The only two differences that we found in both, pET-ATT11 and pET-ATT18, respect to the attacin gene sequence were in the 3' untranslated region. Firstly, a TA tract in our sequence corresponds to an AT in the gene (double underlined at nucleotide positions 629-630 in Fig 6). Secondly, these clones contained an extra T within a run of 9 T that follows the TA change just mentioned (arrow arbitrarily located on top of the nucleotide 631 in Fig 6).

Expression of the attacin gene in Escherichia coli BL21(DE3)

Starting from clone pET-ATT18/BL21, a preparative amount of plasmid DNA was obtained using the alkaline lysis method and further purification through a QIAGEN-tip 500 column (Plasmid Maxi kit, QIAGEN, Chatsworth, CA, USA). This plasmid was used for transformation of strain *Escherichia coli* BL21(DE3) required for expression experiments. After isolation of Amp^R transformants, expression studies were followed by [^{35}S]-methionine labeling of plasmid encoded proteins detected in bacterial lysates by PAGE-SDS. After 3 hour growth in LB-80 µg/ml ampicillin media at 37°C, three colonies of the pET-ATT18 plasmid and one of the pET3d vector in BL21(DE3) cells were transferred to M9 minimal media and grown for 90 min. Then, T7 RNA polymerase was induced with 1 mM IPTG for 30 min, host RNA polymerase inhibited by incubation with rifampicin for 20 min and proteins labeled with [^{35}S]-methionine for 5 min (details in methods). The protein profile was analyzed by 15% PAGE-SDS and fluorographic exposure (Fig 7A). After IPTG induction, four main bands (29, 27, 22.5 and 18 kDa) were seen in the three attacin-expressing colonies (lanes 6, 9 and 12) and only one band (27 kDa) in the case of the vector (lane 3). The larger ones (29

