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ARCHIVES OF CLINICAL MICROBIOLOGY

2013
Vol. 4 No. 5:4
doi: 10.3823/275

Isolation, heterologous expression and gene duplication of certain ribostamycin biosynthetic genes from *Streptomyces ribosidificus* NRRL B-11466

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Abstract

Background: To elucidate the biosynthetic pathways of ribostamycin and related antibiotics, cloning, expression and functional determination of certain genes out of the respective gene clusters have to be carried out.

Methods and findings: Analysis of the respective antibiotic biosynthetic gene clusters showed that the RibN (encoding a putative 5'''-epimerase) protein had a remarkable difference in its primary structure relative to NeoN, ParN, and LivN homologous proteins. In this study, the *rib*C from the ribostamycin biosynthetic gene cluster of *Streptomyces ribosidificus* NRRL B-11466, and the *par*N gene from the paromomycin biosynthetic gene cluster of *Streptomyces rimosus* subsp. *paromomycinus* NRRL 2455 were amplified using PCR, cloned into the cloning plasmid pUCPU21 producing pUCRC and pUCPN recombinant plasmids, respectively. RibC protein was expressed in *E. coli* JM109(DE3) under the control of *T7* promoter. The expressed RibC protein was analyzed and shown to produce the functional protein 2-deoxy-*scyllo*-inosose synthase which catalyzes the formation 2-deoxy-*scyllo*-inosose from glucose-6-phosphate. Alignment of RibN and its homologous proteins, by multiple amino acid alignment sequences, revealed a stretch of non-conserved amino acid sequence at the positions from 185 to 261 in the RibN amino acid sequence. Both *rib*C and parN were cloned into pUWL201PW shuttle vector producing pUWRC and pUWPN, respectively. The resulted recombinant plasmids were transformed into *S. ribosidificus* for the purpose of gene duplication and studying their influence on ribostamycin production.

Conclusion: RibC was successfully cloned, and heterologously expressed in *E. coli*. The produced protein was biochemically proven to be involved in the conversion of Glucose-6-phosphate (G-6-p) to 2-deoxy-*scyllo*-inosose. RibN was proven to have an endogenous frame-shift mutation which was proposed to be the reason for the formation of ribostamycin as an end product instead of neomycin. RibN was cloned into pUWL201PW shuttle vector as a prerequisite step for its influence on ribostamycin production.

Key words: 2-deoxy-*scyllo*-inosose, ribostamycin, *Streptomyces ribosidificus* NRRL B-11466, gene duplication, *rib*C, *par*N.

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2013
Vol. 4 No. 5:4
doi: 10.3823/275

Introduction

Ribostamycin (RIB) is a core compound and is regarded as an evolutionary ancestor in the neomycin (NEO) family of 2-deoxystreptamine-containing aminocyclitol aminoglycoside antibiotics (2DOS-ACAGAs); as it is a common precursor of all other family members including neomycin, lividomycin (LIV), paromomycin (PAR). The ribostamycin biosynthetic gene cluster has been cloned from *S. ribosidificus* by two independent research groups [1, 2]. The putative biosynthetic pathway to ribostamycin and related antibiotics was full described [1].
The original postulate that RIB is the precursor to all NEO-like ACAGAs led us to expect that the *rib*-cluster would be deficient in a *glycosyltransferase* gene for the third glycosylation (2nd hexosaminyltransfer step). This postulate also established the expectation that this *rib*-cluster would perhaps lack other genes needed to complete the subsequent steps in the modification pathway, which in turn would lead to the pseudotetrasaccharidic members of the neomycin family.

Therefore, it was surprising to find that the *neo*- and *rib*-gene clusters are practically identical in gene number and order (**Figure 1**).

Both gene clusters comprise 20 genes and are flanked by the two resistance genes encoding ACAGA modifying enzymes aminoglycoside phosphotransferase APH(3′) (*aphA, rph*) and aminoglycoside acetyltransferase AAC(3) (*aacC*). The remaining 18 genes should be sufficient to express the functional NEO pathway, in which RIB is an intermediate. Genes "C,S,E,M,D" and "Q,B" were shown to be involved in paromamine biosynthesis [3, 4, 5] and in the 6′-/6‴-transamination of hexosamine units [6], respectively. Therefore, they are also conserved in the gene clusters for all other 2-DOS/paromamine-ACAGAs, such as the kanamycin/gentamicin and fortamicin/istamycin families. Of the remaining 11 *rib*-genes, those conserved in all five clusters for NEO-type ACAGAs (including butirosin) are involved in the remaining steps common in all pathways and which constitute 5 -ribosyltransfer to the 2DOS unit. These are the "L, P" proteins and putatively also the "A" protein [3, 7, 8]. The "G, H, I" genes are postulated to encode a sensor/response regulator system, and the "T, U" genes are postulated to encode a drug exporter belonging to the ABC transporter superfamily. Thus, three genes remain to differentiate the close relatives of the NEO-family pathways (PAR, LIV, NEO, RIB); these are the genes "F, N, X". The encoded "F" protein was found to be involved in the glycosylation of RIB to give NEO [5]; surprisingly, the *rib*F gene does not show any indication for being nonfunctional or not being expressed. Therefore, the reason for the absence of the fourth sugar moiety from RIB has to be sought elsewhere. The "X" (possibly a regulatory protein) proteins do not show any evidence for being different between the NEO- and RIB-biosynthetic tools. Therefore, only the "N" proteins, being members of the Fe–S cluster-containing "radical SAM" oxidoreductases (suggesting a biosynthetic function; e.g., for 5‴-epimerization), remain for differentiating the NEO- and RIB-pathways from each other. Following the analysis of the biosynthetic gene clusters of the NEO family ACAGAs, the aim was to study the two genes *rib*C and *rib*N.

