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Review

The multigene families of actinoporins (part II): Strategies for heterologous production in *Escherichia coli*

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ABSTRACT

The sea anemone venom contains pore-forming proteins (PFP) named actinoporins, due to their purification from organisms belonging to *Actiniaria* order and its ability to form pores in sphingomyelin-containing membranes. Actinoporins are generally basic, monomeric and single-domain small proteins (~20 kDa) that are classified as α-type PFP since the pore formation in membranes occur through α-helical elements. Different actinoporin isoforms have been isolated from most of the anemones species, as was analyzed in the first part of this review. Several actinoporin full-length genes have been identified from genomic-DNA libraries or messenger RNA. Since the actinoporins lack carbohydrates and disulfide bridges, their expression in bacterial systems is suitable. The actinoporins heterologous expression in *Escherichia coli* simplifies their production, replaces the natural source reducing the ecological damage in anemone populations, and allows the production of site-specific mutants for the study of the structure-function relationship. In this second part of the review, the strategies for heterologous production of actinoporins in *Escherichia coli* are analyzed, as well as the different approaches used for their purification. The activity of the recombinant proteins with respect to the wild-type is also reviewed.

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1. Introduction

Pore-forming proteins (PFP) are present in sea anemone venoms and are named actinoporins, considering their purification from these organisms (order *Actiniaria*) and its ability to form pores in membranes (Kem, 1988). Actinoporins are monomeric and single-domain small proteins (~20 kDa), and are classified as α-type PFP due to their way to form pores in membrane through α-helical elements (Anderluh and Maček, 2002; Alegre-Cebollada et al., 2007a). Their isoelectric point (pI) is generally basic (above nine), with the exception of two acid actinoporins: epiactin-A from *Epiactis prolifera* and SrcI from *Sagartia rosea* (Bernheimer and Avigad, 1982; Jiang et al., 2002). Actinoporins are cysteine-less proteins (Anderluh and Maček, 2002) and exhibit high affinity for sphingomyelin (SM)-containing membranes (Belmonte et al., 1993; Tejuca et al., 1996; Álvarez et al., 2001; Kohno et al., 2009). Consequently, site-specific mutagenesis by introducing a Cys residue has been a useful strategy for understanding the structure-function relationship in this family of proteins (Malovrh et al., 2003; Kristan et al., 2004; Tejuca et al., 2009; Valle et al., 2011). Nevertheless, different actinoporins with Cys residues have been described for the sea anemones *Heteractis magnifica* (Wang et al., 2008), *Anthopleura asiatica* (Kohno et al., 2009), and *Heteractis crispa* (Tkacheva et al., 2011). More comprehensive information about the actinoporin structures, the relevant functional regions, the multistep pore-formation mechanism, the three transmembrane pore models and the molecular and functional characteristics of isoforms is available in the first part of this review (Valle et al., 2015).

The actinoporins lack carbohydrates and intramolecular or intermolecular disulfide bridges (Anderluh and Maček, 2002), therefore their expression in bacteria has been a successful approach (Anderluh et al., 1996; Jiang et al., 2003a; Pazos et al., 2006; Bellomio et al., 2009; Tkacheva et al., 2011). The actinoporins heterologous expression in *Escherichia coli* simplifies their production (Alegre-Cebollada et al., 2007b) and replaces the natural sources, reducing the ecological damage to anemone populations (Anderluh and Maček, 2002; Pazos et al., 2006). The availability of the genetic constructions for actinoporins heterologous expression allows the site-specific mutagenesis for structure-function relationship studies (Anderluh et al., 1999a; Malovrh et al., 2003; Kristan et al., 2004; Bakrač et al., 2008; Valle et al., 2011; García-Linares et al., 2014) and it could avoid the protein micro-heterogeneity reported for different actinoporin isoforms that can not be resolved by the chromatographic procedures (Valle et al., 2015). The heterologous expression in *E. coli* has also the advantage of achieving high levels of target protein with low costs (Hannig and Makrides, 1998; Baneyx, 1999). For this reason, in this review the different strategies for actinoporins productions in *E. coli* have been analyzed and discussed.

2. Strategies for heterologous expression of actinoporin isoforms in *E. coli*

The genetic approach most widely used to extract the full-length actinoporin isoforms genes is based on complementary deoxyribonucleic acid (cDNA) libraries from sea anemones, constructed in λgtl1-phage or plasmids (Tables 1 and 2) (De los Ríos et al., 2000; Jiang et al., 2003a; Uechi et al., 2005; Pazos et al., 2006; Alegre-Cebollada et al., 2007a; Wang et al., 2000, 2008; Bellomio et al., 2009; Tkacheva et al., 2011). These libraries have been produced by one-step reverse transcription polymerase chain reaction (RT-PCR) of total ribonucleic acid (total-RNA) purified from the body or tentacles of anemones. The screening have been performed by radioisotope-labeled or PCR-derived probe hybridization methodologies (Tables 1 and 2) (Anderluh et al., 1996, 1999b; Pungerčar et al., 1997; Jiang et al., 2002 and 2003a).

For known actinoporins primary structure, the isoform genes were amplified by high-fidelity PCR from cDNA or genomic DNA libraries with specific oligonucleotides primers (Tables 1 and 2). For unknown primary structures, the actinoporin genes were obtained by PCR rapid amplification of cDNA end (PCR-RACE) which allows to obtain cDNAs without constructing or screening a cDNA library (Tables 1 and 2). The principle of PCR-RACE is simple: an anchor sequence is added to the end of the cDNA to be used as PCR primer binding template. A complementary primer to the anchor template is coupled with a gene-specific primer (based on a single short known sequence within the mRNA of interest) in a PCR to amplify the unknown sequence regions (Wang and Young, 2003). Several strategies have been developed to isolate full-length cDNA of actinoporin isoforms using this anchored PCR technology (Uechi et al., 2005; Wang et al., 2000 and De los Ríos et al., 2000).

2.1. Genetic elements for heterologous expression in E. coli

In order to express a eukaryotic gene in *E. coli*, such as actinoporin genes, it is necessary to place the gene in a plasmid with all genetic elements configured for optimal expression. These elements are: an origin of replication (ori), an antibiotic resistance marker, bacterial regulated transcriptional promoters (P), ribosome-binding site (RBS) that include a *Shine Dalgarno* (SD) sequence, a multiple cloning site region (MCS) for the insertion of the genes, and transcriptional-translational terminators (TT) (Fig. 1) (Sorensen and Mortensen, 2005).

The origin of replication (replicon) controls the plasmid copy number per chromosome. The plasmids are divided into four classes based on their copy number: (i) very high (more than 100 copies, e.g. pUC vectors), (ii) high (15–60 copies, e.g. pBR322), (iii) medium (about 10 copies, e.g. pACYC177, pACYC184 and pSC101 vectors), and (iv) low (1–2 copies, e.g. mini-F vectors). The copy number is one of the elements that determines the amount of recombinant protein expressed by the cell (Schumann and Ferreira, 2004).

Most plasmids used for the expression of recombinant proteins in *E. coli* replicate by ColE1 and p15A replicons (Makrides, 1996; Sorensen and Mortensen, 2005). The ColE1 replicon is derived from the pBR322 and pUC plasmid families, whereas the p15A replicon is derived from pACYC184 (Sorensen and Mortensen, 2005). An important element to consider for using two or more plasmids in a single cell is the "plasmidic incompatibility", defined as the inability of two plasmids to stably be maintained in the same cell. For this reason, when multiple plasmids are employed for the co-expression of gene products, it is necessary to use plasmids with different replicons and drug-resistence markers (Sorensen and Mortensen, 2005). Plasmids containing ColE1 and p15A replicons are often combined for this purpose since they are compatible (Sorensen and Mortensen, 2005).

The most common antibiotic resistance markers in expression plasmids confer resistance to ampicillin, kanamycin, chloramphenicol or tetracycline. The plasmid-mediated resistance to ampicillin or its structural-stable analog "carbenicillin" is accomplished by the expression and secretion of β-lactamase (*bla* gene) into the periplasm, where this enzyme inactivates the β-lactam ring by hydrolysis. Kanamycin, chloramphenicol and tetracycline interfere with protein synthesis by binding to critical areas of the ribosome (Russell and Chopra, 1990; Garrett et al., 2000; Sorensen and Mortensen, 2005). Kanamicin is inactivated through phosphorylation by an aminoglycoside phosphotransferase enzyme in the periplasm, whereas chloramphenicol is inactivated by acetylation through a cytoplasmic chloramphenicol acetyl transferase

Table 1
Strategies for heterologous production of actinoporins by phage-T7 RNA polymerase expression systems.

Anemones	Recombinant isoforms	Genetic procedures for cloning isoforms	Expression plasmids	*E. coli* expression strains	Expression broths	Induction	Observations
Actinia equina	**Equinatoxin II (rEqtII)** (Anderluh et al., 1996)	Construction and screening of cDNA library from body of a single specimen of anemone in λgtl1-phage and amplified using the *E. coliY1090hsdR* strain. Gen cloned into the pUC19 plasmid.	pT7-7	*BLR(DE3)*	M-9/LBA, 37 °C	IPTG 0.4 mmolL^{-1} (5 −6 h)	The successful cloning of rEqtII represents the first molecular cloning of actinoporins from sea anemones
	Equinatoxin II 6His-tag (rEqtII-TolAIII-6His) (Anderluh et al., 2003)	Production of DNA by PCR from rEqtII gen Anderluh et al. (1996).	pET8c	*BL21(DE3) pLysE*	M-9/LBAC, 37 °C	IPTG 1.0 mmolL^{-1} (4 −5 h)	The majority was expressed in the non-soluble fraction, but was significantly more expressed in soluble cytoplasmic fraction with 6His-tag and TolAIII solubility-enhancing tag respect to rEqtII.
	Equinatoxin IV (rEqtIV) (Anderluh et al., 1999b)	cDNA λgtl1-phage library screened as described Anderluh et al. (1996). Genes cloned into the pUC19 plasmid.	pT7-7	*BLR(DE3)*	LBA; 37 °C	IPTG 1 mmolL^{-1} (5 −6 h)	The expression level of rEqtIV was lower than in the case of rEqtII or rEqtV, but it was possible to prove the existence by hemolysis of bovine red blood cells
	Equinatoxin V (rEqtV) (Pungerčar et al., 1997)				M-9/LBA, 37 °C	IPTG 0.4 mmolL^{-1} (5 −6 h)	The expression was similar as described by Anderluh et al. (1996)
Sagartia rosea	**SrcI hemolytic toxin (rSrcI-Trx-6His)** (Jiang et al., 2003b)	Production of cDNA by RT-PCR and PCR from mRNA of sea anemone tentacles.	pTRX	*BL21(DE3)*	LBA, 37 °C	IPTG 0.1 mmolL^{-1} (4 h)	cDNA was placed in-frame, downstream of the thioredoxin containing GSGSG, 6His-tag, and enterokinase recognition sequences into the vector pTRX constructed in the laboratory (Yang et al., 2003). The great majority of Trx-SrcI was expressed as inclusion bodies.
Actineria villosa	**AvtI hemolytic toxin (rAvtI)** (Uechi et al., 2005)	Production of cDNA by RT-PCR and PCR-RACE from mRNA of sea anemone.	pETDuet1	*BL21(DE3)*	M-9Y, 37 °C	IPTG 1 mmolL^{-1} (4 h)	A large amount of recombinant protein was expressed as inclusion bodies. A 6His tag at the C-terminus protein was expressed almost exclusively as inclusion body and was not functional.
Stichodactyla helianthus	**Sticholysin I (rStI)** (Pazos et al., 2006)	Production of cDNA by RT-PCR and PCR from mRNA of sea anemone body.	pET3a	*BL21(DE3) pLysS*	LBAC, 37 °C	IPTG 0.1 mmolL^{-1} (4 h)	The recombinant protein was expressed soluble as well as inclusion bodies.
Actinia fragacea	**Fragaceatoxin C (rFraC)** (Bellomio et al., 2009)	Production of cDNA by RT-PCR and PCR from mRNA of a single specimen of anemone.	pBAT-4	*BL21(DE3)*	LBA, 37 °C	IPTG 1 mmolL^{-1} (5 −6 h)	Ser 1 was replaced by Met1 for preventing to the formation of mRNA secondary structures.
Heteractis crispa (*Radianthus macrodactylus*)	**Heteractis crispa hemolytic toxin S family (rHct-S5 and rHct-S6)** (Tkacheva et al., 2011)	Production of cDNA by RT-PCR and PCR from mRNA of sea anemone tentacles.	pET41a (+)	*Rosetta(DE3)*	2xYT-KC, 30 °C	IPTG 0.1 mmolL^{-1} (3 h)	Recombinant proteins were expressed in the form of hybrid proteins containing the GST protein, 6His-tag, and mature actinoporin separated with the enterokinase restriction site.

cDNA: complementary deoxyribonucleic acid (DNA); **mRNA:** messenger ribonucleic acid; **PCR:** polymerase chain reaction; **RT-PCR:** reverse transcription-PCR; **PCR-RACE:** PCR-rapid amplification of cDNA end; **M-9**: minimal broth #9; **M-9Y**: M-9 broth supplemented with 5 gL^{-1} yeast extract; **LB**: Luria Bertari broth; with 100 μg mL^{-1}of ampicillin (**LBA**) and with 34 μg mL^{-1} of chloramphenicol additionally (**LBAC**); **2xYT-KC**: 2xYT broth with 50 μg mL^{-1} of kanamycin and 34 μg mL^{-1} of chloramphenicol; **IPTG**: isopropyl-1thiol-βD-galactopyranoside; **6His:** six-His tag; **TolAIII**: solubility-enhancing tag third domain of the periplasmic protein TolA from *E. coli*; **Trx**: solubility-enhancing tag from thioredoxin. **GST:** glutathione S-transferase tag.

