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ORIGINAL PAPER

Novel Expression Vectors Based on the pIGDM1 Plasmid

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Abstract

Escherichia coli is one of the most widely used hosts for the production of heterologous proteins. Within this host, the choice of cloning vector constitutes a key factor for a satisfactory amplified expression of a target gene. We aimed to develop novel, unpatented expression vectors that enable the stable maintenance and efficient overproduction of proteins in *E. coli*. A series of expression vectors based on the ColE1-like pIGDM1 plasmid were constructed. The vectors named pIGDMCT7RS, pIGDM4RS and pIGDMKAN carry various antibiotic resistance genes: chloramphenicol, ampicillin or kanamycin, respectively. Two derivatives contain the inducible *T7* promoter while the third one bears the constitutive *pms* promoter from a clinical strain of *Klebsiella pneumoniae*. The pIGDM1-derivatives are compatible with other ColE1-like plasmids commonly used in molecular cloning. The pIGDMCT7RS and pIGDM4RS vectors contain genes encoding AGA and AGG tRNAs, which supplement the shortage of these tRNAs, increasing the efficiency of synthesis of heterologous proteins. In conclusion, pIGDMCT7RS, pIGDM4RS and pIGDMKAN vectors, with significantly improved features, including compatibility with vast majority of other plasmids, were designed and constructed. They enable a high-level expression of a desired recombinant gene and therefore constitute a potential, valuable tool for pharmaceutical companies and research laboratories for their own research or for the production of recombinant biopharmaceuticals.

Keywords Expression vectors · Recombinant protein production · Bacterial expression system · *E. coli*

Abbreviations

bla gene	AMPICILLIN RESISTANCE GENE
cat gene	Chloramphenicol resistance gene
kan gene	Kanamycin resistance gene
MCS	Multiple cloning site
SD	Shine Dalgarno sequence
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
CPH	The yeast cyclophilin gene
IFNA13	The interferon alpha 13 gene
Ubi	*HGH*: ubiquitin-human growth hormone fusion gene

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Introduction

Escherichia coli plasmids are widely used in biotechnology as vectors for the overproduction of proteins. Even though standard cloning procedures have become routine and a large variety of host/vector systems for the expression of recombinant genes is available, difficulties are usually encountered when theoretical strategies for overproduction of proteins are put into practice. Issues such as manipulation of the regulatory mechanisms for gene expression and the host/vector interactions usually require extensive research if a high-level expression of a desired recombinant gene is to

be achieved [1–4]. The choice of an expression system for the high-level production of recombinant proteins depends on many factors. These include cell growth characteristics, expression levels, intracellular and extracellular expression, post-translational modifications and biological activity of the protein of interest, as well as regulatory issues in the production of therapeutic proteins [2, 5]. However, there are some limitations such as intracellular accumulation of heterologous proteins, improper folding of the polypeptides, lack of post-transcriptional and post-translational modifications, potential product degradation due to trace of protease impurities and production of endotoxin [6]. In general, it is difficult to decide which host and promoter system is the best for the heterologous protein production. It depends often on the target protein itself.

The choice of expression vector is a key factor in the successfully amplified expression of a target gene in *E. coli*. A variety of expression vectors are available, but the most commonly used ones are plasmid vectors that contain a number of sequence elements, including an origin of replication, a promoter, a multi-cloning site, affinity tags, a transcription terminator and selection markers [7]. Choosing a suitable vector requires detailed understanding of these features and their usefulness can be carefully evaluated according to the characteristics of the target protein [8]. For selection of anticipated cells carrying the desirable plasmid, and to prevent the growth of plasmid-free cells, a resistance marker is generally included in the plasmid. Vectors contain genes that confer antibiotic resistance on the host to aid in plasmid selection and propagation.

Resistance to ampicillin is commonly used for this purpose, however, for the production of human therapeutic proteins other antibiotic resistance markers are preferable to avoid the potential allergic reactions [5, 9]. Therefore, it is important to construct expression vectors with other antibiotic resistance genes, e.g. tetracycline, kanamycin or chloramphenicol. The stability of vectors in antibiotic-free cultures is a significant factor for their use in the biopharmaceutical industry [10].

Considering the tremendous advances in vector engineering, it becomes evident that even a complex set of expression vectors constructed so far does not solve all the problems existing in this field. Therefore, it is important to develop novel, unpatented expression vectors that enable stable maintenance and efficient overproduction of proteins in *E. coli*. For these reasons pharmaceutical companies and research laboratories are interested in exploiting novel commercially available expression systems.

This is why it is necessary to construct completely new expression vectors, which can be freely used for their own research or for the production of recombinant biopharmaceuticals. To obtain novel bacterial co-expression vector systems adapting more than two plasmids, each with a different combination of a compatible replicon and an antibiotic selection marker, could be also valuable. Co-expression of genes is an important objective for biochemical and structural analysis of protein complexes because it often increases authenticity of biological activity and increases solubility of protein partners. The bacterial co-expression method is a useful gene expression technique to reconstitute a hetero-oligomeric protein complexes in vitro [11–13].

In this study, we constructed a series of novel expression vectors based on the ColE1-like pIGDM1 plasmid [14]. Each of these vectors carries various antibiotic resistance genes (ampicillin (*bla* gene), chloramphenicol (*cat* gene) or kanamycin (*kan* gene)). Two derivatives contain the inducible *T7* promoter while the third one bears the constitutive *pms* promoter from a clinical strain of *Klebsiella pneumoniae* (GenBank accession no. AY543071). The pIGDM1-derivatives are compatible with other vectors, including ColE1-like plasmids, commonly used in molecular cloning. They can also be used in the creation of co-expression system in *E. coli* cells [15].

Materials and Methods

Bacterial Strains and Plasmids

E. coli strains and plasmids used in this work are listed in Table 1. All *E. coli* strains were propagated in LB broth (tryptone 10.0 g/L, yeast extract 5.0 g/L, NaCl 5.0 g/L, pH 7.2–7.5), supplemented with ampicillin (100 µg/mL), chloramphenicol (20 µg/mL) or kanamycin (50 µg/mL).