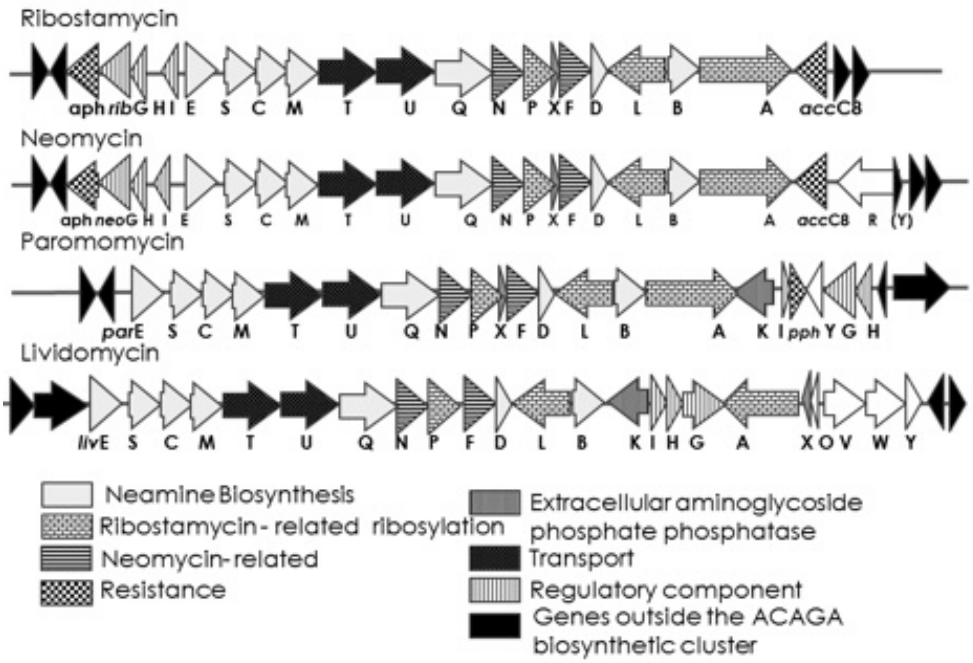

Figure 1. The Biosynthetic gene clusters of the Neo family of ACAGAs.[1.: Ribostamycin biosynthetic gene cluster (Accession code = AJ744850.1); neomycin biosynthetic gene cluster (Accession code = AJ629247.1); paromomycin biosynthetic gene cluster (Accession code = AJ628955.2); lividomycin biosynthetic gene cluster (Accession code = AJ748832.1).

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*rib*C was chosen as it is homologous to *btr*C and *neo*C which encode 2-deoxy-*scyllo*-inosose synthase (DOIS); the enzyme which catalyzes the conversion of Glucose-6-phosphate (G-6-P) to 2-deoxy-*scyllo*-inosose (DOI) [9, 10]. This is the first step in the biosynthetic pathway of all classes 2DOS ACAGAs. DOI is a unique cyclitol found only in these antibiotics and thus, a typical product of microbial secondary metabolism [11]. Therefore, DOIS catalyzes a key and critical step linking between primary and secondary metabolism in the cell. And since primary metabolites are abundant in the cell, this step may be rate limiting in the biosynthetic process. Consequently, it was proposed that homologous *rib*C expression in *S. ribosidificus* (i.e, gene duplication) may increase the antibiotic production. Homologs of *rib*C are conserved in the biosynthetic gene clusters of all 2-DOS-ACAGAs as well as Isatmycins [1, 12]. So if this proposal was proven to be true, this may be of significant effect on the production of a wide range (almost all clinically used) of ACAGAs which are mainly produced through fermentation.

The cloning of biosynthetic genes is a bottleneck for studying biosynthetic pathways or for their modulations [13]. Therefore, to test the two postulates (*rib*C gene duplication and *rib*N effect on production) and to heterologously express RibC, the aim was to clone the two genes *rib*C and *par*N from their corresponding strain.

Materials & Methods

Strains and culture conditions

The strains used in this study are listed in **Table 1.** *Streptomyces* strains were grown in ISP medium 2 (Bacto yeast extract, 4; malt extract, 5; glucose, 2g/l) and Tryptone soya broth (TSB) (Pancreatic digest of casein, 15; Enzymatic digest of soya bean, 5; Sodium chloride 5g/l). *E. coli* strains were grown on LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplied 100 µg/ml ampicillin (Sigma) when required. *E. coli* DH5α was used as the general cloning host. *E. coli* JM109 (DE3) was the host used for heterologous expression. *E. coli* ET12567 was used for the preparation of demethylated DNA for transformation into *Streptomyces*.