(*cat* gene) enzyme (Russell and Chopra, 1990; Garrett et al., 2000). All these enzymes are continuously secreted outside of the cells and the antibiotics could be depleted in a few hours, allowing the increase of antibiotic non-resistent cells during cultivation (Rosano and Ceccarelli, 2014). Tetracycline is a highly stable antibiotic, probably because its resistance is based on an active efflux of the

Table 2
Strategies for heterologous production of actinoporins by phage-T5 and *E. coli* RNA polymerases expression systems.

Promotor expression systems	Anemones	Recombinant isoforms	Genetic procedures for cloning isoforms	Expression plasmids	*E. coli* expression strains	Expression broths	Induction	Observations
Phage-T5 RNA polymerase	*Heteractis magnifica*	**Magnificalysins III (rHMgIII-6His)** (Wang et al., 2000)	Production of cDNA by RT-PCR and PCR-RACE from mRNA of sea anemone tentacles.	pQE30	*M15 pREP4*	LBAK, 37 °C	IPTG 0.1 mmolL^{-1} (3–4 h)	Thrombin cleavage site was inserted between the 6His-tag and HMgIII sequence for selective removal of the tag. The HMgIII was also expressed as two forms of GST fusion proteins with very high levels of expression, but predominantly as inclusion bodies and without hemolytic activity.
		Magnificalysins (rHMgs-6His: rHMg2F′, rHMgs D3 to **D7** and **rHMgs E1** to **E4)** (Wang et al., 2000, 2008)	Production of cDNA by RT-PCR from mRNA and PCR from genomic DNA of sea anemone body and tentacles cells.					
	Stichodactyla helianthus	**Sticholysin I (rStI)** (De los Ríos et al., 2000; Alegre-Cebollada et al., 2007a)	Production of cDNA by RT-PCR and PCR-RACE from mRNA of body sea anemone.	pQE60	*RB791*	LBA, 37 °C	IPTG 1 mmolL^{-1} (4 h)	Overproduction of both proteins was only possible after introducing silent mutations within the 5′-end of their original cDNA by PCR amplification. These mutations would prevent the formation of RNA secondary structures blocking the ribosome-binding site and the initiation codon.
		Sticholysin II (rStII) (Alegre-Cebollada et al., 2007a)	Production of DNA by PCR from **rStII-6His**.					
		Sticholysin I (rStI-6His) (Alegre-Cebollada et al., 2007a)	Production of DNA by PCR from **rStI**.	pQE30			IPTG 1–2 mmol L^{-1} (4 h)	The endopeptidase restriction site was not used for eliminate the 6His tag.
		Sticholysin II (rStII-6His) (De los Ríos et al., 2000; Alegre-Cebollada et al., 2007a)	Production of cDNA by RT-PCR and PCR-RACE from mRNA of sea anemone body.					
***E.coli* RNA polymerase**	*Sagartia rosea*	**SrcI hemolytic toxin (rSrcI)** (Jiang et al., 2002, 2003a)	Screening of cDNA library from sea anemone tentacles and production of cDNA by RT-PCR and PCR.	pBV220	*BL21(DE3)*	LBA, 30 °C	heat 42 °C (4 h s.)	Induction by heat, the plasmid contains P_RP_L tandem promoters of λ bacteriophage.

cDNA: complementary deoxyribonucleic acid; **mRNA:** messenger ribonucleic acid; **PCR:** polymerase chain reaction; **RT-PCR:** reverse transcription-PCR; **PCR-RACE:** PCR-rapid amplification of cDNA end; **LB**: Luria Bertari broth; with 100 μg mL^{-1} of ampicillin (**LBA**) and with 25 μg mL^{-1} of kanamycin additionally (**LBAK**); **IPTG**: isopropyl-1thiol-βD-galactopyranoside; **6His:** six-His tag. **HMg2F′**and **HMgs D3 to D7**: protein sequences of isoforms D3 to D7 obtained from DNA by using *DyNazyme^{TM} II DNA polymerase*; **HMgs E1 to E4**: protein sequences of isoforms E1 to E4 obtained from DNA by using *Expand^{TM}high-fidelity PCR system*.

antibiotic outside of the cell (Rosano and Ceccarelli, 2014).

Most of the regulable promoters of *E. coli* include those derived from gram-positive bacteria and bacteriophages and are located approximately 10–100 bp upstream of the RBS (Fig. 1). The promoters are under the control of a regulatory gene (R) (Fig. 1), which may be present on the vector itself or integrated in the host chromosome and acts on a promoter control region (operator) (Makrides, 1996). The regulated promoters most commonly used in *E. coli* are those derived from lactose operator (*lac*), tryptophan operator (*trp*), combination of both operators (*tac*), *recA* operator, or leftward operator (λ*PL*) from bacteriophage λ (Slater and Williams, 2002).

The actinoporin genes have been cloned into different expression plasmids in order to produce these proteins in bacteria. The plasmids used for expression of recombinant actinoporins in *E. coli* can be classified in three systems considering the different promoter/operator combinations: (i) phage T7 promoter with *lac* operator system, (ii) phage T5 promoter with *lac* operator system, and (iii) promoter of *E. coli* RNA polymerase with *lac* operator system. Below we analyze and discuss each one of the actinoporin

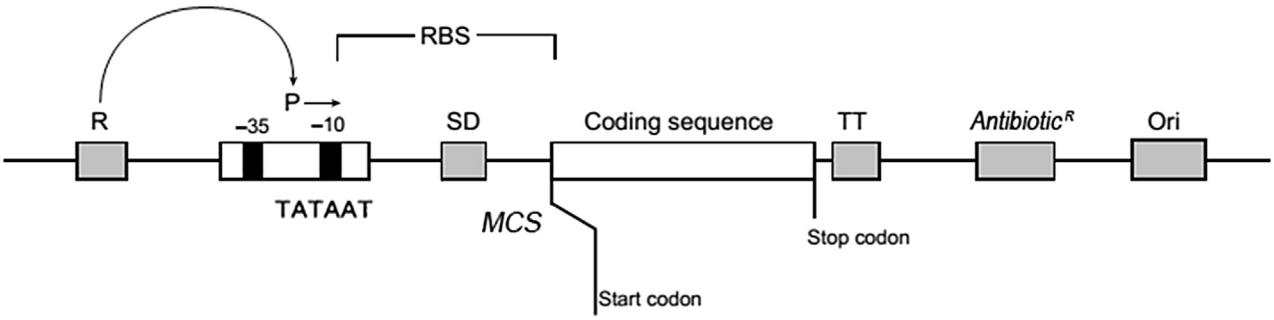

Fig. 1. Schematic representation of the sequence elements of a prokaryotic expression vector. Schematic representation of the features and sequence elements of a prokaryotic expression vector: The repressor protein is encoded by a regulatory gene (R), which modulates the activity of the promoter. The promoter (P) consisting of the −35 and −10 sequences, which are separated by a 17-base spacer. The straight arrow indicates the direction of transcription. The ribosome-binding site (RBS) consists of the *Shine Dalgarno* (SD) sequence that interacts with the 3′ end of the 16S rRNA during translational initiation. The start and stop codons positions and coding sequence are shown. The transcription terminator (TT) serves to stabilize the mRNA and the vector. An antibiotic resistance gene (*antibiotic*^{R}) facilitates phenotypic selection of the vector. Replication origin (Ori) determines the vector copy-number. Figure was modified from Hannig and Makrides (1998).

expression strategies used by different research groups.

2.2. Heterologous expression of actinoporins in E. coli by the phage T7 system

The phage-T7 expression system is one of the most widely used for membrane proteins or target genes that are toxic to the cells (Studier, 1991, 2005). This expression system has five advantages: (i) the phage-T7 RNA polymerase consists of a single subunit (Schumann and Ferreira, 2004); (ii) the phage-T7 RNA polymerase elongates chains about five times faster than the *E. coli* RNA polymerase ("high processing") (Schumann and Ferreira, 2004; Sorensen and Mortensen, 2005); (iii) the use of two polymerases that recognize different promoters can be employed for selective expression (Studier, 1991, 2005); (iv) the basal expression levels can be controlled by a phage-T7 RNA polymerase inhibitor (Sorensen and Mortensen, 2005) and, (v) the phage-T7 RNA polymerase is not affected by rifampicin, which binds to bacterial RNA-polymerase preventing the transcription. Adding this antibiotic about 10 min after induction of the gene coding for the T7 RNA polymerase enhance the amount of recombinant protein (Schumann and Ferreira, 2004).

Table 1 summarizes the procedures for heterologous expression in *E. coli* of recombinant actinoporins by RNA-polymerase promoter of phage-T7 with lac operator system. Ten actinoporins variants were expressed by this system: Equinatoxin II (rEqtII), Equinatoxin II six-His tag (6His) fusion protein (rEqtII-TolAIII-6His), Equinatoxin IV (rEqtIV) and Equinatoxin V (rEqtV) from *Actinia equina* (Anderluh et al., 1996, 2003 and 1999b; Pungerčar et al., 1997), SrcI fusion protein (rSrcI-Trx-6His) from *Sagartia rosea* (Jiang et al., 2003b), AvtI toxin (rAvtI) from *Actineria villosa* (Uechi et al., 2005), Sticholysin I (rStI) from *Stichodactyla helianthus* (Pazos et al., 2006), Fragaceatoxin C (rFraC) from *Actinia fragacea* (Bellomio et al., 2009) and hemolytic S-family toxins (rHct-S5 and rHct-S6) from *Heteractis crispa* (also called *Radianthus macrodactylus*) (Tkacheva et al., 2011).

The plasmids carrying phage-T7 promoter used for the actinoporins heterologous expressions are diverses (Table 1) and include: *pT7-7* and *pET8c* for expression of equinatoxins (rEqts); *pET3a* for rStI; *pETDuet1* for rAvtI; *pBAT-4* for rFraC, *pTRX* for rSrcI, and *pET41a* for S-family toxins from *H. crispa* (rHct-Ss) (Table 1). For the expression systems based on the phage-T7 RNA polymerase, the use of *E. coli (DE3)* strains is necessary. These strains have a bacteriophage lambda-lysogen (λDE3) integrated into the *int* chromosome gene inactivating it (Fig. 2). Disruption of the *int* gene prevents excision of the phage (i.e. lysis) in the absence of a helper phage (Daegelen et al., 2009). The λDE3 gene encodes for the phage-T7 RNA polymerase (MW = 98.854 kDa) under control of the *lacUV5*-promoter in the *E. coli* chromosome, which carries also a *lac I* gene with its *E. coli* native promoter (Fig. 2) (Studier, 1991, 2005). Many commercial vectors contain the full-length *lac* control region (*lac* operator) (Slater and Williams, 2002), which consists of a promoter/operator region preceded by the *lac I* regulatory gene that encodes the Lac I repressor protein (Fig. 3). Many promoter mutants have been identified from the sequence of the wild-type *lac* promoter, one of them was named *lacUV5* (Grossman et al., 1998; Pan and Malcolm, 2000). This promoter contains three nucleotidic point mutations that distinguish it from the wild-type *lac* promoter (Fig. 3) (Schumann and Ferreira, 2004; Terpe, 2006). Two point mutations are in the −10 region, which increases 2.5-fold the promoter strength, and a third point mutation is present at −66 region within the CAP (catabolite gene activator protein) binding site (Fig. 3). The three mutations allow stronger induction of the expression (Terpe, 2006).