DNA Manipulation, Transformation and Sequencing

DNA restriction, ligation and gel electrophoresis were performed using standard techniques [21]. All transformations of bacterial cells with plasmid DNA were carried out by electroporation, using 1 mm cuvettes (BTX) and Micro-Pulser™ electroporator (BioRad, US). Electrocompetent *E. coli* DH5α and BL(DE3)21 cells were prepared using standard techniques [21]. Plasmid DNA was isolated using the Plasmid Mini Isolation Kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. All restriction enzymes, ligase and DNA ladder were purchased from Fermentas MBI (US) and were used according to the manufacturer's instructions. The amplification of DNA was conducted on PTC-100 cycler (MJ Research, US) using Biotools DNA Polymerase (Biotools B&M Labs, Spain) according to the manufacturer's instructions. A prestained protein molecular weight marker was purchased from GE Healthcare (UK). DNA sequences were determined at the

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Sources/references
Strains		
Escherichia coli BL21(DE3)	F^- *ompT* *hsd*S_B (r_B^-, m_B^-) *gal dcm* (DE3)	Stratagene
Escherichia coli DH5α	F⁻ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17 (r_K^-, m_K^+) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1	ATCC no. 27117
Plasmids		
pIGDM1	A natural plasmid isolated from *Enterobacter agglomerans*	GenBank: AF014880
pBR328	A pMB1- derived replicon bearing ampicillin-, tetracycline-, and chloramphenicol resistance genes	[16]
pBS(+)	Phagemid cloning vector, bearing ampicillin resistance gene	[17]
pBlueSK(-)	Standard cloning vector, bearing ampicillin resistance gene	[18]
pKK223-3	An expression vector bearing ampicillin resistance gene, and T1, T2 transcription termination sequences	Pharmacia
pT7RS	An expression vector bearing ampicillin resistance gene T7 promoter, T7 transcription terminator and $tRNA^{Arg}$ gene	GenBank: AY923866
pUC19	A pMB1- derived replicon bearing ampicillin resistance gene	[19]
pIGRK	A natural plasmid isolated from clinical strain CZD 2324 bearing *pms* promoter	[20]
pIGDMC	As pIGDM1 but bearing a chloramphenicol resistance gene	This work
pIGDMC-1	As pIGDMC with inserted MCS region	This work
pIGDMCT7RS	An expression vector derived from pIGDMC-1 with inserted fragment containing T7 promoter, T7 transcription terminator and $tRNA^{Arg}$ gene	GenBank: DQ485721
pIGDM2	As pIGDM1 but bearing ampicillin resistance gene	This work
pIGDM3	As pIGDM2 with inserted MCS region	This work
pIGDM4	As pIGDM3 with inserted MCS region with inserted fragment containing T7 promoter, T7 transcription terminator and $tRNA^{Arg}$ gene	This work
pIGDM4RS	An expression vector derived from pIGDM4 with inserted T7 transcription terminator	GenBank: HQ845200
pIGDMK-1	As pIGDM1 with inserted EZ::TN < KAN-2 > , KAN-R	This work
pIGDMK-2	An expression vector derived from pIGDMK-1 with inserted *pms* promoter	
pIGDMKAN	As pIGDMK-2 with inserted T1, T2 transcription termination sequences	This work
pIGDMKUH	As pIGDMKAN with inserted the Ubi::HGH fusion gene	GenBank: CS136259
pIGAL/IFNA13	An expression vector bearing ampicillin resistance gene, *pms* promoter and gene coding *IFNA13*	This work
pIGDM5/IFNA13	An expression vector bearing ampicillin resistance gene, *ptcB* promoter and gene coding *IFNA13*	This work

Institute of Biotechnology and Antibiotics (Warsaw, Poland) using the commercially available sequencing facility. The primers used in this study were synthesized at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Poland), and their sequences are provided in Table 2. PCR products were separated on agarose gels and purified using a Gel-out Kit (A&A Biotechnology, Poland) according to the manufacturer's instructions.

Table 2 Sequences of primers used in this study. This restriction sites are underlined

Primer	Primer sequence (5′→3′)	Restriction enzyme sites (underlined)
DM1	5′ GGGAGGCCTAAGGGATTTTGGTCATGAG	StyI
DM2	5′ GGGGTCGACGGTACCTTTGATATCCCTCAGGTGGCACTTTTCG	SalI
RAPR1	5′ ATGCAAGCTTCAGGGTTGAG	HindIII
RAPR2	5′ AATGGATCCTCTAGAGTCGACCATATGAAACCTCCTTAAAGTTAA	BamHI, Xba I, SalI, NdeI
TERM1	5′ GGGTCTAGACAAATAAAACGAAAGGCTCAGTCG	XbaI
TERM2	5′ GGGGATCCAAACGCAAAAAGGCCATCCGTC	BamHI

Construction of the Expression Plasmid pIGDMCT7RS

The expression plasmid pIGDMCT7RS is a derivative of plasmid pIGDM1 [14]. Plasmid pIGDMCT7RS was constructed by cloning the HincII-HincII fragment of pBR328 plasmid (ATCC), including the *cat* gene, into the corresponding site of pIGDM1. The resulting plasmid, named pIGDMC, was cleaved with StuI/HindIII and then, 72 nucleotides with short multiple cloning site (MCS), originating from pBluescribe M13(+)—pBS(+) plasmid (Stratagene), were inserted into the cleaved plasmid to give pIGDMC-1. This short MCS was digested with EcoRI, filled-in with the Klenow, and then cleaved with HindIII so that the cleavage sites matched each other. The pIGCM-1 plasmid was further treated with HindIII, filled-in with the Klenow, then cleaved with BamHI, and ligated with 716 bp ClaI (filled-in with the Klenow) and BglII insert comprising the *T7* promoter, T7 transcription terminator and $tRNA^{Arg}$ gene with anticodon UCU/CCU. Therefore, the plasmid can be used to express eukaryotic sequences containing frequent arginine codons. This insert contains a SD sequence (AGGA) at position -13 relative to the start codon. This fragment originated from the pT7RS expression vector (GenBank accession no. AY923866). Finally, the sequence of the entire plasmid was confirmed, and the resulting construct was named pIGDMCT7RS. Figure 1 illustrates the strategy employed for the construction of the expression vector pIGDMCT7RS.