DNA isolation and manipulations

Genomic DNA extractions from *S. ribosidificus* and *S. rimosus* were done according to Kieser *et al.,* 2000 [16] based on the method developed by Pospiech and Neumann, 1995 [17]. Cloning, preparation and transformation of competent *E. coli* cells and *in vitro* DNA manipulations were carried out according the standard protocols [18].

Table 1. List of strains used in this study.

Strain	Relevant markers// product	Source / Reference
E. coli DH5α	F-, o80Δ lacZ ΔM15, endA1, recA1 hsdR17 (r-, m+), gyrA96, thi, relA1// General cloning host	[14]
E. coli JM109(DE3)	F-, recA1, supE44, endA1, hsdR17, gyrA96, thiΔ (lac-p roAB), Expression host	Novagen, Darmstadt, Germany
E. coli ET12567	F-, dam13, dcm6, hsdM, hsdR, recF143, galT22, ara14, lacY1, hisG4// To obtain demethylated DNA	[15]
S. ribosidificus	ribostamycin producer	NRRL B-11466
S. rimosus subsp. paromomycinus	paromomycin producer	NRRL 2455

PCR amplification

The *rib*C gene was amplified by polymerase chain reaction (PCR) using *S. ribosidificus* genomic DNA as a template, and the primers: RC-F (5′-AGGGCATATGCAGGTCACGC-3′) and RC-R (5′-CCCGTGCGGATCCACCGGCTA-3′). Likewise the gene *par*N was PCR amplified using *S. rimosus* genomic DNA as a template, and the primers PN-F (5′ CACCCCGCATATGACCACC-3′) and PN-R (5′-ACGGGCGTACCGGATCCGTCCGT-3′). The PCR was performed in thermal cycler, Biocycler TC-S (Boeco, Germany); using 2.5 U Go *Taq*® Flexi DNA polymerase (Promega). The reaction mixtures (50 µl) contained 250 ng of genomic DNA, 1µM of each primer, 200 µM of each dNTP, 3 mM $MgCl_2$, and 1X *Taq* buffer. The following conditions were used for the PCR reaction: the enzyme was added after an initial denaturation for 1 minute at 98°C, followed by 30 cycles [98°C for 45 seconds, (66°C *rib*C; 65°C *par*N) for 45 seconds, 72°C for 1 minute] and final elongation at 72°C for 5 minutes.

Plasmid construction

The PCR-amplified products, *rib*C and *par*N, were double digested using Fast digest® *Nde*I and *Bam*HI (Fermentas), and were cloned into the appropriate site of pUCPU21, and a resulting plasmids (pUCRC and pUCPN, respectively) were isolated. After DNA sequencing, the insert DNA (*rib*C) was

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Vol. 4 No. 5:4
doi: 10.3823/275

Table 2. Plasmids used and generated in this study.

Plasmids	Properties/Scope of use	Source/reference
pUCPU21	bla lacZ' ColE1-ori / cloning in *E. coli*	U. Wehmeier, Wuppertal, Germany
pET16b	bla lacI P_{T7-10} His10 tag ColE1-ori / expression in *E. coli*	[19]
pUWL201PW	bla tsr P_{emrE} ColE1-ori PIJ101-ori / shuttle vector, cloning and expression in Streptomyces spp.	[20]
pUCRC	1.183 kb NdeI/BamHI PCR fragment (primer ribC-F & ribC-R) in pUCPU21 (NdeI/BamHI)	This work
pUCPN	0.904 kb NdeI/BamHI PCR fragment (primer ribC-F & ribC-R) in pUCPU21 (NdeI/BamHI)	This work
pETRC	1.183 kb NdeI/BamHI fragment from pUC.ribC in pET16b (NdeI/BamHI)	This work
pUWRC	1.183 kb NdeI/BamHI fragment from pUC.ribC in pUWL201PW (NdeI/BamHI)	This work
pUWPN	0.904 kb NdeI/BamHI fragment from pUC.parN in pUWL201PW (NdeI/BamHI)	This work

isolated and purified after digestion with *Nde*I/*Bam*HI, and was again ligated into the *Nde*I-*Bam*HI sites of PET16b and pUWL201PW generating the recombinant plasmids pETRC and pUWRC, respectively. Likewise, the insert DNA in pUCPN (*par*N) was isolated, double digested (*Nde*I/*Bam*HI), purified and ligated into the *Nde*I-*Bam*HI sites of pUWL201PW generating the recombinant plasmids pUWPN. Moreover, the DNA inserts of the respective recombinant plasmids were also verified using DNA sequencing. **Table 2** shows the plasmids used in this study.