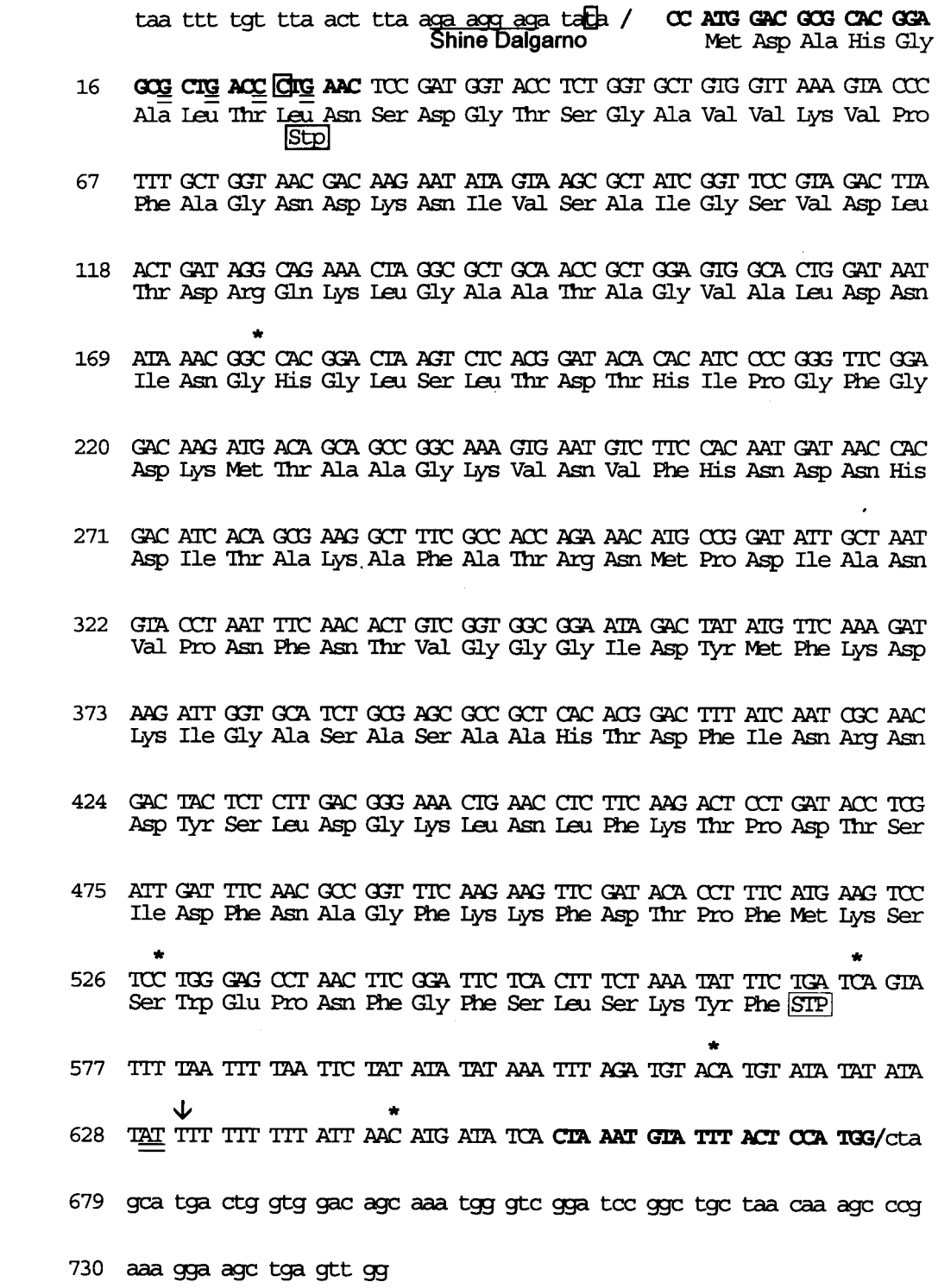

Fig 6. Nucleotide sequence of clones pET-ATT11 and pET-ATT18. Supercoiled plasmid DNA was sequenced according to Methods. Nucleotide sequence is numbered starting at first ATG codon. The deduced amino acid sequence for pET-ATT18 is also included. Boxed nucleotides indicate deletions occurring at pET-ATT11.Vector sequences are shown in lower case. Nucleotides indicated by asterisks correspond to the differences respect to the acidic attacin cDNA sequence (Kockum et al, 1984). Nucleotide differences respect to the gene sequence (Sun et al, 1991) are double underlined. The arrow indicates absent nucleotide in the gene sequence. Stop codons (Stp and STP) are boxed and sequences corresponding to those used for the design of the Att-1 and Att-3 primers are displayed in bold letters.