Construction of the Expression Plasmid pIGDM4RS

Plasmid pIGDM4RS was constructed by PCR amplification of the *bla* gene from pUC19 (ATCC) using primers DM1 and DM2 (Table 2), digestion of the amplified product by

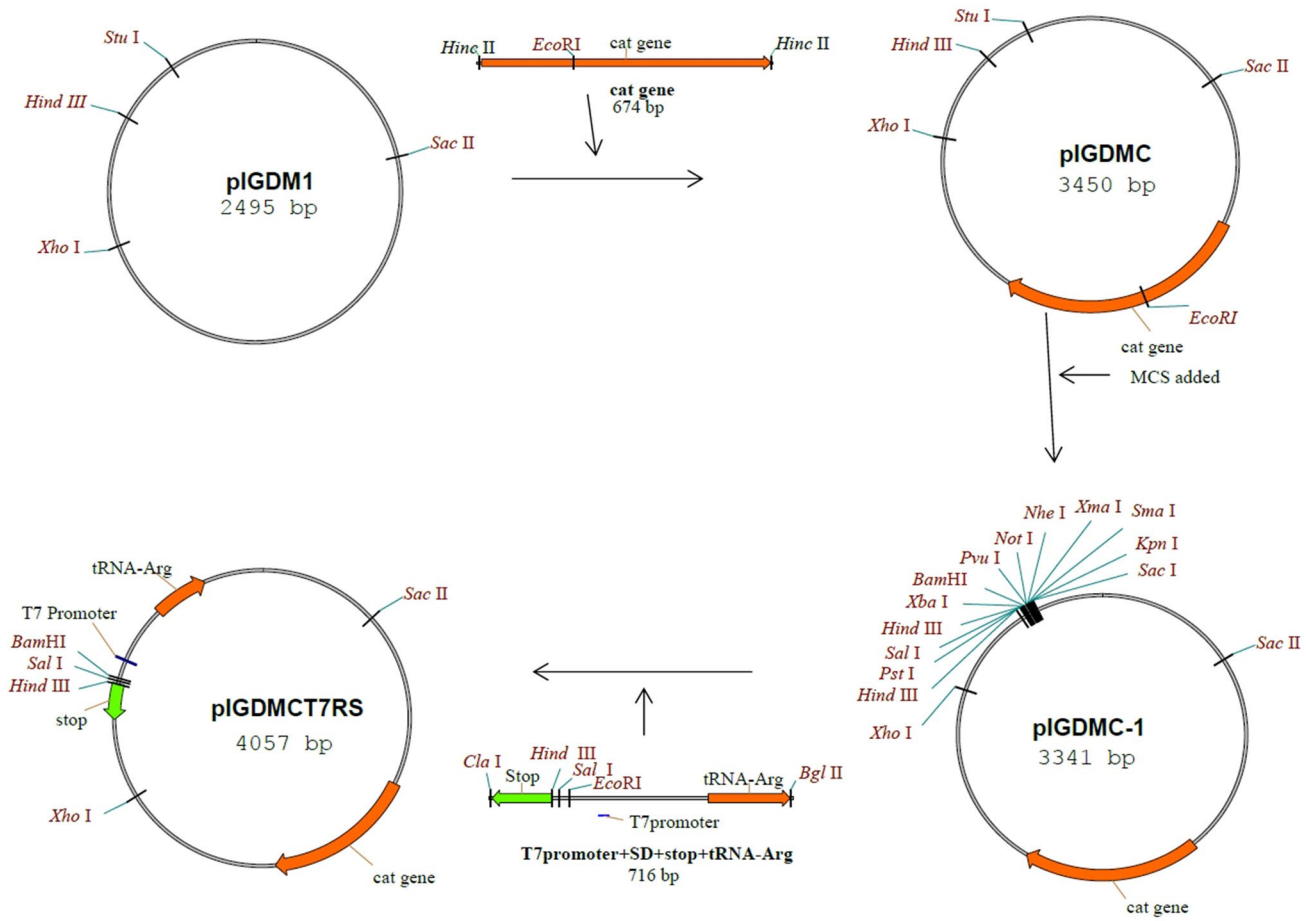

Fig. 1 The construction scheme of the pIGDMCT7RS expression vector. Abbreviations used: *cat gene* chloramphenicol resistant gene, *MCS* molecular cloning sites, *T7* T7 promoter, *SD* Shine-Dalgarno sequence, *Stop* T7 transcription terminator, *tRNA-Arg* $tRNA^{Arg}$ gene with anticodon UCU/CCU

StuI and SalI, and cloning it into pIGDM1 plasmid digested with StuI and XhoI. The resulting plasmid, named pIGDM2, was cleaved with KpnI and EcoRV and subsequently, 82 nucleotides with MCS originating from pBluescript SK(−)—pBlueSK(−) plasmid (Stratagene), treated with XbaI, filled-in by Klenow and KpnI, were inserted into the cleaved plasmid to give pIGDM3. The pIGDM3 plasmid was further treated with EcoRI and BamHI, ligated with 543 bp EcoRI and BglII insert comprising the *T7* promoter and the $tRNA^{Arg}$ gene with anticodon UCU/CCU, and a SD sequence (AGGA) at position -13, relative to the start codon, originating from the pT7RS expression vector, to form the pIGDM4 plasmid. The ultimate vector pIGDM4RS was obtained by digesting with HindIII and ClaI and cloning in the insert containing the T7 transcription terminator, cleaved with appropriate enzymes. Figure 2 illustrates the strategy employed for the construction of the expression vector pIGDM4RS.