Overexpression of the *rib*C gene in *E. coli*

The recombinant plasmid, pETRC, was transformed into *E. coli* JM109 (DE3). The preculture of *E. coli* JM109(DE3)/pETRC was grown in LB medium (100µg/ml ampicillin) overnight at 37°C/300 rpm (xg= 1.12 x R x (RPM/1000)² to saturation. The preculture was used to inoculate a fresh LB/amp growth culture medium (1/100 of volume). The growth culture was grown at 37°C/300 rpm until the OD_{550} reached 0.5, at which point IPTG was added (final concentration of 1mM) and the incubation was continued at 30°C/55 rpm for 12 hours. About 1 ml aliquots were taken at zero, 1 hr, 2 hr, 4 hr and 12 hr after IPTG induction. For each aliquot, the cells were harvested by centrifugation (13,000 rpm/2-3minutes, 4°C), washed twice with ice-cold 25 mM Tris-HCl pH 7.5, and resuspended in 6 ml cell lysis buffer (25 mM Tris-HCl, pH 7.5; 1 mM Dithiothreitol; 3 mM β-mercaptoethanol; 10 mM $MgCl_2$) per gram *E. coli* cells. Cells were disrupted by sonication on ice (3 times, each for 45 sec with 30 sec intervals at 60 watt). The cell-free extract was clarified by centrifugation 13000 rpm/15 min at 4°C. Aliquots of the cell-free extracts were run on a 12% SDS-PAGE.

Assay of the expressed RibC protein

The activity of the 2-deoxy-*scyllo*-inosose synthase (RibC) was determined according to the protocol developed for the AcbC enzyme assay described by Stratmann *et al.,* 1999 [21] with minor modifications. The enzyme assay was performed at 30°C for 6 - 12 hr. The assay was done in a 100 µl reaction mixture containing 12mM G-6-P, 2.5 mM NAD, 4 mM NaF, 0.08 mM $CoCl_2$, 20 mM phosphate buffer (pH 7.5) and 30 µl of the soluble fraction of the cell free extract of RibC harvested 4 and 12 hr after IPTG induction. After incubation the reaction was stopped by heating at 95-100°C for 5 min and centrifuged at 13,000 rpm for 2-3 min. 4-6 µl of the reaction solution was analyzed by TLC and detected with cer reagent. Standard 2-deoxy-*scyllo*-inosose was kindly provided by Dr. Udo Wehmeier, Wuppertal, Germany.

Transformation of *rib*C and *par*N into *S. ribosidificus*

S. ribosidificus protoplasts were prepared according to Sambrook and Russell, 2001 [18] originally based on the work of Okanishi *et al.*, 1974 [22] which was optimized for high transformation frequency by Bibb *et al.*,1978 [23]. Mycelia were grown at 28°C/200 rpm for 36-40 hr in 25 ml TSB supplemented with 5% PEG 6000, 0.5% glycine and 5mM $MgCl_2$ with a stainless steel spring. The mycelia were pelleted by centrifugation at 3000 rpm/10 min, washed twice with 10.3% sucrose solution and resuspended in 4 ml L-buffer or lysozyme solution (lysozyme (MP biomedicals) dissolved in P-buffer at a concentration of 1 and 2 mg/ml) and incubated at 30-37°C for 15-60 min till protoplast formation was evident. 5 ml P-buffer (sucrose, 103; K_2SO_4, 0.25; $MgCl_2{\cdot}6H_2O$, 2.02

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g/800 ml, trace elements solution 2 ml/800 ml; dispense in 80 ml aliquots and autoclave; Before use add to each aliquot 0.5% KH2PO4, 1ml; 3.68% CaCl2.2H2O, 10ml; 5.73% TES buffer pH7.2, 10 ml. Trace elements solution: $ZnCl_2$, 40 mg; $FeCl_3.6H_2O$, 10; $CaCl_2$ $2H_2O$, 10; $MnCl_2.4H_2O$, 10; $Na_2B_4O_7.10H_2O$, 10; $(NH_4)6Mo_7O_{24}.4H_2O$, 10mg/l) was then added and the protoplast suspension was filtered through sterile cotton wool. The protoplasts were sedimented by centrifugation at 3000 rpm/7 min and resuspended in 1 ml P-buffer. The protoplasts were transformed according to Babcock and Kendrick, 1988 [24]. 5 µl of the demethylated recombinant DNA (isolated from the methylation-deficient strain *E. coli* ET12567) was added to 50 µl freshly prepared protoplast suspension and mixed immediately by finger tapping. 200 µl T-buffer (or 25% PEG 1500 in P-buffer) was immediately added and mixed gently by pipetting up and down several times. After the addition of 1.3 ml P-buffer, the cells were plated out onto pre-dried protoplast regeneration medium (SpMR). The cells were allowed to recover by an overnight incubation at 30°C, after which they were flooded with 1ml of dilute thiostrepton (MP biomedicals). Cells were allowed to grow under the appropriate selection pressure (50 µg/ml thiostrepton). Plates were checked for transformants after 2-5 days.