The Lac I repressor protein is responsible for maintaining a negative control over the *lacUV5* and *T7-lac* promoters, avoiding the phage T7 RNA polymerase and target gene expression (Fig. 2) (Studier et al., 1990; Dubendorff and Studier, 1991). Since there are around 10–20 molecules of Lac I per each *E. coli* cell it is not enough to control the expression of proteins encoded in plasmids of high copy number. Hence, it is recommended to use host strains carrying the *lac I*^{q} mutant gene in order to achieve a more efficient repression (Studier et al., 1990; Dubendorff and Studier, 1991). The *lac I*^{q} mutant has a 15 bp deletion in the *lac I* promoter that replaces the native −35 hexamer by the consensus sequence for σ70-dependent promoters. This mutation leads to an increase in the strength of the *lac I* promoter and therefore in the number of Lac I molecules per cell (around ten-fold) (Calos, 1978). For plasmids of very high copy number, the *lac I* or *lac I*^{q} genes are typically cloned into the expression plasmid or provided in *trans* on a compatible plasmid (Schumann and Ferreira, 2004).

In the absence of inducer, the Lac I repressor binds to *lac* O_1 operator site, located immediately downstream of the promoter (P) (Figs. 2 and 3A). If full-length *lac* promoter/operator is present, a stable DNA loop structure is folded from the binding of Lac I repressor to O_1 site and from the interaction with one of the two auxiliary operator sites O_2 (downstream of coding region) or O_3 (upstream of CAP binding site) (Fig. 3A) (Saïda, 2007). The homotetramer of Lac I protein is formed during the interaction with DNA and it prevents the target-gene transcription by the RNA polymerase (Fig. 3A) (Schumann and Ferreira, 2004). The addition of a substance that avoids the binding of the Lac I repressor to the *lac*

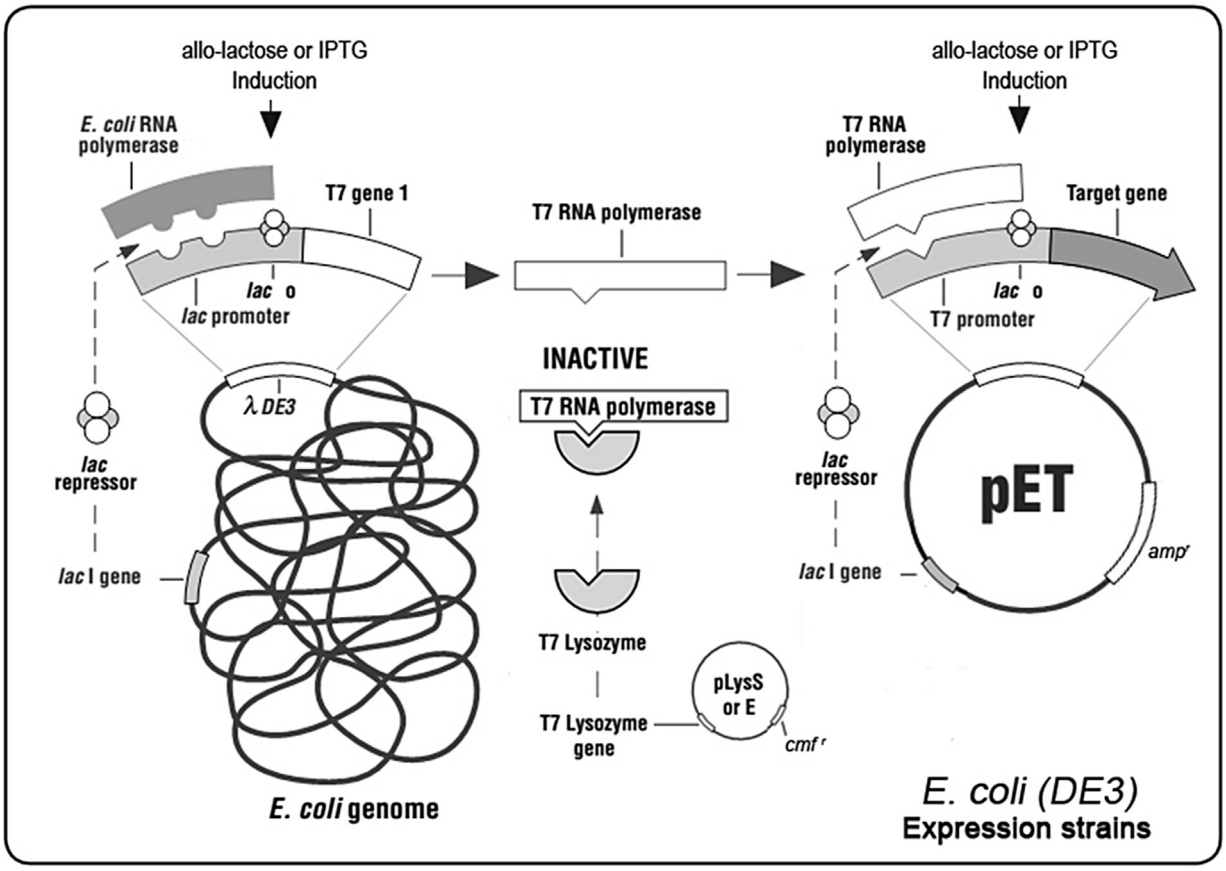

Fig. 2. Schematic representation of the genetic and protein regulatory elements in the phage-T7 RNA polymerase expression systems. *E. coli(DE3)* strains have a bacteriophage lambda-lysogen (λ**DE3**) integrated into the chromosome, coding for the phage-T7 RNA polymerase (**T7 gen 1**) under control of the *lacUV5*-promoter. These strains also carry a ***lac I* gene** in their chromosome. The **lac repressor** protein binds to operator (**lac O**) and prevents the *E. coli* or T7 RNA polymerase activities. Allo-lactose or IPTG binds to the lac I repressor and inactivates it, and then the **T7 RNA polymerase** can transcribe the **target-gene**. T7 lysozyme binds to T7 RNA polymerase as a natural inhibitor of transcription and the basal expression of target genes is repressed. The T7 lysozyme is provided to the strains by *pLysS* and *pLysE* plasmids that offer resistance to chloramphenicol (*cmf* ^{r}). Figure was modified from Novagen pET Manual 10th edition (www.novagen.com).

operator sites can induce the target protein expression.

Lactose induces expression in *lac* system after its conversion to allo-lactose by beta-galactosidase (*lacZ* gene). The allo-lactose or the non-hydrolysable structural analog of lactose, isopropyl-β-D-1-thiolgalactopyranoside (IPTG), binds to the Lac I repressor and changes its conformation in a way that it is no longer able to interact with *lac* operator sites (Figs. 2 and 3B). Once the Lac I repressor is inactivated the RNA polymerase can transcribe the sequences downstream of the promoter and the transcripts produced are translated into the recombinant protein (Figs. 2 and 3B) (Terpe, 2006). All plasmids used for actinoporin expression by phage-T7 promoter with *lac* operator system were inducible with 0.1–2.0 mmolL^{-1} of IPTG (Tables 1 and 2) or 0.2 mol L^{-1} of lactose in bacterial culture (Valle et al., 2011).

The full-length *lac* promoter has also a positive control known as catabolite repression, which acts in stationary phase of the cultures when lactose and glucose are present. Expression from *lac* promoter is not completely induced until glucose is depleted and adenosine 3′, 5′-cyclic monophosphate (cAMP) is produced (Wanner et al., 1978; Postma and Lengeler, 1985; Pan and Malcolm, 2000; Rosano and Ceccarelli, 2014). The cAMP and CAP complex binds to the −66 region and allows the recognition of promoter by RNA polymerase (Fig. 3B) (Schumann and Ferreira, 2004). Additionally, glucose abolishes lactose uptake by inactivation of lactose permease (Winkler and Wilson, 1967; Rosano and Ceccarelli, 2014). To achieve the protein expression in presence of glucose the lacUV5 promoter is used in comercial plasmids (Silverstone et al., 1970; Lanzer and Bujard, 1988; Rosano and Ceccarelli, 2014). The point mutation of lacUV5 promoter, within the CAP binding site, makes it less liable to catabolite repression by glucose because it decreases its dependence to the cAMP-CAP complex (Fig. 3B). For this reason, the T7 RNA polymerase production can be induced with IPTG even in the presence of glucose (Grossman et al., 1998; Pan and Malcolm, 2000; Schumann and Ferreira, 2004; Terpe, 2006).

E. coli BL21(DE3) and *BLR(DE3)* derivatives strains are defficient in *lon* and *omp-t* proteases, which is convenient for the stability of target proteins (Phillips et al., 1984; Studier, 1991). These strains have been used for expression of differents recombinant actinoporins (Tables 1 and 2). The *BLR* strains are *recA*$^{-}$ derivative of *BL21* that improves plasmid monomer yields and may help stabilize target plasmids. The *recA* mutation prevents recombination of introduced DNA with host DNA, which avoids the loss of the *DE3* prophage by recombination, increasing stability of inserts (Stewart et al., 1998). The *BLR* strains have been used for rEqtII, rEqtIV and rEqtV expressions (Anderluh et al., 1996 and 1999b; Pungerčar et al., 1997). The *E. coli BL21(DE3)* cells and their derivatives have been especially designed for the overexpression in T7 system of membrane proteins (Miroux and Walker, 1996) and have been used for expressions of the most actinoporin isoforms (Tables 1 and 2).

The *E. coli (DE3)* strains always show some basal level expression of phage T7 RNA polymerase because Lac I repressor is in reversible binding equilibrium with the *lac* operator region (Fig. 2). Since the