Construction of the Expression Plasmid pIGDMKAN

To obtain the pIGDMKAN, the kanamycin resistance gene (*kan*), originating from transposon EZ::TN < KAN-2 > using EZ::TN™ < KAN-2 > Insertion Kit (Epicentre Biotechnologies, US) was inserted, according to the manufacturer's instructions, into the pIGDM1 plasmid. As a result, several plasmids differing with the insertion site of the *kan* gene were obtained. The sequences of all resulting plasmids were checked and one, named pIGDMK-1, was chosen for further modification. To modify pIGDMK-1 into an expression vector, it was cleaved with BamHI/HindIII and then the 172 bp length DNA fragment containing the constitutive *pms* promoter [20] and a SD sequence (AGGAGG) at position -12, relative to the start codon, was inserted. The fragment was amplified by PCR, using the primers RAPR1 and RAPR2 and exploiting the pIGRK plasmid as a template. The *pms* promoter was ligated in the complementary orientation to give pIGDMK-2 plasmid. The pIGDMK-2 plasmid was further treated with XbaI/BamHI and ligated with 202 bp XbaI/BamHI insert comprising two prokaryotic transcription termination sequences T1 and T2, amplified by PCR using the primers TERM1 and TERM2, derived from pKK223-3 plasmid, yielding pIGDMKAN. Figure 3 illustrates the strategy employed for the construction of the expression vector pIGDMKAN.

Cloning and Expression of the Model Genes in the New pIGDM1-Derived Vectors

In order to verify the usefulness of plasmids as expression vectors, the genes coding for various proteins were cloned in. The yeast cyclophilin gene—*CPH* (assembled from cDNA of *Saccharomyces cerevisiae*) was cloned into pIGDMCT7RS vector using the restriction enzymes NdeI and HindIII (Fig. 4). The gene coding for interferon alpha (*IFNA13*) was cloned into pIGDM4RS vector, digested with the restriction enzymes NdeI and HindIII (Fig. 5). The fusion gene coding for ubiquitin-human growth hormone was cloned into pIGDMKAN vector, cleaved with NdeI and SalI (Fig. 6). The genes in pIGDMCT7RS vector and pIGDM4RS vector are under control of the inducible *T7* promoter which requires addition of IPTG for induction in the *E. coli* BL21(DE3) strain. Gene expression in pIGDMKAN vector contains the constitutive *pms* promoter in *E. coli* DH5α strain and requires no addition of IPTG.

Protein Overproduction and SDS-PAGE

To test the recombinant protein overproduction, *E. coli* BL21(DE3) or DH5α were transformed with plasmids, and 3 mL LB cultures were started from single-transformant colonies. At optical density (OD_{600}) of 0.6, IPTG was added to a final concentration of 1 mM to induce expression of the recombinant genes in *E. coli* BL21(DE3). Cultures were incubated for further 2 h with intensive shaking. Culture samples were collected before and after induction for SDS-PAGE. The method used for expression in *E. coli* DH5α was essentially the same, except that cultures were propagated to OD_{600} of 1.0 before samples were collected. SDS-PAGE of all samples was performed in 15% polyacrylamide gels, prepared using standard techniques [21]. Samples for SDS-PAGE were prepared by suspending bacterial pellets in 200 µL of R1 solution (25 mM Tris–HCl, 10 mM EDTA, 50 mM glucose) and adding 100 mL of Laemmli buffer [21]. Prior to electrophoresis, samples were boiled for 5 min. Quantitative analyses of the proteins CPH, IFNA13 and Ubi::HGH were performed using the UN-SCAN-IT gel Analysis Software (USA).

Results

Designing of the pIGDM1 Vector Family

The previously described plasmid pIGDM1 from *Enterobacter agglomerans* [14] has been used as a basis for the creation of a series of new expression vectors (Table 1). Three expression vectors, each carrying a different antibiotic resistance gene (*bla*, *cat* or *kan* gene) and various promoters (*T7* or *pms*), were constructed for protein overproduction in *E. coli.*

The Expression Vector pIGDMCT7RS

The expression plasmid pIGDMCT7RS is a derivative of plasmid pIGDM1. The pIGDMCT7RS vector was constructed by insertion of the chloramphenicol resistance