Results & Discussion

For an elucidation of the genetics and the biosynthetic pathways for the production of the ribostamycin and related antibiotics, the biosynthetic gene clusters of the respective antibiotics were fully isolated, sequenced, analyzed and annotated [1, 4, 25]. Analysis of the biosynthetic gene clusters for the major 2DOS-ACAGAs revealed greater similarities in the both gene/enzyme sequences and arrangement particularly to those involved in the biosynthesis of 2DOS moiety, the basic aglycone unit [1, 4, 25]. The similarity of the chemical structures of the regarded antibiotics was mirrored by their greater similarity in the respective gene clusters, gene content and sequence similarity in the individual genes/enzymes [1]. Basically, the biosynthesis of these antibiotics in their producers exhibits a common biosynthetic pathway which is then followed by further unique biosynthetic steps that will lead to the different members of this subclass [1]. Furthermore, both the rib- and neo-clusters are highly conserved and the only major difference found was that in the rib-cluster, a natural frame shift mutation created by two compensating frame shifts (+1 bp in position 544 and -1 in position 771) was seen in the *rib*N open reading frame (ORF) (encoding a putative 5'''-epimerase; **Figure 2**) [1].

Alignment of RibN and homologous proteins

The RibN protein shows a remarkable difference in its primary structure relative to those of the NeoN, ParN, and LivN proteins: A stretch of nonconserved amino acid sequence is observed at the positions from 185 to 261 in the RibN amino acid sequence as determined by multiple alignment sequences of the respective proteins (**Figure 2**). Moreover, this deviating sequence is created by two compensating frame-shifts (+1/–1) in the nucleotide sequence of *rib*N between which the sequence shows the same degree of conservation as that of the *neo*N gene as outside this region [1]. These results can be interpreted by either one or a combination of the following possibilities: (i) the RibF protein has lost its function; however, its production cannot be excluded because there are no features to be seen in the DNA sequence which would indicate its exclusion from transcription or translation; (ii) the natural frame-shifts occurring in the ribN ORF lead to an inactive RibN protein which could negatively affect this glycosylation process (RibN could be involved in other, as yet, unknown accompanying function(s) affecting the fidelity of the third glycosylation step); (iii) the trisaccharidic nature of ribostamycin could be a favorite substrate for the exporter system (RibT/U) and therefore, compete with the third glycosylation (RibF) which leads to pumping of ribostamycin outside the cell before being further glycosylated [1]. Therefore, The gene *par*N (without frame-shift mutation) from *Streptomyces rimosus* subsp. *paromomycinus* NRRL 2455 was also amplified using PCR and cloned into the cloning pUCPU21 and shuttle pUWL201PW vectors in an attempt for transformation and heterologous expression in *S. ribosidificus* and to study its role in ribostamycin production.

Construction of recombinant plasmids

Both *ribC* and *parN* were amplified using PCR from the genomic DNA of *S. ribosidificus* and *S. rimosus*, respectively. The forward primers were designed to introduce an *Nde*I site (underlined) in place of the natural start codon and for the ability to create a start codon fusion of *rib*C and *par*N downstream from the ribosome-binding-site of cloning pUCPU21, expression pET16b, and shuttle pUWL201PW vectors. On the other hand, the reverse primers were designed for the introduction of an alternate restriction site located immediately downstream of the natural stop codon in order to allow orientated cloning into the cloning and expression vectors. The recombinant plasmids pUCRC, pUCPN, pETRC, pUWRC, and pUWPN constructed in this work before and after *Nde*I/*Bam*HI digestion were tested and analysed using agarose gel electrophoresis (**Figure 3**, **Figure 4**).