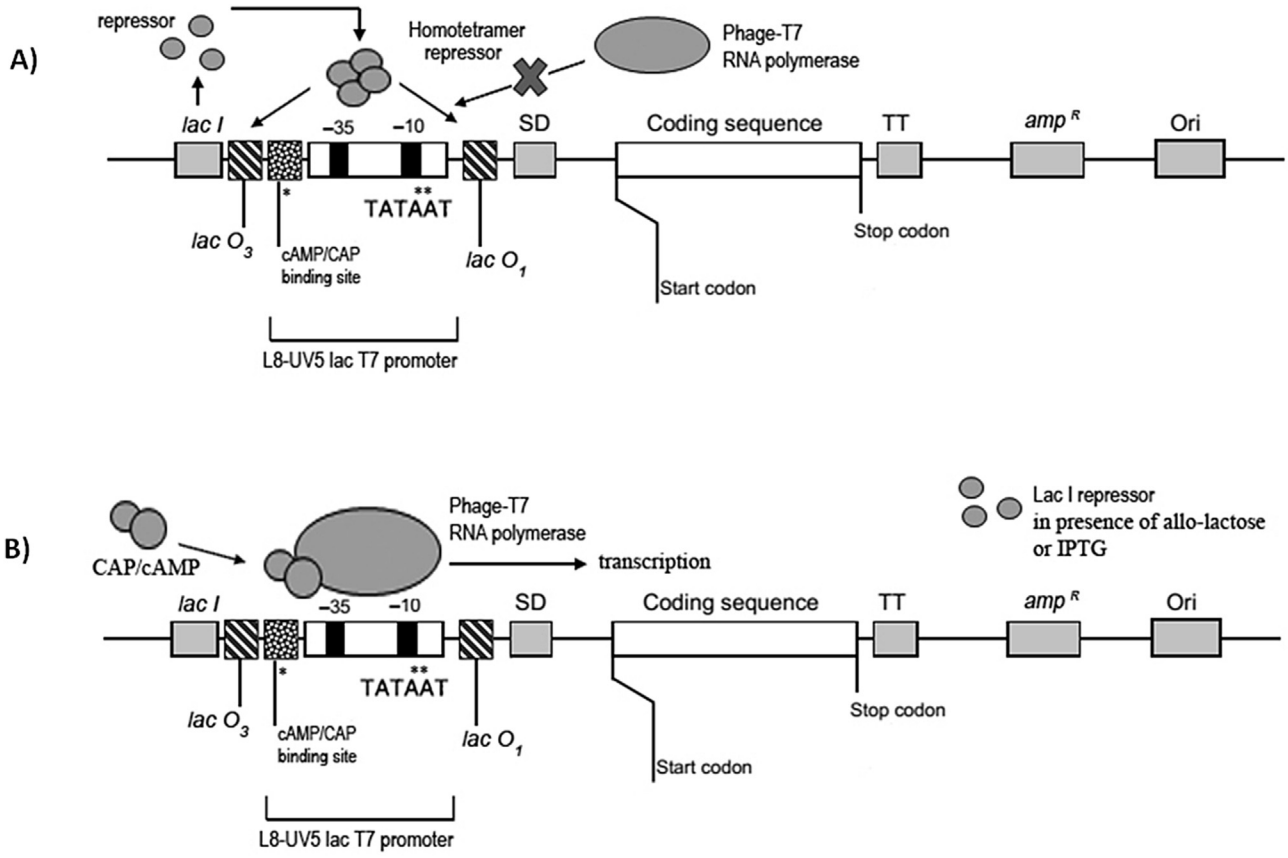

Fig. 3. Schematic representation of the genetic sequences and protein regulatory elements of a phage-T7 expression vector. **(A)** Negative-control over the lacUV5 promoter: the Lac I repressor protein is encoded by a ***lac I*** regulatory gene and is responsible for preventing the phage-T7 RNA polymerase activity by binding to O_3–O_1 **operators**. **(B)** Positive-control over the lacUV5 promoter: Allo-lactose or IPTG bind to the Lac I repressor inactivating, and the RNA polymerase can transcribe the sequences downstream from the promoter. The **cAMP** with catabolite gene activator protein (**CAP**) forms a complex that binds to the −66 region and induces promoter recognition by the RNA polymerase. (*) One point mutation is present at −66 region within the CAP binding site that generates a promoter less sensitive to glucose since decreases its dependence to cAMP-CAP complex. (**) Two point mutations in the −10 region which increases 2.5-fold the promoter strength. The three mutations allow a strong induction of the gene expression. **SD**: *Shine Dalgarno* sequence, **start** and **stop codons** positions, **coding sequence**, **TT**: transcription terminator, ***amp*^{R}**: ampicillin resistance gene, **Ori**: origin of replication, are shown. Figure was modified from Hannig and Makrides (1998) and Novy and Morri (2001).

reversible binding equilibrium, the high processivity of the T7 RNA polymerase, and the presence of multiple copies of the plasmid encoding to target gene in each cell, the target-toxic genes cloned downstream to T7 promoter also could show basal expression. The basal expression of toxin target gene may lead to reduced growth rates, cell death, or plasmid instability (Doherty et al., 1993; Dong et al., 1995; Rosano and Ceccarelli, 2014). Additionally, the expression of genes cloned in vectors from the pET series show a partial control under repressive conditions due to using only O1 operator site to control the very strong T7 promoter (Saïda, 2007).

One of the best ways to control the basal expression of target protein has been to introduce phage T7 lysozyme into the system. Phage T7 lysozyme is a bifunctional protein: cuts a specific bond in the peptidoglycan layer of the *E. coli* cell wall and binds to T7 RNA polymerase by a 1:1 protein-protein complex as a natural inhibitor of transcription (Zhang and Studier, 1997; Samuelson, 2011; Terpe, 2006). Therefore, the basal expression of target genes under the control of the T7 promoter is repressed by phage T7 lysozyme presence (Moffatt and Studier, 1987; Studier, 1991; Zhang and Studier, 1997; Huang et al., 1999; Terpe, 2006). *E. coli (DE3)pLysS* and *E. coli (DE3)pLysE* are strains carrying a plasmid (*pLys*) derived from *pACYC184* that provides resistance to chloramphenicol and encodes T7 lysozyme (Fig. 2) (Chang and Cohen, 1978). The *pLys* plasmids have a *p15A* replicon compatible with plasmid carry out *ColE1* or *pMB1* origins (e.g. *pUC*, *pBR322* or derived) (Baneyx, 1999).

The T7 lysozyme is provided to the *E. coli (DE3)* strains from a clone of the phage T7 lysozyme gene cloned in the *Bam*HI site of the *pACYC184* plasmid (Chang and Cohen, 1978) with a φ3.8-promoter for phage T7 RNA polymerase immediately following the lysozyme gene (Dunn and Studier, 1983). When this fragment is oriented so that the lysozyme gene is expressed from the *tet* promoter of *pACYC184* (*pLysE* plasmid) substantial levels of lysozyme are accumulated in these cells. However, when the fragment is orientated in opposite sense so that the lysozyme gene is expressed under control of the φ3.8-promoter by phage T7 RNA polymerase (*pLysS* plasmid) the cells accumulate much lower levels of lysozyme with respect to *pLysE*. The *BL21(DE3)* strains with *pLysE* or *pLysS* plasmid have been used for expression of rEqtII-TolAIII-6His (Anderluh et al., 2003) and rStI (Pazos et al., 2006), respectively.

The expression of lysozyme in *pLysS* strains depends on culture conditions. High amounts of lysozyme have been observed in stationary phase cultures, such that levels of the target protein are decreased. This is likely due to the fact that the gene promoter of chloramphenicol acetyl transferase is also sensitive to stimulation by cAMP in the absence of glucose and is upstream of the T7 lysozyme gene in pLysS (Novy and Morri, 2001). The active lysozyme expressed in *pLysS* or *pLysE* strains can be used for lyse cell spontaneously by several freeze-thaw cycles prior to purification, and it could be the reason why growth rates with these strains on occasion diminish (Terpe, 2006). If this feature is not desired, *pLysY*

strains are a good alternative. The *pLysY* plasmid contains a phage T7 lysozyme variant that lacks amidase activity (K128Y mutation) but retains full function in inhibiting T7 RNA polymerase activity (Cheng et al., 1994). The *pLysY* plasmid never has been used for the actinoporin expression, but it could be interesting to consider its use in the future.

In summary, when the increasing of T7 RNA polymerase level (as result of the inductor presence) exceeds the limited inhibitory capacity of T7 lysozyme, the target-gene expression is enhance (Fig. 2). The phage T7 RNA polymerase/lysozyme system has been satisfactorily used for rStI expression in *E. coli BL21(DE3)pLysS* (Pazos et al., 2006) and rEqtII-TolAIII-6His in *E. coli BL21(DE3) pLysE* (Anderluh et al., 2003) (Table 1).

For rAvtI expression has been of *pETDuet1* plasmid (Uechi et al., 2005) which has a T7 promoter with *lac* operator, designed to coexpress two target-proteins in *E. coli*. The *pETDuet-1* plasmid carries the *ColE1* replicon, have a *bla* gene (ampicillin resistance) and each vector carries two expression units controlled by a T7-lac promoter. Each promoter in the expression units is followed by a RBS and MCS region with a T7 terminator (Novy et al., 2002). Coexpression of multiple target genes in *E. coli* is advantageous for studying protein complexes, often achieves optimal yield, solubility, and activity, and may protect individual subunits from degradation (Li et al., 1997; Tan, 2001). Uechi et al. (2005) not used the *pETDuet-1* plasmid for rAvtI expression by coexpression. A large amount of rAvtI was expressed in inclusion bodies by this procedure (Table 1).

The expression of a stretch of amino acids (peptide-tag) or a large polypeptide (fusion partner) in tandem with the target-protein to form a chimeric protein allow easily purify it from the *E. coli* cellular milieu (Nilsson et al., 1997; Rosano and Ceccarelli, 2014). Since simple purification schemes have been described for proteins fused at either end to tags or fusion partner which bind affinity resins, several recombinant actinoporins have been expressed by this procedure (Table 1). A rEqtII-TolAIII-6His actinoporin variant with a His-tag and a solubility-enhancing fusion partner-tag (third domain of the periplasmic protein TolA of *E. coli*) was expressed by T7 system with *pET8c* plasmid (Anderluh et al., 2003) (Table 1). Although, rEqtII-TolAIII-6His was expressed in the soluble fraction, the highest amount was obtained in inclusion bodies (Anderluhet al., 2003) (Table 1). A rSrcI-Trx-6His actinoporin variant with a solubility enhancing Trx fusion partner-tag (thioredoxin protein, LaVallie et al., 1993) and a 6His-tag was expressed by T7 system with *pTRX* plasmid, but the majority of target protein was expressed in inclusion bodies (Jiang et al., 2003b) (Table 1). Also, a 6His-tag rAvtI actinoporin variant at the C-terminus was expressed almost exclusively as inclusion body and no activity was observed (Uechi et al., 2005). rHct-S5 and rHct-S6 actinoporins were expressed in the form of hybrid proteins containing the solubility enhancing GST (glutathione S-transferase) fusion partner (Smith and Johnson, 1988) and 6His-tag (Tkacheva et al., 2011) (Table 1). Despite the repeated efforts to actinoporins expressed with solubility enhancing fusion partner have been not possible avoid the inclusion bodies formation.

The difference between the codon usage in prokariotic (*E. coli*) and eukariotic cells could be a problem during translation in the heterologous expression, because some tRNAs may be rare or lacking in the host cell (Kane, 1995; Goldman et al., 1995; Terpe, 2006). It is well-known that amino acids are encoded by more than one codon, and each organism carries its own bias in the usage of the 61 available amino acid codons (Dong et al., 1996; Terpe, 2006). Insufficient tRNA pools lead to translational stalling, premature translation stop, translation frame shift, and undesirable amino acid incorporation (Kurland and Gallant, 1996; Terpe, 2006). Many *E. coli* strains such as *Rosetta* were engineered to deal with this issue (Terpe, 2006). The *Rosetta* strains are *BL21* derivative and were designed to enhance the expression of eukaryotic proteins by the introduction of tRNAs for the rare codons AUA, AGG, AGA, CUA, CCC, and GGA on the compatible chloramphenicol-resistant plasmid *pRARE* (Novy et al., 2001; Rosano and Ceccarelli, 2014). The *E. coli Rosetta(DE3)* strain was satisfactorily used for rHct-Ss protein expression (Table 1) (Tkacheva et al., 2011).

2.3. Heterologous expression by phage T5 RNA polymerase systems

Table 2 summarizes the procedures for heterologous expression in *E. coli* of recombinant actinoporins by phage T5 RNA polymerase promoter with *lac* operator system. More than seven actinoporin isoforms were expressed by this system (Table 2). For the expression of 6His recombinant actinoporins-tagged (rHMgIII-6His and sticholysins-6His (Sts-6His)) the plasmid employed was *pQE30* (Table 2) (Wang et al., 2000, 2008; De los Ríos et al., 2000; Alegre-Cebollada et al., 2007a).

In contrast to T7 system, T5 promoter is recognized by *E. coli* RNA polymerase (Rosano and Ceccarelli, 2014) and can be used with any *E. coli* strain (Fig. 4A). The extremely high constitutive transcription rate of phage-T5 promoter can be controlled and repressed only by high levels of the Lac I protein (Rosano and Ceccarelli, 2014). The *pQE30* and *pQE60* expression vectors (*QIAexpress System*) contain an optimized promoter-operator element, consisting of a phage T5 promoter and two-lac operator sequences that increases the binding of Lac I to ensure efficient repression of T5 promoter (Rosano and Ceccarelli, 2014) (Fig. 4B).