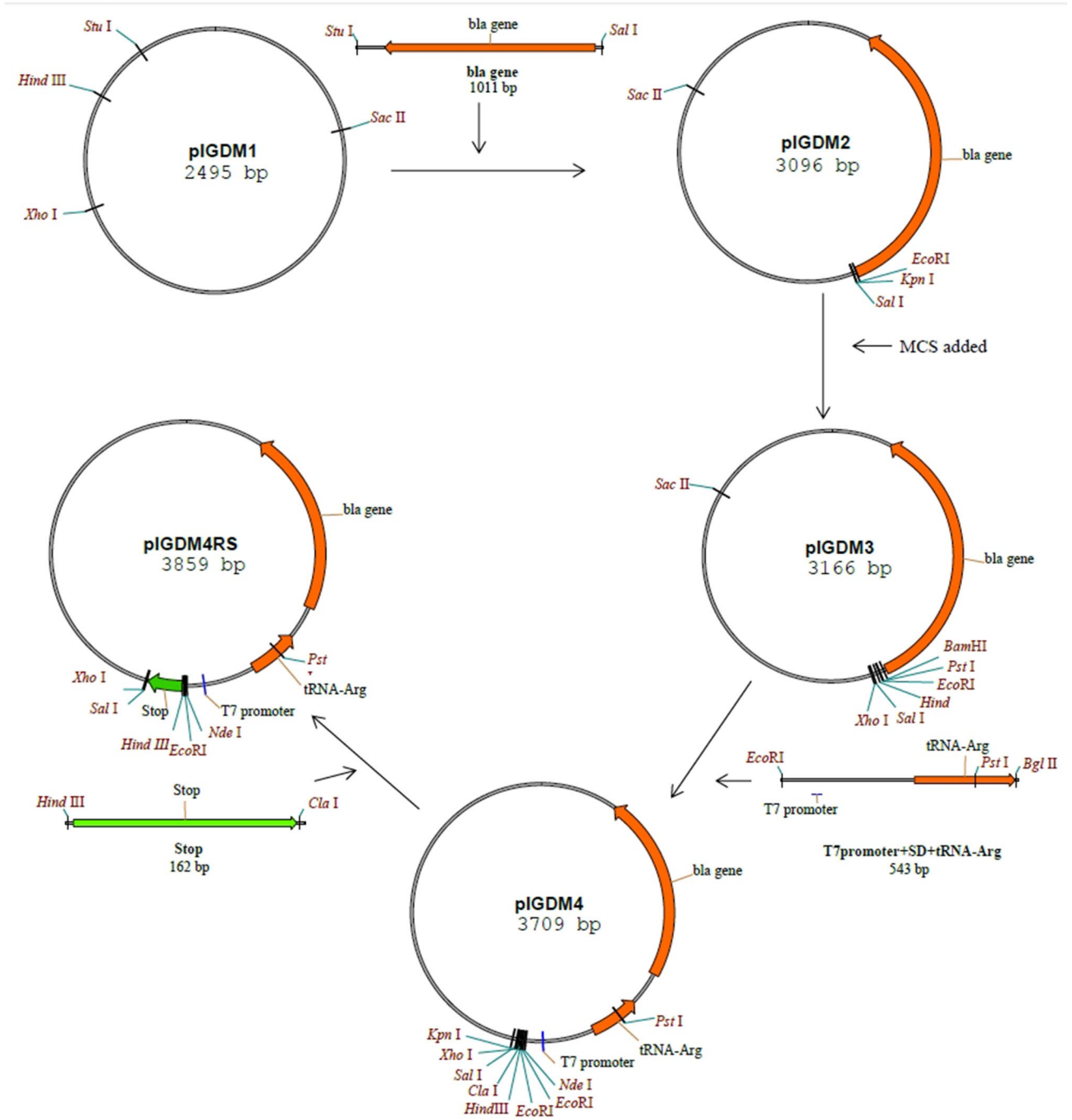

Fig. 2 The construction scheme of the pIGDM4RS expression vector. Abbreviations used: *bla gene* ampicillin resistant gene, *MCS* molecular cloning sites, *T7* T7 promoter, *SD* Shine-Dalgarno sequence, *Stop* T7 transcription terminator, *tRNA-Arg* $tRNA^{Arg}$ gene with anticodon UCU/CCU

gene (*cat*), originating from pBR322 (the HincII-HincII fragment), into the corresponding site of pIGDM1 vector. Then, the short nucleotide fragment containing multiple cloning site (MCS) from pBS(+) plasmid was cloned. The next step was adding the insert comprising the *T7* promoter, Shine Dalgarno sequence (SD), T7 transcription terminator and $tRNA^{Arg}$ gene (Fig. 1). The nucleotide sequence of pIGDMCT7RS was submitted to GenBank and the accession number is DQ485721. The obtained expression vector was verified as a useful tool for protein overproduction in *E. coli* cells. For this purpose, the yeast cyclophilin gene (*CPH*) was cloned into the NdeI