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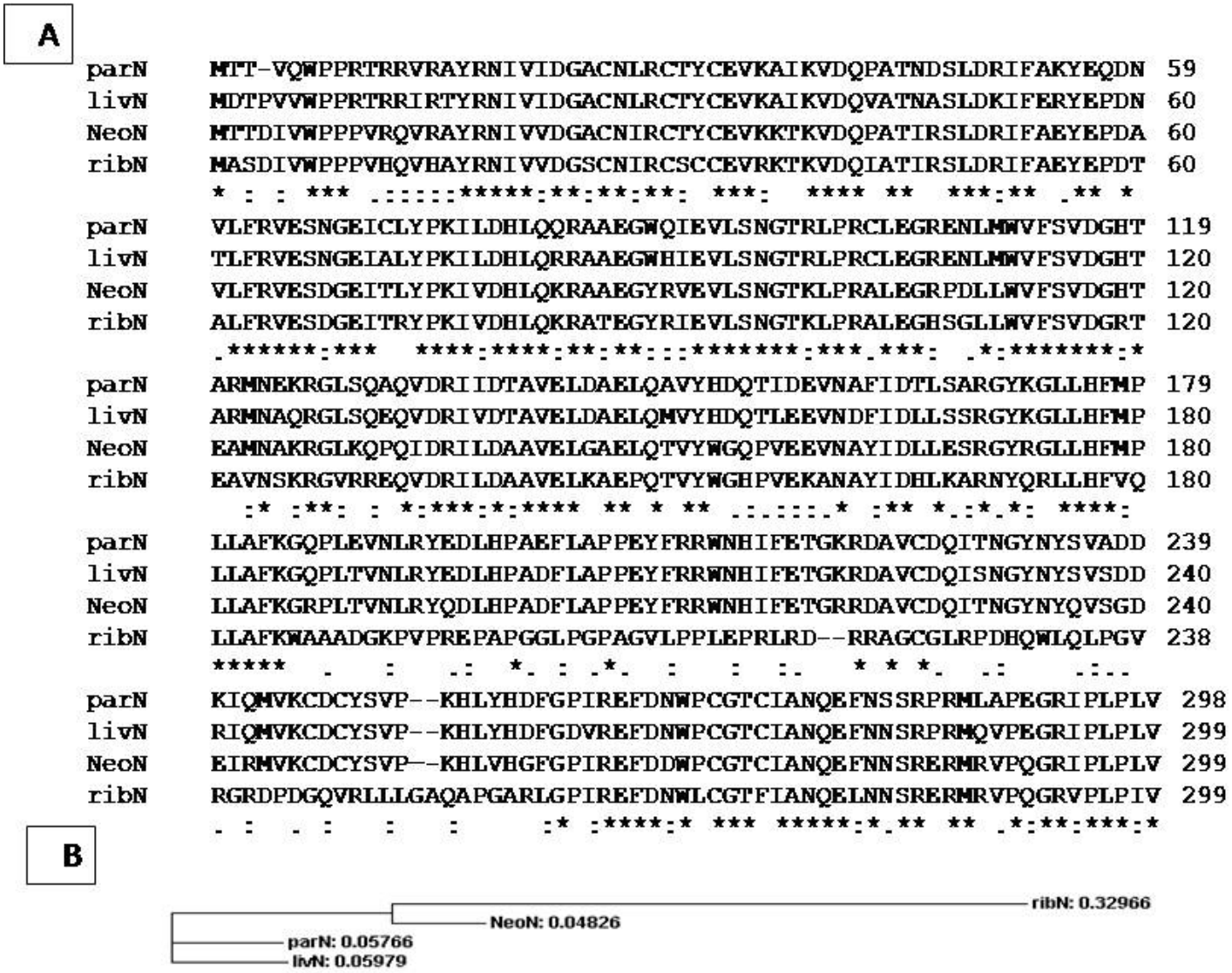

Figure 2. (A) Alignment of RibN and its homologous proteins (accession code = CAG34032) and the homologous proteins: ParN (accession code = CAF32379), livN (accession code = CAG38702and NeoN (accession code = CAF33317) and **(B)** their phylogram.

Expression of *rib*C in *E. coli* JM109(DE3)

As shown in **Figure 5**, RibC was overproduced as soluble N-terminal His-tagged protein in *E. coli* JM109 (DE3) as determined by SDS-PAGE. An additional band of about 42 kDa corresponding to the expected molecular mass of the His-tagged RibC protein was observed in the soluble fraction of the cell-free extracts. Maximal expression of RibC was 4h after IPTG induction at 30°C/55 rpm. In this work, the soluble N-terminally His-tagged RibC protein was only achieved via heterologous expression in the *E. coli* strain.

RibC catalyzed the formation of DOI

The soluble fractions containing His-tagged RibC protein [JM109(DE3)/pETRC] 4 and 12 hr after IPTG induction were used for analysis. Conversion of G-6-P into 2-deoxy-scyllo-inosose was detected by TLC using cer-reagent. A new spot on the TLC sheet with an Rf-value corresponding to the migration of the standard 2-deoxy-scyllo-inosose was observed in the enzymatic reaction containing the soluble 4 hr His-tagged RibC protein and to a lower extent in that containing the soluble 12 hr protein. In control reactions, containing the empty vector (pET16b) or containing no G-6-P. no conversion of G-6-P to 2-deoxy-scyllo-inosose was observed (**Figure 6**).

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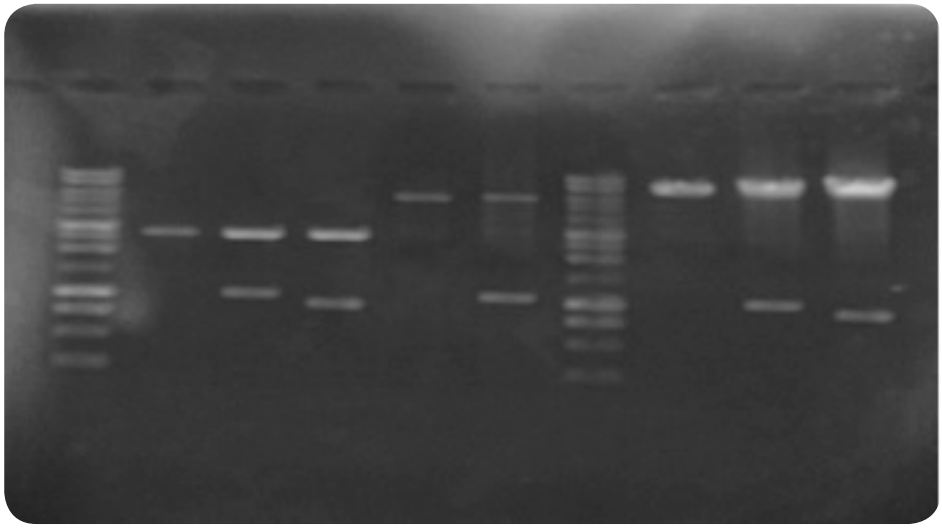