E. coli host strains employed in the *QIAexpressSystem* use a *lac* repressor gene in *trans* or *cis* to the target-gene (Fig. 4). In the *trans* control system, the host strains are transformed with *pREP4* plasmid (low copy number) which confers kanamycin resistance and constitutively expresses the Lac I repressor protein (Fig. 4B) (Farabaugh, 1978). The *pREP4* plasmid is derived from *pACYC* and contains the p15A replicon, compatible with ColE1 carrying plasmids (Baneyx, 1999). Multiple copies of *pREP4* are present in the host strains (e.g., *M15pREP4 E. coli* strain) to ensure the high level production of Lac I repressor and a tightly regulation of recombinant protein expression (Fig. 4). This control system was used during Magnificalysins expression (Table 3) (Wang et al., 2000 and Wang et al., 2008). The production of recombinant proteins encoded in *pQE* vectors can be rapidly induced by the addition of IPTG (Fig. 4). Once the Lac repressor is inactivated by IPTG, the host RNA polymerase can transcribe the sequences downstream of the promoter to synthesize the recombinant protein (Fig. 4).

2.4. Heterologous expression by E. coli RNA polymerase systems

Another approach for heterologous expression of actinoporins was the cloning of rSrcI gene under control of the regulated phage promoter P_LP_R in the plasmid pBV220 (Table 2) (Jiang et al., 2002, 2003b). This system uses the *E. coli* RNA polymerase promoter with the bacteriophage λ repressor cI which prevents expression of all lytic genes by interacting with two operators termed O_L and O_R (Fig. 5) (Terpe, 2006; Villaverde et al., 1993; Menart et al., 2003; Dodd et al., 2005). When the host SOS response is triggered by DNA damage, the expression of the protein RecA is stimulated and catalyzes the cleavage of λcI repressor, allowing transcription of P_L-controlled genes (Johnson et al., 1981; Galkin et al., 2009; Rosano and Ceccarelli, 2014). λcI857, a λcI mutant, is unstable at temperatures higher than 37 °C. The λcI857 gene can be introduced in *E. coli* host cells by two ways: as a portion of the λ genome (λ prophage) integrated in the *E. coli* chromosome (Fig. 6) and does not provide the lytic components, or included into a plasmid. This modified host strains are first grown at 28–30 °C until desired

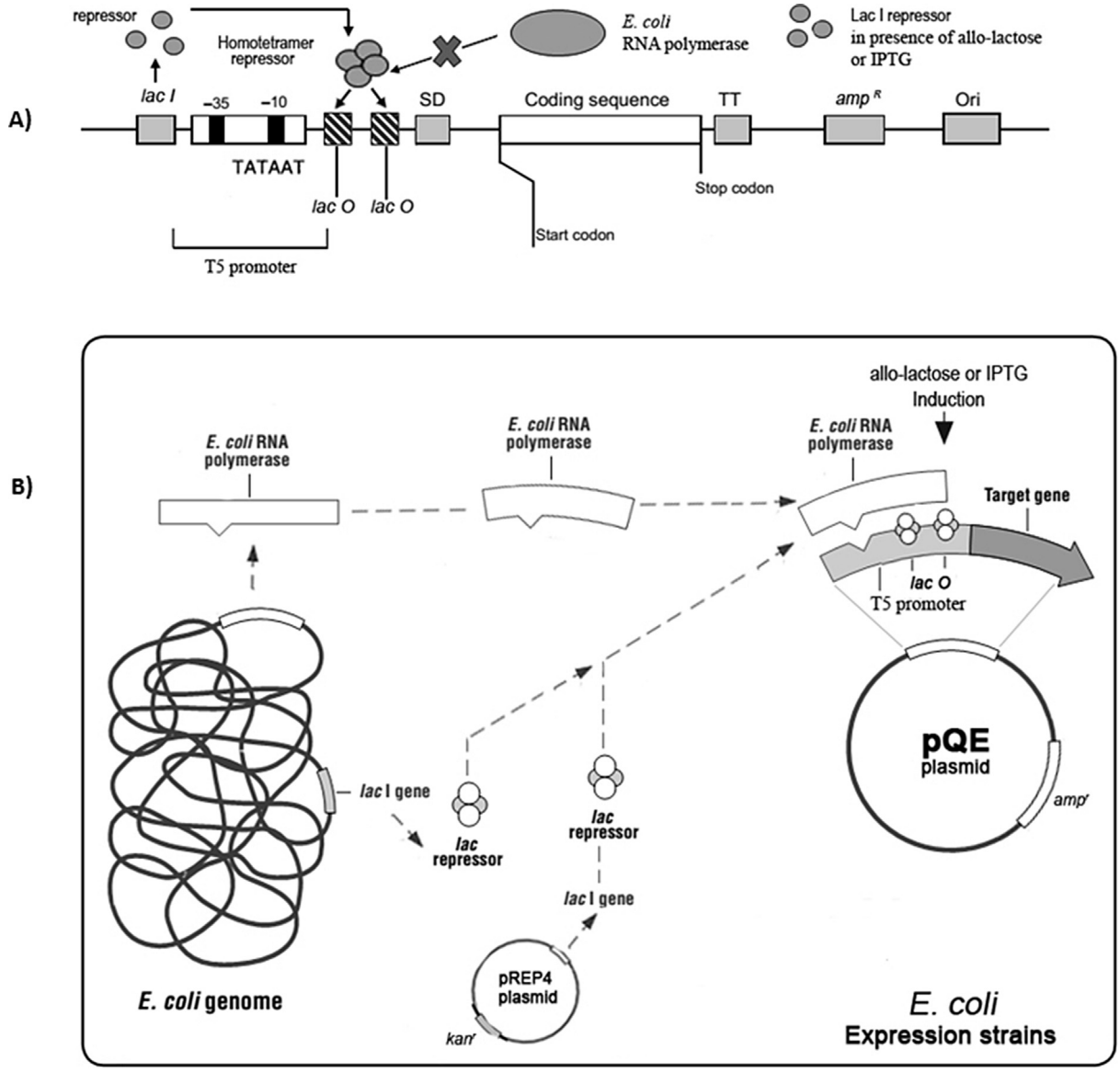

Fig. 4. Schematic representation of the regulatory elements of phage-T5 expression system. **(A)** Negative-control over the T5 promoter: the Lac I repressor proteins are encoded by the ***lac I*** regulatory gene and are responsible for preventing the *E. coli* RNA polymerase activity by binding to O operators (***lac O***). **SD**: *Shine Dalgarno* sequence, **start** and **stop codons** positions, **coding sequence**, **TT**: transcription terminator, ***amp*^{R}**: ampicillin resistance gene, **Ori**: replication origin, are shown. **(B)** Genetic and protein regulatory elements in the phage-T5 expression systems. ***E. coli* RNA polymerase** can transcribe the **target genes** downstream from the **T5 promoter**, but the **lac I repressor** proteins bind operators (**lac O**) and prevent polymerase activity. Once the lac I repressor is inactivated by bind to allo-lactose or IPTG the polymerase can transcribe. In the *trans* negative-control the host strains are transformed with the low-copy plasmid ***pREP4*** which confers kanamycin resistance (*kan* ^{r}) and constitutively produces the Lac I repressor proteins. Figures were adapted following the same sketch of Fig. 2 and 3.

density, and protein expression is induced by temperature shift to 40–42 °C (Menart et al., 2003; Valdez-Cruz et al., 2010; Rosano and Ceccarelli, 2014).

The main advantage of the P_L promoter is to exhibits an extremely low basal expression in host strains carrying a chromosomal copy of the phage cI and a high level expression when is induced (Villaverde et al., 1993; Menart et al., 2003). Besides, induction by heat shift is a low cost method. However, the $\lambda P_L/P_R$-cI system requires specific host strains and uses relatively low copy number vector to maintain a tight regulation (Terpe, 2006).

The protein expressions in thermo-regulated $\lambda P_L/P_R$-cI system is based on the insertion of the target-gene into a vector containing the leftward (P_L) and rightward (P_R) promoters, a ribosome recognition and a translation initiation sites (Valdez-Cruz et al., 2010) (Fig. 5A). The O_L and O_R operators overlap with the two strong P_L and P_R promoters while cI repressor proteins bind to O_L and O_R operators to prevent the RNA polymerase activity (Fig. 5A). The temperature shift-up induction is performed in bacterial strains carrying the temperature-sensitive cI857 repressor gene or by addition of mitomycin C or nalidixic-acid to strains carrying the wild-type cI repressor (Villaverde et al., 1993) (Fig. 5B). Nalidixic acid inhibits the DNA gyrase leading to DNA damage and induces RecA protease production by SOS response. The wild-type cI repressor could be cleaved by this protease (Johnson et al., 1981; Galkin et al., 2009). The main difference between both induction ways is the time needed for product accumulation: 0.8–1.5 h by heat-mediated induction and 5–6 h by nalidixic acid-mediated induction of protein expression. The wild-type cI repressor can also be inactivated by UV-irradiation or by mitomycin C addition (Lewin et al., 1989; Shatzman et al., 2001).

Table 3
Actinoporins purification strategies by non-affinity chromatography systems.

Sea anemones	Isoforms	Procedures and purification conditions			Protein yield mg L^{-1}
		Step 1	Step 2	Purity assessment	
Actinia equina	**Equinatoxin II (rEqtII)** (Anderluh et al., 1996)	Usually, bacterias were harvested, frozen and disrupted by osmotic shock and sonication. **IE** (*CM cellulose*): 0.05 mol L^{-1} NH_4Ac, pH 6.8. Linear gradient elution 0 −0.5 mol L^{-1} NaCl.	**GF** (*Superdex HR75*): 0.05 mol L^{-1} Na_2HPO_4/NaH_2PO_4; 0.1 mol L^{-1} NaCl, pH 6.5.	SDS-PAGE and HPLC-RP (*C-18*)	~1
	Equinatoxin IV (rEqtIV) (Anderluh et al., 1999b)				n.d.
	Equinatoxin V (rEqtV) (Pungerčar et al., 1997)				n.d.
Sagartia rosea	**SrcI hemolytic toxin (rSrcI)** (Jiang et al., 2002, 2003a)	Bacterias were harvested and lysed by sonication. The inclusion bodies were collected and used for the rSrcI renaturation. **IE** (*Q-Sepharose Fast Flow*) 0.02 mol L^{-1} Tris-HCl; 0.02 mol L^{-1} NaCl, pH 8.0. Washing: 0.05 mol L^{-1} Tris-HCl; 0.35 mol L^{-1} NaCl, pH 8.0. Elution: 0.05 mol L^{-1} Tris-HCl; 0.8 mol L^{-1} NaCl, pH 8.0.	**HIC** (*Phenyl-Sepharose 6 Fast Flow*) 0.05 mol L^{-1} Tris-HCl; 2.0 mol L^{-1} $(NH_4)_2SO_4$, pH 8.0. Linear gradient elution: 2–0 mol L^{-1} $(NH_4)_2SO_4$. Desalting in 0.05 mol L^{-1} NH_4HCO_3 by G-25 column	HPLC-RP (*C-18*)	n.d.
Actineria villosa	**AvtI hemolytic toxin (rAvtI)** (Uechi et al., 2005)	**IE** (*CM-Sephadex, TSK-GEL CM-5PW*): 0.01 mol L^{-1} Na_2HPO_4/NaH_2PO_4; pH 6.0. Elution steps: 0–1 mol L^{-1} $NaCl_2$.	**GF** (*Superdex HR75*):0.01 mol L^{-1} Na_2HPO_4/NaH_2PO_4; pH 6.0.	SDS-PAGE	0.8–1.9
Stichodactyla helianthus	**Sticholysin I** (**rStI**) (Pazos et al., 2006)	Bacterias were harvested and lysed by sonication. **IE** (*CM cellulose 52*): 0.02 mol L^{-1} Na_2HPO_4/NaH_2PO_4, pH 7. Linear gradient elution 0 −0.5 mol L^{-1} NaCl.		HPLC-RP (C-4)	n.d.
	Sticholysin I (rStI[a]) (De los Ríos et al., 2000; Alegre-Cebollada et al., 2007a)	Bacterias were harvested and lysed by sonication. **IE** (*CM cellulose 52*) 0.05 mol L^{-1} Tris-HCl; pH 6.8. Linear gradient elution 0–0.3 mol L^{-1} NaCl.		SDS-PAGE	12.8
	Sticholysin II (rStII[a]) (Alegre-Cebollada et al., 2007a)	**IE** (*CM cellulose 52*) 0.05 mol L^{-1} Tris-HCl, pH 7.8. Linear gradient elution 0–0.5 mol L^{-1} NaCl.		SDS-PAGE	5.2
Actinia fragacea	**Fragaceatoxin C (rFraC)** (Bellomio et al., 2009)	Bacterias were harvested, frozen and disrupted by sonication. **IE** (*SP-Sepharose*): milliQ water. Gradient elution 0 −0.7 mol L^{-1} NaCl, 0.05 mol L^{-1} Na_2HPO_4/NaH_2PO_4; pH 7.5.	**GF** (*HiPrep 16/60 Sephacryl S-300 HR*): 0.05 mol L^{-1} Tris-HCl; 0.2 mol L^{-1} NaCl, pH 7.5.	SDS-PAGE	0.5

[a] Overproduction of St I and St II was only possible with silent mutations within the 5′-end of original cDNA sequence for preventing mRNA secondary structure formation that blocks the ribosome-binding site and/or the initiation codon. **IE**: ion exchange chromatography; **GF**: gel filtration chromatography; **CM**: carboxymetyl cation weak exchanger, **SP**: sulfopropyl cation strong exchanger, **HIC**: hydrophobic interaction chromatography; **RP-HPLC**: reverse phase-high performance liquid cromotography; **SDS-PAGE**: sodium dodecylsulphate-polyacrilamide gel electrophoresis; **mg L^{-1}**: mg of pure protein per liter of culture.