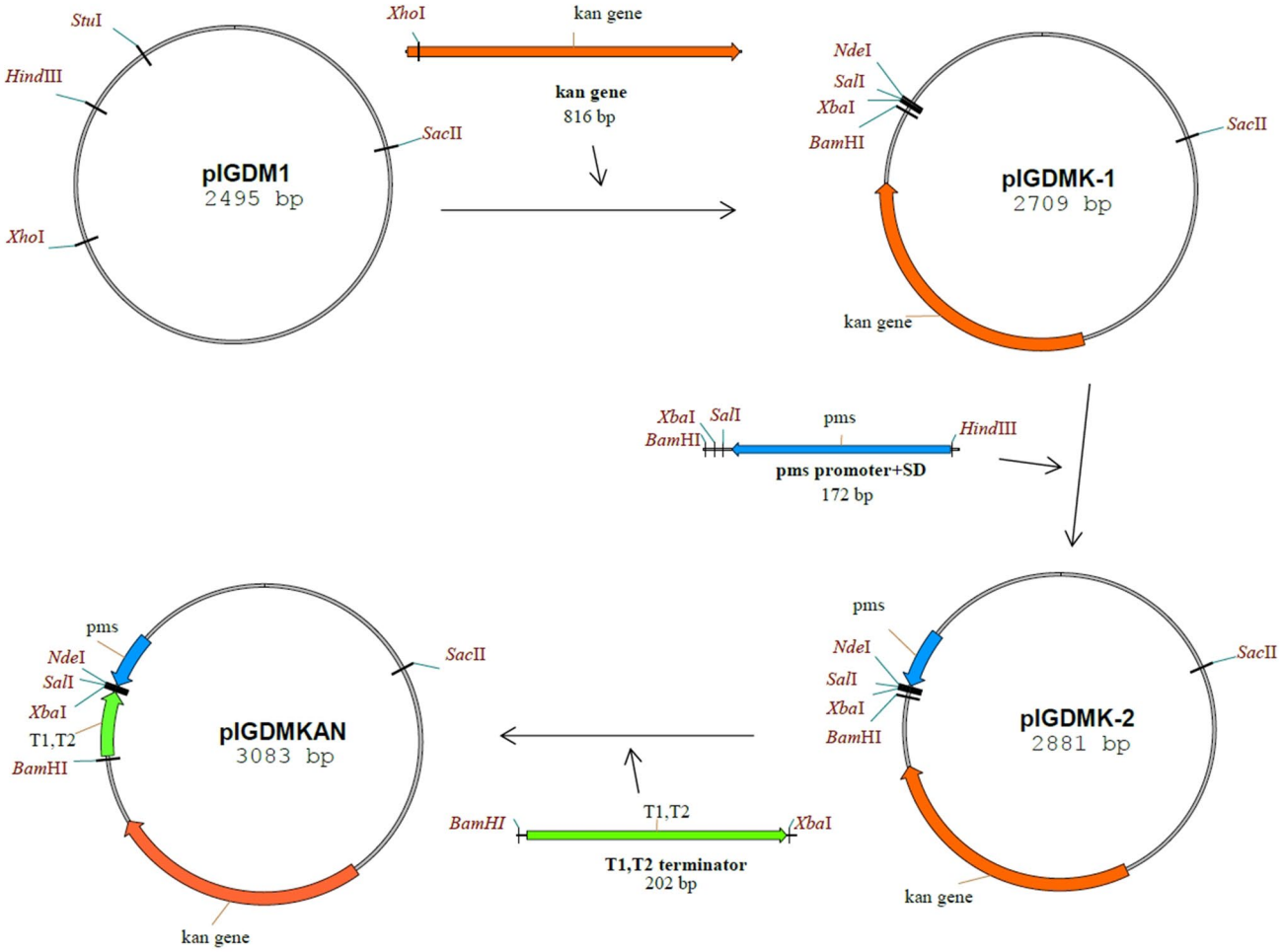

Fig. 3 The construction scheme of the pIGDMKAN expression vector. Abbreviations used: *kan gene* kanamycin resistant gene, *pms* pms promoter, *SD* Shine-Dalgarno sequence, *T1,T2* transcription terminators

and HindIII sites and is under control of the inducible *T7* promoter. The high efficiency of expression of the cloned cyclophilin gene was achieved after supplementation of IPTG (isopropyl ß-D-1-thiogalactopyranoside) in the culture medium. The results of these experiments are shown in Fig. 4. We carried out the comparison of the expression level of the gene coding for the yeast cyclophilin gene (*CPH*) in the pIGDM4RS + CPH plasmid and the pT7RS (GenBank: AY923866) vector, based on the commercial vector pT7. The results are shown in Fig. 7. The level of the gene overexpression was comparable in both cases. Densitometric analysis of the CPH protein in SDS-PAGE indicated protein productivity at the level of 8.5 mg/mL in the case of the plasmid, respectively. Therefore, the expression level from the newly constructed vector was as high as from the commercial one, while the new vector possesses all other advantages, described in this report.

The pIGDMCT7RS was used also as a backbone to clone the expression cassettes in a novel system for stable expression from *T7* promoter [22].

The Expression Plasmid pIGDM4RS

The primers DM1 and DM2 (Table 2) were used to amplify the beta-lactamase gene (*bla* gene, conferring ampicillin resistance) by PCR (polymerase chain reaction). The StuI site was introduced upstream of the *bla* gene, and the SalI site was designed downstream of the *bla* gene. This gene was inserted into the pIGDM1 plasmid to generate the pIGDM2 vector. Then, the short MCS, originating from pBlueSK(−) plasmid, was cloned yielding the pIGDM3 vector. The next vector, named pIGDM4, was constructed by ligation with the insert containing the *T7* promoter, SD sequence and the $tRNA^{Arg}$ gene with anticodon UCU/CCU. Finally, the ultimate pIGDM4RS expression vector was obtained by cloning the T7 transcription terminator sequence (Fig. 2). The

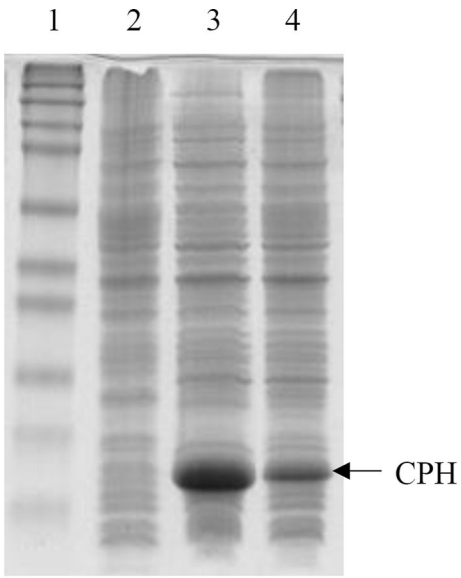

Fig. 4 SDS-PAGE analysis of *E. coli* BL21(DE3) producing the yeast cyclophilin (CPH). Proteins were separated in 15% acrylamide gel and stained with Coomassie Brilliant Blue. Lanes: 1—protein molecular marker (12–225 kDa) (Rainbow Marker—Full Range, Amersham, UK); 2—lysate of *E.coli* BL21(DE3) control; 3—lysate of *E. coli* BL21(DE3) transformed with pIGDMCT7RS + CPH vector induced with 1 mM IPTG for 2 h; 4—lysate of *E. coli* BL21(DE3) transformed with pIGDMCT7RS + CPH vector without IPTG

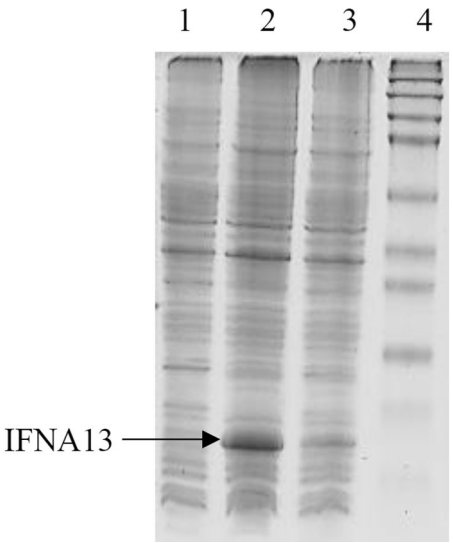

Fig. 5 SDS-PAGE analysis of *E. coli* BL21(DE3) producing the interferon alpha 13 (IFNA13). Proteins were separated in 15% acrylamide gels and stained by Coomassie Brilliant Blue. Lanes: 1—lysate of *E.coli* BL21(DE3) control; 2—lysate of *E. coli* BL21(DE3) transformed with pIGDM4RS + IFNA13 vector induced with 1 mM IPTG for 2 h; 3—lysate of *E. coli* BL21(DE3) transformed with pIGDM4RS + IFNA13 vector without IPTG; 4—protein molecular marker (12–225 kDa) (Rainbow Marker - Full Range, Amersham, UK)