Figure 3. Agarose gel (0.8%) of *rib*C and *par*N clones. M: 1kb DNA ladder (Promega);Lane 1: pUCPU21 (*Nde*I/*Bam*HI); lane 2: pUCRC (*Nde*I/*Bam*HI); lane 3: pUCPN (*Nde*I/*Bam*HI); lane 4: pET16b (*Nde*I/*Bam*HI); lane 5: pETRC (*Nde*I/*Bam*HI); lane 6: pUWL201PW (*Nde*I/*Bam*HI); lane 7: pUWRC (*Nde*I/*Bam*HI) , lane 8: pUWPN (*Nde*I/*Bam*HI)

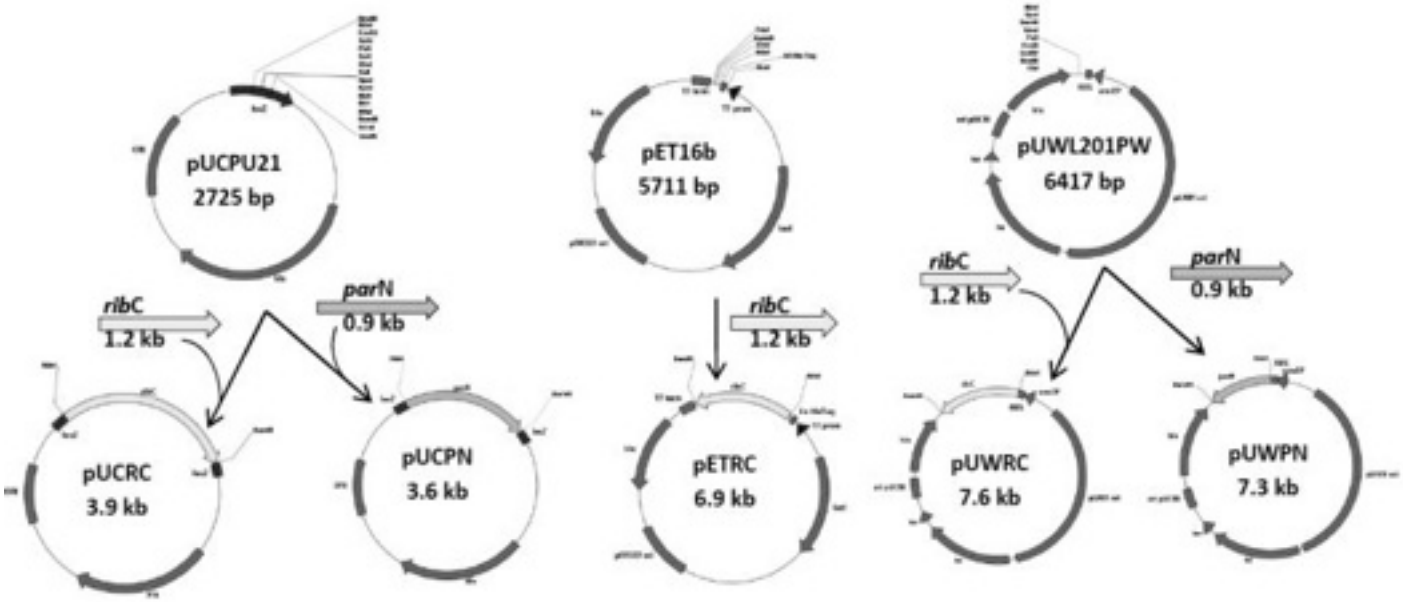

Figure 4. Schematic diagram of *rib*C and *par*N clones generated in this study.

Role of RibC in ribostamycin biosynthesis

DOI synthase switches the flux of G-6-P from the intercellular primary metabolite pool to a secondary metabolite biosynthetic route through the synthesis of a non-aminogenous cyclitol, DOI [26]. Thus, it is a key enzymatic step in the biosynthetic pathway of 2-DOS-containing ACAGAs. RibC was verified biochemically to be involved in the conversion of G-6-P into 2-deoxy-scyllo-inosose. In this context, a promising strategy that could lead to the higher production of ribostamycin, and probably all 2-DOS-containing ACAGAs, would be the construction of a cassette for the DOI synthase gene and its subsequent transformation into *S. ribosidificus* or other producing strains. Also, DOI is valuable as a starting material for the benzene-free synthesis of catechol and other benzenoids [27, 28, 29]. Protoplasts of *S. ribosidificus* were successfully transformed with pUWRC (PUW201PW + *ribC* gene, 1.2 kb) for the purpose of ribC gene duplication (one cope was located in the chromosome and other copy was on the recombinant shuttle vector, pUWRC). Results showed that the pUWRC transformant was able to grow on thiostrepton (50µg/ml)-containing SpMR plates, while the wild S. ribosidificus was unable to grow under these conditions. The pUWRC transformants were selected to be tested for their ribostamycin production in comparison to the wild strain.