The regulation of $\lambda P_L/P_R$-cI857 system allows the gene expression control by changing the growth temperature instead of chemical inducer addition (Terpe, 2006). *E. coli* cells carrying a λ-P_LP_R and cI857-based expression system are grown to middle-exponential phase at temperature below 37 °C where the *c*I857 repressor is functional (28–32 °C) and it turns off expression (Fig. 5A). By increasing the temperature above 37 °C (up to 42 °C), the repressor is inactivated and the gene expression is induced (Love et al., 1996; Valdez-Cruz et al., 2010; Schumann and Ferreira, 2004) (Figs. 5B and 6). Since cI857 repressor becomes completely active above 29 °C and P_L promoter is constitutive (Lowman and Bina, 1990), low temperatures could be employed to produce proteolytically susceptible proteins (Menart et al., 2003).

3. Expression conditions for recombinant actinoporins in *E. coli*

For recombinant actinoporins expression, conventional conditions were used: *Luria-Bertani* (LB) or minimal number 9 (M-9) broths supplemented with appropriate antibiotics, culture temperature of 37 °C and continuous stirring (Tables 1 and 2). LB broth is commonly employed for *E. coli* culturing because is easy to prepare, has rich nutrient contents and its osmolarity is optimal for the early phase of growth (Rosano and Ceccarelli, 2014). Despite being a

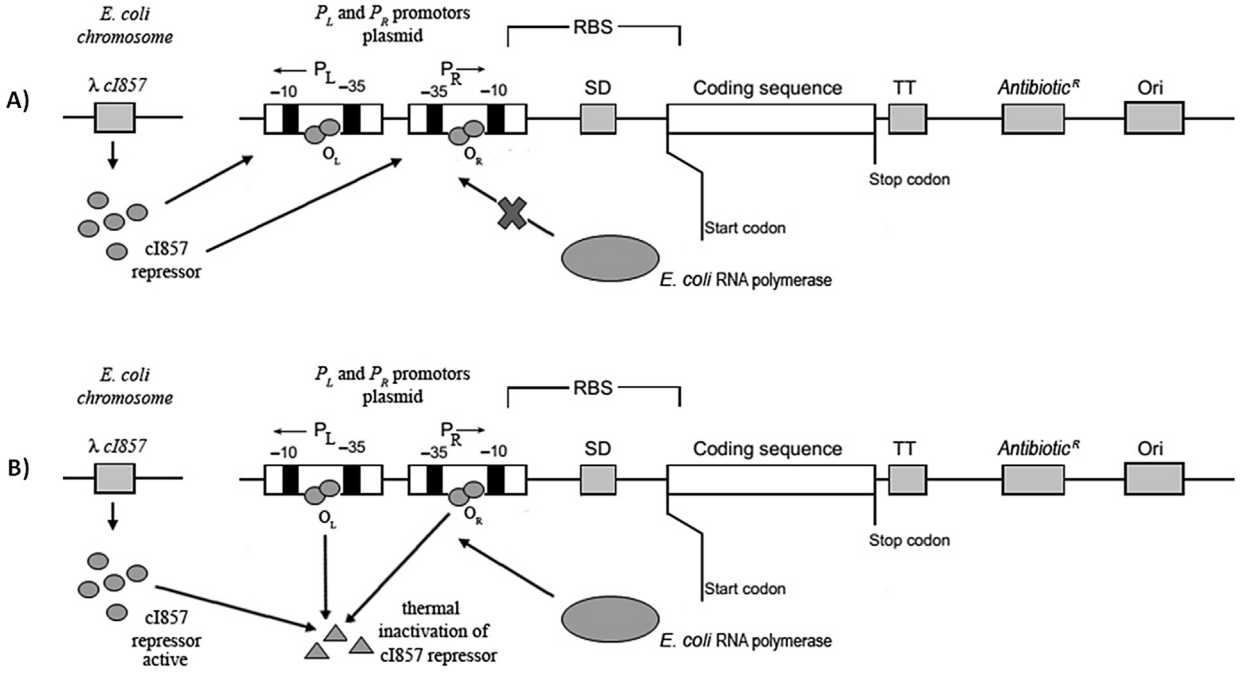

Fig. 5. Genetic and protein regulatory elements in the thermo-regulated λP_L/P_R-cI expression system. **(A)** Negative-control over the P_L and P_R promoters: the thermo-stable **cI857 repressor** proteins are encoded by a λ**cI857** regulatory gene in the *E. coli* chromosome. cI857 repressors proteins bind to O_L and O_R operators and prevent the *E. coli* RNA polymerase activity. **(B)** The induction is performance by temperature shift-up which inactive the cI857 repressors and the *E. coli* polymerase can transcribe. **SD**: *Shine Dalgarno* sequence, **start** and **stop codons** positions, **coding sequence**, **TT**: transcription terminator, ***Antibiotic*^{R}**: antibiotic resistance gene, **Ori**: replication origin and **RBS**: ribosome binding site, are shown. Figures were adapted following the same sketch of Fig. 3.

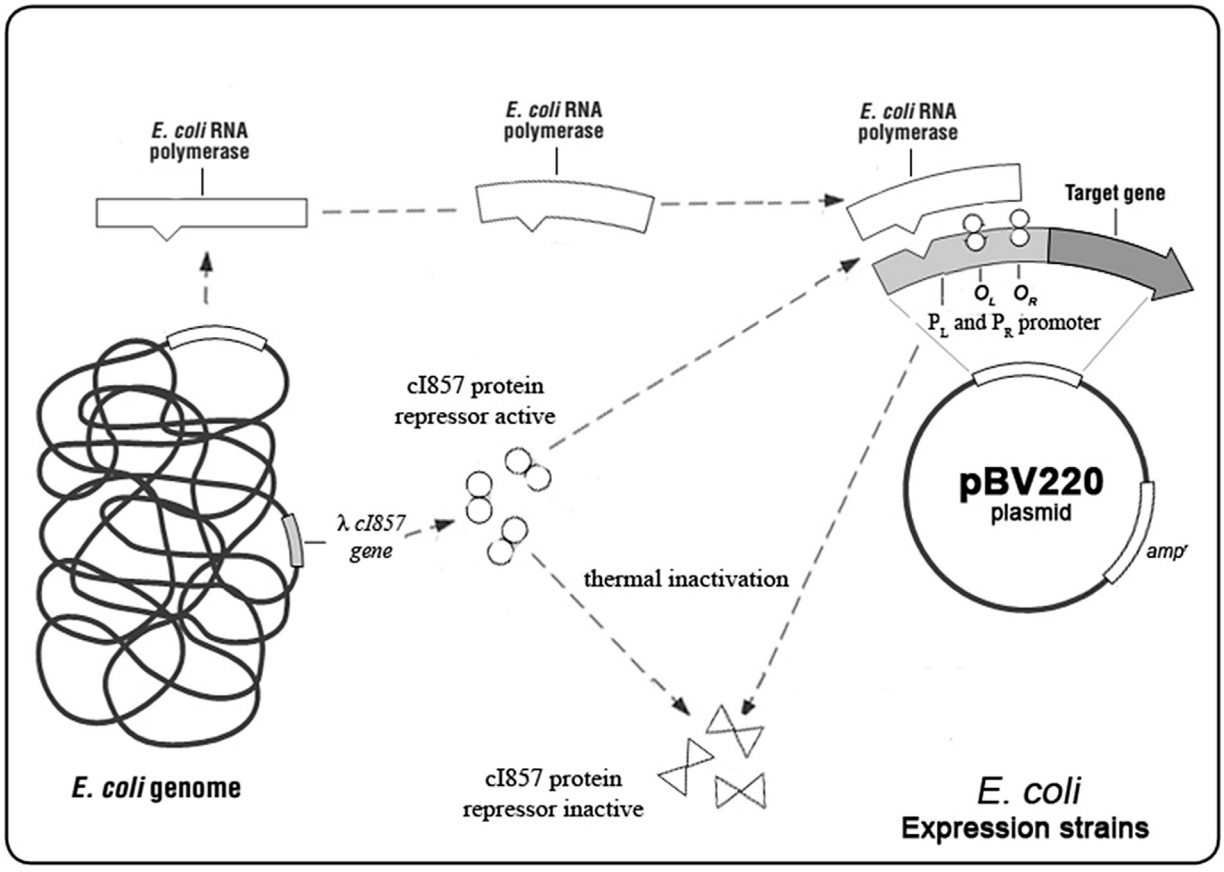

Fig. 6. Schematic representation of the genetic and protein regulatory elements in the λP_L/P_R-cI *E. coli* expression system. *E. coli(DE3)* strains have a lambda-lysogen regulatory gene (λ**cI857**) integrated into the chromosome that encoded thermo-stable **cI857 repressor** proteins. The **cI857 repressor** proteins active (circles on the figure) bind to operators (O_L and O_R) in the plasmid pBV220 and prevent the *E. coli* polymerase activity. The cI857 repressors are inactivated (hexagon on the figure) by temperature shift-up (37–42 °C) and the ***E. coli* RNA polymerase** can transcribe the **target gene**. Figure was adapted following the same sketch of Fig. 2.

rich broth, culture growth stops at a relatively low density since LB contains scarce amounts of carbohydrates (and other carbon sources) and divalent cations (Sezonov et al., 2007). Higher cell densities can be achieved by increasing twice the amount of peptone and yeast extract (2xYT broth), and by supplementing the medium with divalent cations in a millimolar range (Studier, 2005; Rosano and Ceccarelli, 2014). The 2xYT broth was used for rHct-S5 and rHct-S6 actinoporins expression (Table 1). TB (terrific broth) and SB (super broth) media have also been employed to reach higher cell densities (Madurawe et al., 2000; Atlas, 2004; Studier, 2005).

In 2005, the auto-induction medium composed of an optimized mixture of glucose, lactose, and glycerol was proposed (Studier, 2005). The auto-induction expression system could be used with *lac* operon systems and has gained popularity, especially in T7-lac expression systems. By this procedure, the cells are cultured in a defined media containing a mix of carbon sources: glucose, lactose, and glycerol. Glucose is the preferential carbon source and is metabolized preferentially during growth, which prevents uptake of lactose until glucose is depleted usually in middle of late log phase (Rosano and Ceccarelli, 2014). In this situation the cells change their metabolism to use glycerol and lactose, which induces expression of the T7 RNA polymerase (Studier, 2005). The auto-induction methodology has been successfully used in our group for the expression of recombinant and single-Cys mutants of sticholysin (Hervis et al., 2014).

A protein overexpression drawback is the formation of inclusion bodies in the cytoplasm of *E. coli*. The concentration increase of nascent polypeptide chains is enough to induce aggregates of recombinant target-protein and other contaminants such as: host proteins (e.g., RNA polymerase, outer membrane proteins), ribosomal components, circular and nicked forms of plasmid DNA, and small heat shock proteins (IbpA and IbpB) (Terpe, 2006). The recombinant proteins could be purified from these aggregates (inclusion bodies) by solubilization and refolding procedures (Singh and Panda, 2005; Terpe, 2006).