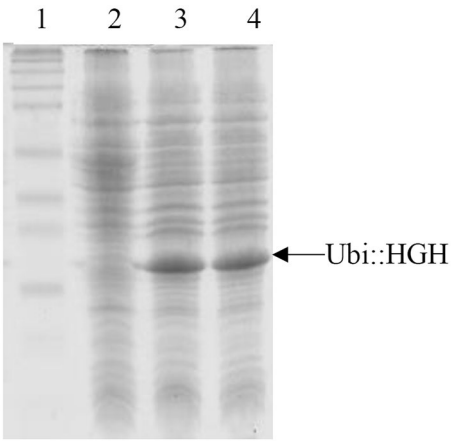

Fig. 6 SDS-PAGE analysis of *E. coli* DH5α producing a fusion protein of the ubiquitin-human growth hormone (Ubi::HGH). Proteins were separated in 15% acrylamide gels and stained by Coomassie Brilliant Blue. Lanes: 1—protein molecular marker (12–225 kDa) (Rainbow Marker—Full Range, Amersham, UK); 2—lysate of *E.coli* DH5α control; 3,4—lysate of *E. coli* DH5α transformed with pIGDMKUH vector (constitutive expression)

nucleotide sequence of pIGDM4RS was submitted to GenBank, and its accession number is HQ845200. To investigate the usefulness of these vectors as protein production tools in *E. coli*, the gene coding for interferon alpha 13 (*IFNA13*) was cloned between the NdeI and HindIII restriction sites, which put it under control of the *T7* promoter. The level of expression of the recombinant gene was verified by separation of proteins from cell extract in 15% SDS-PAGE (Fig. 5). Densitometric analysis of IFNA13 protein in SDS-PAGE indicated protein productivity at the level of 7.0 mg/mL. A comparison of the expression level of the gene coding for interferon alpha 13 (*IFNA13*) in two other expression vectors, pIGAL/IFNA13 and pIGDM5/IFNA13, was also carried out. The results of these experiments are shown in Fig. 8. Based on the SDS-PAGE analysis, it was found that only in the case of the use of the pIGDM4RS vector it was possible to obtain efficient expression of the *IFNA13* recombinant gene.

The Expression Plasmid pIGDMKAN

The basic vector pIGDMK-1 was obtained by inserting the kanamycin resistance gene (*kan*), originating from transposon EZ::TN < KAN-2 >, into the pIGDM1 plasmid. Several colonies of *E. coli* DH5α containing the insert were screened by PCR and sequenced. One clone, designated pIGDMK-1, was selected to the continued processing. Afterwards, the expression vector was constructed in two steps. First, the DNA fragment containing the constitutive *pms* promoter and SD sequence, amplified by PCR, was cloned. Then, two prokaryotic transcription termination sequences, T1 and T2,

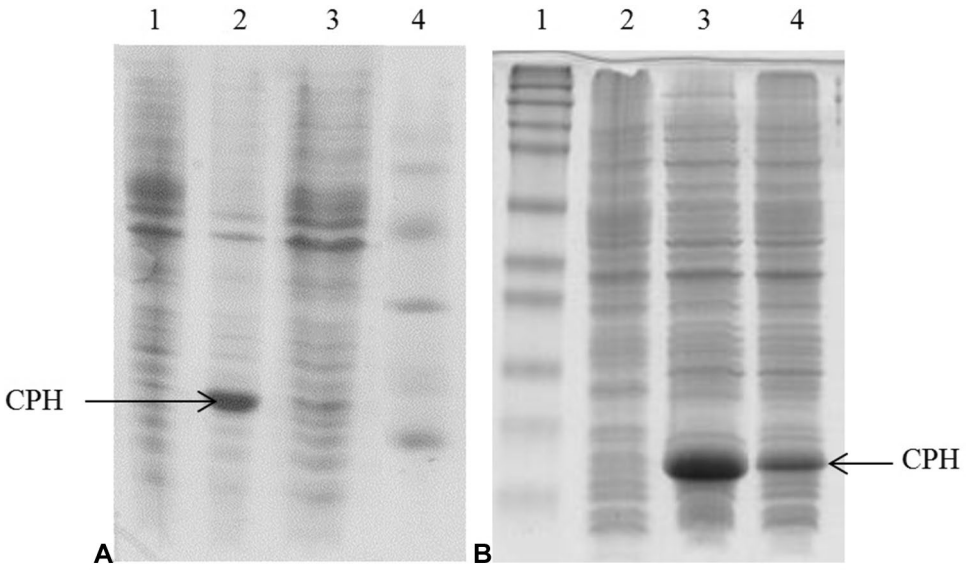

Fig. 7 Comparison of *CPH* gene expression in the SDS-PAGE. Proteins were separated in 15% acrylamide gels and stained by Coomassie Brilliant Blue. **a** Lanes: 1—lysate of *E.coli* BL21(DE3) control; 2—lysate of *E. coli* BL21(DE3) transformed with pT7RS + CPH vector induced with 1 mM IPTG for 2 h; 3—lysate of *E. coli* BL21(DE3) transformed with pT7RS + CPH vector without IPTG; 4—protein molecular marker (14.4-97.0 kDa) (Low Molecular Weight, Amersham, UK). **b** Lanes: 1—protein molecular marker (12–225 kDa) (Rainbow Marker—Full Range, Amersham, UK); 2—lysate of *E.coli* BL21(DE3) control; 3—lysate of *E. coli* BL21(DE3) transformed with pIGDMCT7RS + CPH vector induced with 1 mM IPTG for 2 h; 4—lysate of *E. coli* BL21(DE3) transformed with pIGDMCT7RS + CPH vector without IPTG