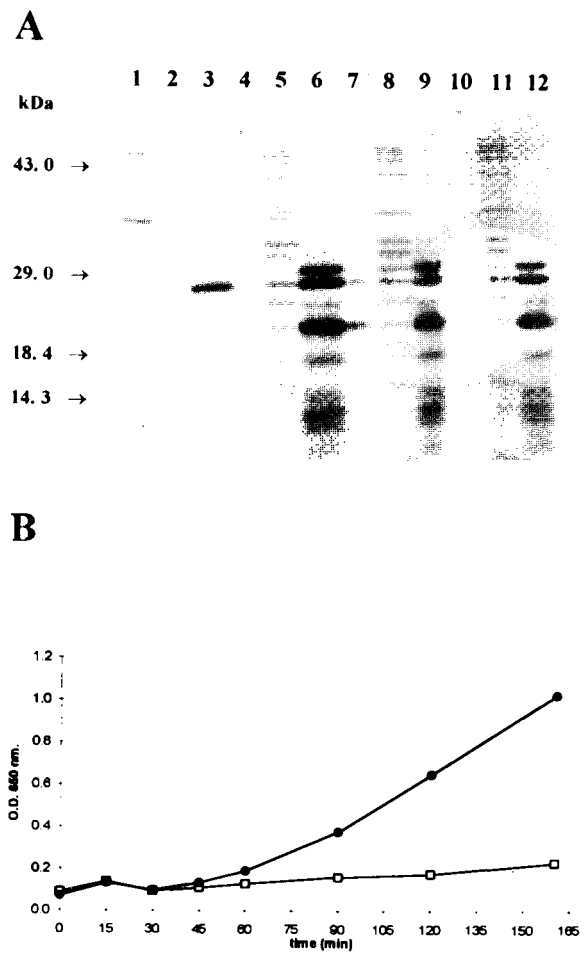

Fig 7. Expression studies of pET-ATT18/BL21(DE3) clones after *in vivo* [^{35}S]-methionine protein labeling analyzed by PAGE-SDS and by following bacterial growth after induction with 1 mM IPTG. **A**: A fluorographic exposure of a 15% polyacrylamide gel on a X-Omat AR film shows 20 µl lysate aliquots of bacterial cultures grown until OD_{600} = 0.4, transferred to minimal media, induced 90 min with 1 mM IPTG, incubated 30 min with rifampicin and then labeled with ^{35}[S]-methionine for 5 min. Lane 1, BL21(DE3) cells uninduced and labeled; lane 2, pET3d /BL21(DE3) cells induced but without labeling; lane 3, pET3d/BL21(DE3) induced and labeled; lanes 4, 7 and 10, three clones derived from pET-ATT18 /BL21 (DE3) induced but not labeled; lanes 5, 8 and 11, same pET-ATT18 clones labeled but uninduced; lanes 6, 9 and 12, lysates from same pET3-ATT18/BL21(DE3) clones after 1 mM IPTG induction and ^{35}[S]-labeling. Position of prestained low molecular weight standard (GIBCO-BRL) and their weights are indicated in kDa by the arrows and the corresponding numbers at the left. **B:** Growth profile of clones pET-ATT18/BL21(DE3) (□—□) and pET3d/BL21(DE3) (•—•) followed by OD_{650} after 1 mM IPTG induction at 0 min. For OD_{650} measurements, 1 ml aliquots were taken from cultures at the indicated times.

and 27 kDa) correspond to β-lactamase precursor and the mature enzyme respectively, as described by Tabor and Richardson (1985). The precursor band was not seen in the cells carrying the vector. The synthesis of these gene products is explained by the absence of transcription terminator signal after the attacin gene. This produces a polycistronic message synthesized by the T7- RNA polymerase, which encodes both attacin and β-lactamase sequences. The third band (22.5 kDa) corresponds to attacin, since it matches the expected molecular weight and appears only after IPTG induction. The smaller bands (18, 14, 12.3 and 8.7 kDa) may correspond to proteolytic cleavage products derived from attacin. In contrast to these expression results, Western blot assays (not shown) using a polyclonal antibodies prepared against the attacin peptide (see methods), did not detect attacin bands after several attempts, probably due to low level of attacin expression or low sensitivity of the antibody.

An estimate of the maximum detectable amount of attacin protein based on the MIC reported value (1 µM) for *Escherichia coli* (Engström *et al*, 1984) suggests that attacin concentration in late exponential culture of pET-ATT18 induced clone should be close to 0.022 µg/µl. Obviously, when attacin is synthesized and kept at the cytoplasm, a lower mass of protein should be required to stop bacterial growth, since the protein is not exported to the culture medium and is produced next to its target, avoiding further dilution. In addition, considering a saturated culture (10^8 cell/ml), the bacterial volume is only a small percentage of the total cell suspension volume. Even though, without taking these considerations, a 5µl lysate aliquot of bacterial cell supposedly grown with attacin concentration close to its MIC value, it would contain, after lysis, approximately 0.11 µg of the protein, amount that hardly doubles the minimum required for visual detection of Coomassie blue stained bands (Breitlow, 1992) or is low down the amount needed for immunological detection using a polyclonal antibody in a dot blot assay (lowest amount detected in dot assay was 0.10 µg of the attacin peptide, which means 0.5 µg of the protein, result not shown).

During the expression studies it was noted that clone pET-ATT18/BL21(DE3)

grew poorly. In order to quantitate this effect bacterial growth was followed by OD_{650} almost 3 hours after 1 mM IPTG induction. Monitoring bacterial growth revealed that only pET3d vector reassumed a normal growth rate 60 min post-IPTG induction, in contrast, pET-ATT18 grew rather poorly if any (Fig 7B).

These results drove us to consider that a low level of attacin expression in clone pET-ATT18/BL21(DE3) is causing an early bacterial death and this amount is enough to stop bacterial growth.

Since attacin have shown a lower bactericidal activity against Gram-positive bacterial species (Engström *et al*, 1984) attempts to express this gene in other bacteria such as *Bacillus* species have been tried but no transformants after electroporation procedure have been isolated so far (not shown).

It remains to be demonstrated how a low level of attacin synthesis (driven by a T7 promoter) may be produced in strain BL21 (which does not produce T7 RNA polymerase) in order to explain the isolation of a plasmid with a nucleotide deletion in the attacin gene (mutation supposedly selected for bacteria survival). Moreover, a problem remains to be solved: how to grow a clone expressing a bactericidal protein at appropriate level without killing most of the bacterial host. *E. coli* mutant strains resistant to attacin have not been yet isolated. This is a necessary step to produce a sufficient amount of protein to undertake future studies.

ACKNOWLEDGMENTS

This research was supported by funds from Fondo Nacional de Ciencia y Tecnología (FONDECYT grant # 1940713) and Fondo de Fomento para Ciencia y Tecnología (FONDEF grant # AN-09).

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