Most authors reported inclusion bodies formation during actinoporins expression (Anderluh et al., 1996; De los Ríos et al., 2000; Wang et al., 2000; Jiang et al., 2002; Uechi et al., 2005; Alegre-Cebollada et al., 2006; Pazos et al., 2006; Wang et al., 2008). The percentage of soluble actinoporin, relative to total protein expressed, changes according to cloning and expression strategies employed. For instance, the percentage of soluble actinoporin in rEqtII expression was about 50% (Anderluh et al., 1996), whereas rHmgIII-6His (Wang et al., 2000) and rStII-6His (De los Ríos et al., 2000) was about 20%. The rAvtI with 6His at the carboxyl end was expressed exclusively as inclusion bodies and not showed hemolytic activity (Uechi et al., 2005). Hence, the recombinant actinoporins are predominantly expressed as insoluble form in *E. coli*.

4. Chromatographic procedures for purification of actinoporin isoforms from *E.coli*

Since heterologous proteins have been expressed in *E. coli* forming inclusion bodies, several authors have performed their purification from these aggregates. These procedures usually involves four step: i) harvest the inclusion bodies from the cell lysate by centrifugation, ii) solubilize the pelleted inclusion bodies in buffer containing a denaturant agent (e.g., 6 M guanidinium chloride or 6–8 M urea) by resuspension, iii) purify the solubilized target-proteins usually by ion-exchange chromatography in presence of nonionic denaturants (e.g., urea), and iv) fold *in vitro* the target-proteins by addition of low-molecular weight folding enhancers (e.g., 1.0–1.3 M guanidium chloride, 2 M urea or polyethyleneglycol). If recombinant protein contains disulfide bridges, reduced and oxidized glutathione must be added to allow their formation (Schumann and Ferreira, 2004). rSrcI acid actinoporin was expressed in *BL21(DE3) E. coli* strain forming inclusion bodies. Its purification required different washing for *in vitro* folding and involved two chromatography steps: an anion exchange chromatography with the strong anion-exchanger Q-sepharose (Jiang et al., 2003a) and a subsequently hydrophobic interaction chromatography (HIC) with phenyl sepharose matrix (Table 3). The relationship between the actinoporins pI values and their function-structure relationship remained to be further studied (Jiang et al., 2003b).

The actinoporin purification from inclusion bodies must be driven with caution since the denaturation-renaturation procedures could originate several conformational populations that differ from native protein. There is an evident risk of "contamination" by partially folded forms of actinoporins due to *in vitro* folding protocols, which could interfere in the conformational and functional studies. For instance, during the renaturation of StII was reported the existence of partially folded states and aggregates formation (Mancheño et al., 2001). A similar phenomenon was reported for EqtII when pH and ionic strength stability studies were developed (Poklar et al., 1997; Ulrih et al., 2004). Probably, most of actinoporins were not purified from inclusion bodies because of this drawback (Tables 3 and 4).

Two different strategies have been performed for recombinant soluble actinoporins purification, according to the presence or not of tags. Considering the basicity of recombinant actinoporins without tags different cation exchange chromatographies have been used during purification (Anderluh et al., 1996; Uechi et al., 2005; Pazos et al., 2006; Alegre-Cebollada et al., 2007a; Bellomio et al., 2009). For rEqts, rSts and rAvtI purification an ion-exchange chromatography step with a weak cation-exchanger (carboxymetyl-cellulose ion exchanger at pH 6.0–8.0) was used. In addition, for rFraC a strong cation-exchanger (sulfopropyl-sepharose) was used (Table 3). In the case of rEqtII, rEqtIV, rEqtV, rAvtI, and rFraC a size exclusion chromatography was used as a second purification step (Table 3). The T7 lysozyme could also elute during cation-exchange chromatographic procedure, when *E. coli* strains with *pLysS* and *pLysE* plasmids are used for expression (unpublished results). T7 lysozyme is a basic protein (pI~9.1) with a molecular weight of 17 kDa.

The rStI and rStII showed the highest yield of purified protein per liter of culture by non-affinity chromatography: 12.8 and 5.2 mgL^{-1}, respectively (Table 3). Alegre-Cebollada et al. (2007a), introduced silent mutations in order to prevent the formation of mRNA secondary structures and to achieve the sticholysins expression. For FraC expression was also necessary to replace the Ser^{1} by Met, however, the *E. coli* Met-aminopeptidase removes this residue causing a decrease in the hemolitic activity of this protein (Bellomio et al., 2009).

Several authors have reported different buffer solutions for recombinant actinoporins purification respect to procedures used from natural sources (Wang et al., 2008; Pazos et al., 2006). Probably, changes in the primary structure (including a single change amino acid) may influence into the different solubility observed between recombinant and natural actinoporins (Wang et al., 2008). Three types of buffer solution have been used to purify basic recombinant actinoporins by cation-exchange chromatography: ammonium acetate, sodium phosphate and Tris-HCl, with neutral or slightly acid pH values (Table 3).

Ammonium acetate is volatile and has rendered excellent purification results at neutral pH in spite of the very low buffering capacity. In general, high capacity buffers are recommended, but many of them (e.g., Tris and phosphate) are non-volatiles. Volatility

Table 4
Actinoporins purification strategies by metal-ion affinity chromatography systems.

Sea anemones	Isoforms	Procedures and purification conditions				Protein yield mg L^{-1}
		Step 1	Step 2	Step 3	Purity assessment	
Heteractis magnifica	**Magnificalysins III (rHMgIII-6His)** (Wang et al., 2000)	Bacterias were harvested and lysed by sonication, and subsequently **MAC** (*HiTrap*): 0.01 mol L^{-1} Na_2HPO_4/NaH_2PO_4 and imidazole; 0.5 mol L^{-1} NaCl; 0.1 mol L^{-1}	Cleavage with thrombin and subsequently **IE** (*Mono-S*): 0.05 mol L^{-1} Na_2HPO_4/NaH_2PO_4; pH4.0. Linear gradient elution: 0–0.25 mol L^{-1} NaCl.		SDS-PAGE and N-terminal amino acid sequencing	n.d.
	Magnificalysins (rHMgs-6His: rHMg2F′, rHMgs D3 to **D7** and **rHMgs E1** to **E4)** (Wang et al., 2000, 2008)	$NiSO_4$; pH 7.4. Gradient elution 0.01 –0.5 mol L^{-1} imidazole.	Cleavage with thrombin and subsequently **IE** (*Mono-S*): 0.05 mol L^{-1} NaAc; pH4.0. Gradient elution 0–0.25 mol L^{-1} NaCl.		HPLC-RP (*C-18*) and N-terminal amino acid sequencing	3.0
Sagartia rosea	**SrcI hemolytic toxin (rSrcI-Trx-6His)** (Jiang et al., 2003b)	Bacterias were harvested and lysed by sonication. The inclusion bodies were collected and used for renaturation, and subsequently **MAC** (Ni^{+2} *Sepharose*) 0.05 mol L^{-1} Tris-HCl; 0.5 mol L^{-1}; NaCl and 0.01 mol L^{-1} imidazole, pH 7.8. Gradient elution 0.01–0.5 mol L^{-1} imidazole.	Fractions containing the Trx-rSrcI fusion protein were pooled and cleavaged with enterokinase and subsequently **IE** (*Q-Sepharose Fast Flow*): 0.02 mol L^{-1} Tris-HCl; 0.02 mol L^{-1} NaCl, pH 8.0. Gradient elution: 0.02–1.0 mol L^{-1} NaCl.	rSrcI and Trx-rSrcI were separated by **GF** (*Sephadex 75*) and exchanged to 0.05 mol L^{-1} NH_4HCO_3 by G-25 column.	HPLC-RP (*C-18*)	20.0
Actinia equina	**Equinatoxin II (rEqtII-TolAIII-6His)** (Anderluh et al., 2003)	Bacterias were harvested, frozen and disrupted by osmotic shock and sonication. **MAC** (*Ni-NTA*) 0.05 mol L^{-1} NaH_2PO_4; pH 8.0; 0.3 mol L^{-1} NaCl; 0.01 mol L^{-1} imidazole, and 0.02 mol L^{-1} 2-mercaptoethanol. Elution: with 0.3 mol L^{-1} imidazole.	Cleavage with enterokinase and subsequently **IE** (*Mono-S*) 0.04 mol L^{-1} Tris-HCl; pH 8.4. Gradient elution: 0–0.5 mol L^{-1} NaCl.		SDS-PAGE	11
Stichodactyla helianthus	**Sticholysin I (rStI-6His)** (Alegre-Cebollada et al., 2007a)	Bacterias were harvested and lysed by sonication, and subsequently **MAC** (*Ni-NTA*) 0.05 mol L^{-1} Tris-HCl; pH 7.8; 0.3 mol L^{-1} NaCl and 1% (v/v) Tween 20. Elution 0.01 mol L^{-1} Mops; pH 8.0; 0.25 mol L^{-1} imidazole (De los Ríos et al., 2000).			SDS-PAGE	15.0
	Sticholysin II (rStII-6His) (De los Ríos et al., 2000; Alegre-Cebollada et al., 2007a)				SDS-PAGE	3–5
Heteractis crispa (*Radianthus macrodactylus*)	**Heteractis crispa hemolytic toxin S family (rHct-S5** and **rHct-S6)** (Tkacheva et al., 2011)	Bacterias were harvested and lysed by sonication, and subsequently **MAC** (Ni^{+2}*-CAM-agarose*) 0.05 mol L^{-1} NaH_2PO_4; 0.3 mol L^{-1} NaCl; pH 8.0 and 0.01 mol L^{-1} imidazole.	Cleavage of hybrid protein with enterokinase, and Ni^{2+}-*CAM-agarose* was centrifuged and the supernatant was incubated with STI-agarose for elimination of enterokinase		HPLC-RP (*C-8*)	4.0

6His: six-His tagged; **HMg2F′**and **HMgs D3-D7**: protein sequences of isoforms D1 to D7 obtained from DNA using *DyNazyme™ II DNA polymerase*; **HMgs E1-4**: protein sequences of isoforms E1 to E4 obtained from DNA using *Expand™high-fidelity PCR system*; **Trx**: thioredoxin; **TolAIII**: fusion protein of the third domain of the bacterial periplasmic protein. **MAC**: metal-ion affinity chromatography; ***Ni-NTA***: Ni+2-nitrilotriacetic acid; ***Ni-CAM-agarose***: affinity resin for metal-ion affinity chromatography; **IE**: ion exchange chromatography; **GF**: gel filtration chromatography; **RP-HPLC**: reverse phase-high performance liquid cromotography; STI-agarose: soybean trypsin inhibitor (STI) cross-linked to agarose. **SDS-PAGE**: sodium dodecylsulphate-polyacrilamide gel electrophoresis; **mg L^{-1}**: mg of pure protein per liter of culture.

allows directly freeze-drying of the pure proteins, while for phosphate and Tris buffers a desalting step in a volatile buffer is necessary before freeze-drying, with the consequent extra work and the lost of material. In case of the acid actinoporin rSrcI, an anion-exchange chromatography with buffer Tris-HCl at basic pH was used (Table 3).

The expression of proteins with peptide tags or fusion partners have at least three advantage: (i) easy detection of desired protein during expression and purification schemes; (ii) attain maxima solubility; and (iii) easily purify it from the *E. coli* cellular milieu by affinity chromatography (Nilsson et al., 1997; Rosano and Ceccarelli, 2014). Generally, the peptide tags impair less the conformation and function of proteins than the fusion partners, which have the extra advantage of enhance the solubility (Hammarstrom et al., 2002; Rosano and Ceccarelli, 2014). In some cases, tags and partners may provoke unwanted effects on the terciary structure or biological function of the protein (Bucher et al., 2002; Klose et al., 2004; Chant et al., 2005; Khan et al., 2012; Rosano and Ceccarelli, 2014). Several expressions plasmids introduce peptide tags or fusion partners on either the N-terminal or the C-terminal end of target-protein. If three-dimensional (3D) structure of target-protein is available, it is recommended to place the tag or partner in the solvent-accesible end (Terpe, 2003; Rosano and Ceccarelli, 2014).