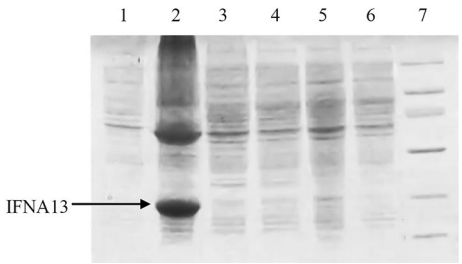

Fig. 8 Comparison of *IFNA13* gene expression in the SDS-PAGE. Proteins were separated in 15% acrylamide gels and stained by Coomassie Brilliant Blue. Lanes: 1—lysate of *E.coli* BL21(DE3) control; 2—lysate of *E. coli* BL21(DE3) transformed with pIGDM4RS + IFNA13 vector induced with 1 mM IPTG for 2 h; 3—lysate of *E. coli* BL21(DE3) transformed with pIGDM4RS + IFNA13 vector without IPTG; 4—lysate of *E. coli* DH5α transformed with pIGAL/IFNA13 vector (constitutive expression); 5—lysate of *E. coli* DH5α transformed with pIGDM5/IFNA13 vector (constitutive expression); 6—lysate of *E. coli* DH5α control; 7—protein molecular marker (14.4–97) (Low Molecular Weight, GE Healthcare)

were introduced. Finally, the recombinant plasmid was verified to confirm the correctness of the nucleotide sequence by DNA sequencing. The resulting expression vector was named pIGDMKAN (Fig. 3). To test the expression ability of pIGDMKAN, the fusion gene coding for ubiquitin-human growth hormone (Ubi::HGH) was cloned into the NdeI and SalI sites, yielding pIGDMKUH plasmid [23]. The nucleotide sequence of pIGDMKUH was submitted to the GenBank and its accession number is CS136259. The overexpression of the *Ubi::HGH* fusion gene was confirmed by separation of cellular proteins using 15% SDS-PAGE (Fig. 6). Densitometric analysis of Ubi::HGH protein in SDS-PAGE indicated protein productivity at the level of 4.3 mg/mL. The pIGDMKAN/Ubi::HGH plasmid was found to remain stable through 80 generations (four passages) in antibiotic-free cultures. This finding is especially important for the use of the vector in production of proteins in the pharmaceutical industry [24].

Discussion

The most widely used host for the overproduction of proteins is undoubtedly *E. coli*. Heterologous expression of proteins in *E. coli* has two great advantages, namely high efficiency of production in strains overexpressing recombinant genes, and easy and low-cost large-scale protein production.

In this study, we constructed a series of expression vectors, based on the ColE1-like pIGDM1 plasmid [14], that can be used in *E. coli* expression system. The pIGDMCT7RS and pIGDM4RS derivatives contain the inducible *T7* promoter, while the pIGDMKAN bears the constitutive *pms* promoter. The plasmids are relatively small, with many suitable cloning sites. They are compatible with

ColE1-like plasmids and they can also be used for creation of co-expression systems in *E. coli* cells. The ability of pIGDM1-derived vectors to co-exist with common replicons within bacterial cells demonstrated the possibility of constructing a two-plasmid system for co-expression of two proteins [15]. Moreover, it was demonstrated previously that copy number of pIGDM1-derived replicons can be easily up- and down-regulated in various host-plasmids systems [14]. For example, under standard laboratory conditions (LB medium, 37 °C), these plasmids occur in *E. coli* at the level of 10–12 copies per cell, while in the *pcnB* mutant, this value drops to 1–2 copies per cell. On the other hand, *rom*-defective derivatives of the plasmid are maintained at 30–40 copies per cell. Moreover, these plasmids can be amplified in vivo, in amino acid-starved *relA* mutants over 3-times, and in chloramphenicol-treated *E. coli* cells over 2.5-times [14]. Therefore, one can easily obtain *E. coli* hosts bearing the constructed cloning vectors with a wide range of copy number per cell.

A desired feature of a cloning vector is its stable maintenance in bacterial cells without a selective pressure. In this light, the frequency of pIGDMKAN vector loss was measured during bacterial growth for approximately 80 generations without antibiotic pressure, and stable maintenance of this plasmid was confirmed.

It has been shown that the AGA and AGG codons for arginine are the least used codons in *E. coli* [25]. The pIGDMCT7RS and pIGDM4RS vectors contain genes encoding AGA and AGG tRNAs, which supplement the shortage of these tRNAs that results from the codon usage in *E. coli.* The introduction of an additional pool of such tRNA molecules increases the efficiency of heterologous protein synthesis.

The recombinant proteins produced in *E. coli* frequently create the inclusion bodies. This was confirmed in the case of the *IFN-13* gene expression in the pIGDM4RS vector. Moreover, every protein needs a specific purification method appropriate to its structure. We have developed a unique and effective method for protein purification that allows to obtain a desired protein with a high purity [26]. Based on our experience, we are able to adjust the individual method of protein purification for various heterologous proteins.

In addition to the above-mentioned elements that have a direct impact on the efficiency of gene expression, the new vectors contain genes that confer antibiotic resistance of the host, which facilitates selection and propagation of plasmid-containing cells. The vectors named pIGDMCT7RS, pIGDM4RS and pIGDMKAN carry various antibiotic resistance genes: ampicillin (*bla* gene), chloramphenicol (*cat* gene) or kanamycin (*kan* gene), respectively. We assume that all developed vectors, because of their features, are appropriate tools for pharmaceutical companies and research laboratories to create a novel prokaryotic expression system.

Conclusions

In conclusion, a new expression system for overproduction of recombinant proteins in *E. coli* has been developed. Each of the described vectors possesses all the components which allow efficient cloning of the target gene, its controlled expression and convenient purification of the gene product (recombinant protein). Copy number of these vectors can be effectively regulated, with a wide range of values easily achievable. Compared to expression vectors reported previously, the novel system encompasses significantly improved features, including easy manipulation and stable maintenance of plasmids in *E. coli* as well as compatibility with vast majority of other plasmids.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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