The 6His peptide tag, which binds to Ni chelated nitrilotriaceticacid agarose (Ni-NTA), were used for purifying recombinant actinoporins. Combinations of 6His tag with a solubility-enhancing partner (e.g, protein TolA or thioredoxin) were used as well (Tables 1 and 2). The purification of 6His tagged HMgs, Sts and rSrcI was performed by metal-ion affinity chromatography (MAC). This procedure rendered the highest amounts of purified protein per liter of culture: rHMgs (3 mg L^{-1}) (Wang et al., 2008), rStI-6His (3 mg L^{-1}), rStII-6His (15 mg L^{-1}) (Alegre-Cebollada et al., 2007a) and rSrcI-Trx-6His (20 mg L^{-1}) (Jiang et al., 2003b) (Table 4). However, any 6His-tagged recombinant actinoporins by N-terminal end lose activity with respect to the wild-type protein (Wang et al., 2000; Pazos et al., 2003; Uechi et al., 2005). The actinoporin N-terminal segment, which includes an amphipathic helix, from several monomers participates in the formation of the transmembrane pore walls (Mancheño et al., 2003; Mechaly et al., 2011; Tanaka et al., 2015). Specific excision sites are usually added between the recombinant protein and the tag or fusion partner, to allow eventual enzymatic or chemical removal (Rais-Beghdadi et al., 1998; Routzahn and Waugh, 2002; Jenny et al., 2003; Waugh, 2011).

Two commonly used proteolytic sites are those recognized by bovine enterokinase and Factor Xa (Jenny et al., 2003). These enzymes are commercially available and are specific for their recognition sequences, allowing cleavage without protein degradation under conditions where protein solubility and activity are preserved. Enterokinase and FactorXa cleave on the C-terminal sides of their recognition sequences (DDDDK and IEGR, respectively), and are widely tolerant for different amino acids in the position following the cleavage site. Thus, the fusion of N-terminus protein to the C-terminus of one of these protease recognition sequences may allow generation of a cleaved protein with an authentic N-terminus (Arnau et al., 2006; Waugh, 2011; Wood, 2014).

In the case of rHMgs-6His, after the MAC, the 6His tag was eliminated by thrombin cleavage and subsequently rHMgs were re-purified by mono-S cation exchange chromatography (Table 4). rHMgs digested with thrombin and re-repurified had a Gly-Ser sequence in the amino end from proteolytic site. This additional sequence is responsible for the lower hemolytic activity respect to natural source protein (Wang et al., 2000) (Table 4). An rAvtI variant with 6His tag at the carboxyl end (rAvtI-6His) was expressed exclusively as inclusion bodies and was no functional (Uechi et al., 2005).

The rSrcI-Trx-6His protein, susceptible to cleavage, was also produced and purified by MAC (Jiang et al., 2003b). Subsequently, the Trx-6His-tag was eliminated by cleavage with enterokinase. rSrcI was re-purified by anion exchange chromatography in Q-sepharose and molecular exclusion chromatography in sephadex-75 (Table 4), and showed hemolytic activity similar to the wild-type protein (Table 3).

5. Changes in the amino acid sequences and hemolytic activities of recombinant actinoporins

The efficient production of actinoporins in bacteria without need of renaturation has allowed the three-dimensional (3D) structure determination by nuclear magnetic resonance and X-ray diffraction (Athanasiadis et al., 2001; Hinds et al., 2002; Mancheño et al., 2003; Kristan et al., 2004; Castrillo et al., 2009; Mechaly et al., 2011; Pardo-Cea et al., 2011; Tanaka et al., 2015; Morante et al., 2015). The 3D structures of equinatoxin II purified by recombinant technology in *E. coli* (PDB: 1KD6) are essentially identical to those obtained from natural source (PDB: 1IAZ), so the heterologous expression does not change the protein structure (Athanasiadis et al., 2001; Hinds et al., 2002). Actinoporins were expressed in *E. coli* without anemone signal peptide and propeptide motif, indicating that at least these elements are not important for the correct folding.

Several recombinant actinoporin variants were expressed with amino acidic changes respect to wild-type protein, such as: rEqtII (S177T), rStI (E16Q) and rFraC (S1M) (Table 5). The changes present into rEqtII and rStI probably are due to isoforms or allelic variants found in anemones populations (Pazos et al., 2006). The S177T substitution in rEqtII did not modify the hemolytic activity respect to wild-type actinoporin (Anderluh et al., 1996), whereas the E16Q substitution in rStI unexpectedly exhibited a greater hemolytic activity than StI upon human red blood cells (Table 5) (Pazos et al., 2006; Valle et al., 2015). The substitution of Ser1 by Met in rFraC was carried out for achieve the heterologous expression and the Met1 elimination by *E. coli* Met-aminopeptidase produced a decrease in hemolytic activity (Table 5) (Bellomio et al., 2009).

The expression of tagged recombinant actinoporins facilitated their solubilization and purification by affinity chromatography (Table 4), but decreased their litic activity with respect to protein from natural sources (Wang et al., 2000 and Pazos et al., 2003; Uechi et al., 2005) (Table 3). The presence of 6His-tag in rStII-6His reduced the hemolytic activity in human red blood cells and the leakage of carboxyfluorescein from the inside of lipid vesicles (Pazos et al., 2003) (Table 5). Therefore, it is advisable to produce recombinant actinoporins avoiding the tags-expression strategy.

By enzymatic or chemical removal of tags or partners, recombinant proteins could be obtain with similar activity to natural variants. However, the use of the enzymatic approach for actinoporins did not prevent the lost of activity due to the presence of some residual amino acids in the ends after removal (Table 5). The N-terminal Gly-Ser sequence remained after 6His-tag removal by thrombin cleavage in rHMgIII-6His, and these additional residues were responsible for the hemolytic activity decrease (Table 5) (Wang et al., 2000, 2008). In addition, the recombinant actinoporins without tags showed the same hemolytic activity respect to protein purificated from sea anemones (Table 5).

6. Remarks and prospects

The actinoporins expression with solubility enhancing fusion partner not avoided the inclusion bodies formation, the hemolitic activities were decreased and the tags elimination increased the costs and the number purification steps. For these reasons, is recommended to avoid the use of tags or partners for heterologous expression of recombinant actinoporins. The production of these proteins has been mainly accomplished by phage T7 system in (DE3) *E. coli* strains. The actinoporins purification should be

Table 5
Changes in the amino acid sequences and hemolytic activities of recombinant actinoporins.

Sea anemones	Isoforms	Structural information	Functional information
Actinia equina.	**Equinatoxin II (rEqtII)** (Anderluh et al., 1996)	Replacement Ser 177 x Thr	Similar HA to wild-type actinoporin from anemone in spite of the Ser 177 x Thr 177 replacement. This indicates that substitution of Ser177 by Thr is not essential for cytolytic activity of EqtII.
	Equinatoxin II 6His-tag (rEqtII-TolAIII-6His) (Anderluh et al., 2003)	Replacement Ser 177 x Thr and the solubility-enhancing tags was cleavage with enterokinase of protein.	HA respect to wild-type actinoporin from anemone not was determined.
	Equinatoxin IV (rEqtIV) (Anderluh et al., 1999b)	n.d.	Similar HA and primary structure to wild-type actinoporin from anemone (Pungerčar et al., 1997; Anderluh et al., 1999b).
	Equinatoxin V (rEqtV) (Pungerčar et al., 1997)	n.d.	HA respect to wild-type actinoporin from anemone not was determined.
Stichodactyla helianthus	**Sticholysin I** (**rStI**) (Pazos et al., 2006)	Replacement Glu16 x Gln.	Higher HA than to wild-type StI from anemone. This indicates that position 16 is implicated in cytolytic activity of StI.
	Sticholysin I (rStI[a]) (De los Ríos et al., 2000; Alegre-Cebollada et al., 2007a)	Replacement Glu16 x Gln.	HA respect to wild-type actinoporin from anemone not was determined.
	Sticholysin II (rStII[a]) (Alegre-Cebollada et al., 2007a)		HA respect to wild-type actinoporin from anemone not was determined.
	Sticholysin I (rStI-6His) (Alegre-Cebollada et al., 2007a)	6His-tag protein.	Lytic activity was decreased for the presence of the 6His tag.
	Sticholysin II (rStII-6His) (De los Ríos et al., 2000; Alegre-Cebollada et al., 2007a)	6His-tag protein.	Lower HA than to wild-type actinoporin from anemone (Pazos et al., 2003).
Heteractis magnifica	**Magnificalysins III (rHMgIII-6His)** (Wang et al., 2000)	The 6His-tag protein with thrombin proteolitic site (-L-V-P-R-G-S-). The N-terminal (Gly-Ser-) sequence was preserved from proteolysis	HA of was decreased for the presence of the N-terminal (Gly-Ser-) sequence after cleavage by Thrombin. The HA decreased with increasing length of the N-terminal tag.
	Magnificalysins (rHMgs-6His: rHMg2F′, rHMgs D3 to **D7** and **rHMgs E1** to **E4)** (Wang et al., 2000, 2008	The 6His-tag protein with trombin proteolitic site (Wang et al., 2000). In 22 of the 52 total clones were observed cysteine residue including in more one position. The rHMgIII isoforms showed very different solubility.	The isoforms showed different activity. HA of wild-type HMgIII was highest compared to the rHMgs due to the presence of the N-terminal (Gly-Ser-) sequence after cleavage by Thrombin. The HA decreased with increasing length of the N-terminal tag.
Sagartia rosea	**SrcI hemolytic toxin (rSrcI)** (Jiang et al., 2002, 2003a)	Renaturation from inclusion bodies was used and rSrcI protein revealed the presence of the expected extra methionine at the N-terminus of the protein. SrcI is an acidic actinoporin.	HA respect to wild-type SrcI from anemone not was determined.
	SrcI hemolytic toxin (rSrcI-Trx-6His) (Jiang et al., 2003b)	Solubility-enhancing tag thioredoxin and 6His-tag were used for protein expression. SrcI is an acidic actinoporin.	HA respect to wild-type SrcI from anemone not was determined (Jiang et al., 2003b).
Actineria illosa	**AvtI hemolytic toxin (rAvtI)** (Uechi et al., 2005)		9.2-fold higher HA in sheep erythrocytes compared to that of wild-type Avt-I from anemone. This result suggests that the activity of wild-type Avt-I from anemone was reduced during purification.
Actinia fragacea	**Fragaceatoxin C (rFraC)** (Bellomio et al., 2009)	Ser 1 was replaced by Met 1 for prevent to the formation of RNAm secondary structures and the *E.coli* methionine aminopeptidase removed the Met 1.	Lower HA than to wild-type actinoporin from anemone because the Ser 1 was replaced by Met 1 and was removed the Met 1.
Heteractis crispa or *Radianthus macrodactylus*	**Heteractis crispa hemolytic toxin S family (rHct-S5** and **rHct-S6)** (Tkacheva et al., 2011)	Hct-S family characterized by presence of N-terminal serine in the mature proteins and were expressed as hybrid proteins containing the GST-protein, 6His-tag and enterokinase restriction site.	HA respect to wild-type SrcI from anemone not was determined, but was one order of magnitude lower than that of natural actinoporins such as EqtII, StnII, HMgI, HMgII, RTX-A, RTX-SII, and rRTX-S3.

[a] Overproduction of St I and St II was only possible with silent mutations within the 5′-end of original cDNA sequence for preventing mRNA secondary structure formation that blocks the ribosome-binding site and/or the initiation codon.**HMg2F**′and **HMgs D3-7**: protein sequences of isoforms D1 to D7 obtained from DNA using *DyNazyme*^{TM} *II DNA polymerase*; **HMgs E1-4**: protein sequences of isoforms E1 to E4 obtained from DNA using *Expand*^{TM}*high-fidelity PCR system*. **6His**: six-His tagged; **TolAIII**: fusion protein of the third domain of the bacterial periplasmic protein; **Trx**: thioredoxin. **HA**: hemolytic activity. **n.d.**: no determined. GST: glutathione S-transferase.

performed from soluble fraction of bacteria homogenate since renaturation from inclusion bodies could bring about different conformational populations. The carboxymetyl-cellulose weak cation-exchanger could allow the purification of the basic actinoporins by a single step chromatography procedure.

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