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Vol. 4 No. 5:4
doi: 10.3823/275

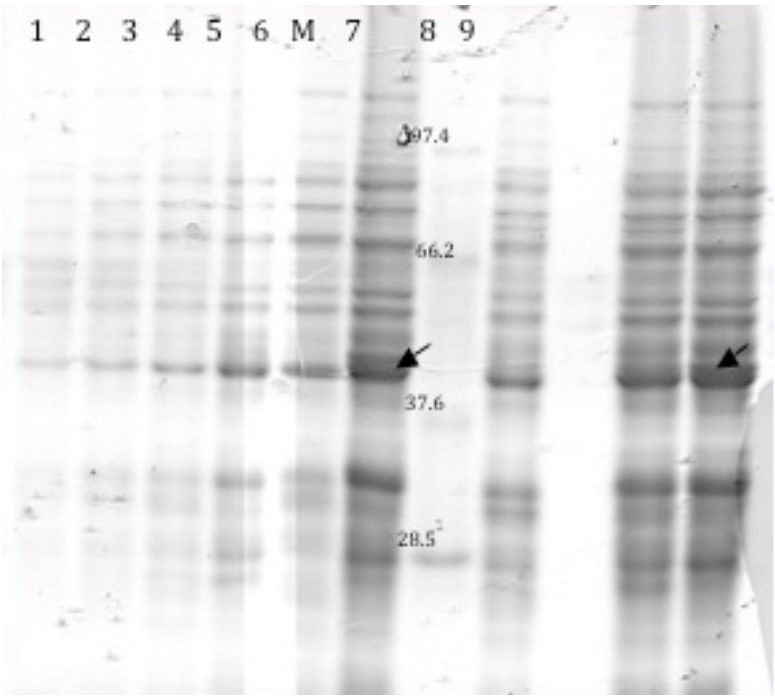

Figure 5. Laemmli SDS-PAGE gels(12%) of the cell-free extracts of *E. coli* JM109(DE3) harboring the following plasmids: pET16b, lane 1 (before induction), lanes 3, 5, 7, 8 were, 2 hr, 4 hr, 12 hr, 12hr after IPTG induction; pETRC (RibC), lane 2 (before induction), lanes 4, 6, 9 were 2 hr, 4 hr, 12hr after IPTG induction. M: protein marker in kDa (Jena Bioscience). Arrows point to the protein bands corresponding to the expected molecular mass of the RibC protein.

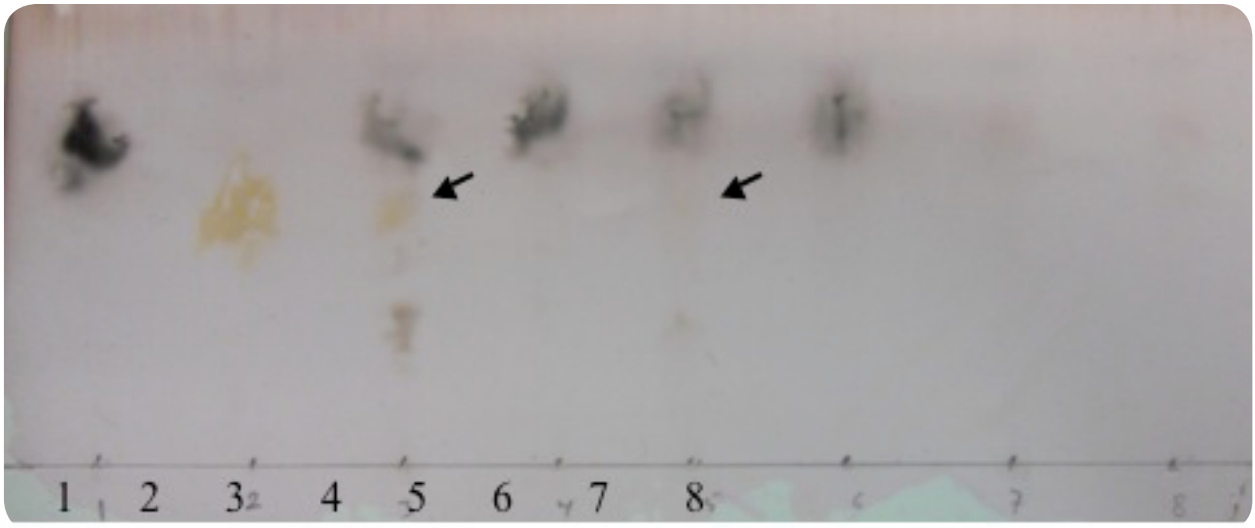

Figure 6. TLC analysis of RibC protein catalyzed conversion of G-6-P into 2-deoxy-scyllo-inosose. Lane 1: standard Glucose-6-phosphate; lane 2: standard 2-deoxy-*scyllo*-inosose; lane 3: pETRC 4 hr; lane4: pET16b 4hr; lane5: pETRC 12 hr; lane 6: pET16b 12hr; lane 7: PETRC 4hr, No G-6-P; lane 8: pET16b 4hr, No G-6-P.

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doi: 10.3823/275

Conclusion and prospective of this work

The detection of DOI synthase activity in the cell-free extract of *E. coli* JM109 (DE3)/pETRC represents the first report of expression of functional 2-deoxy-*scyllo*-inosose from *S. ribosidificus* in *E. coli*. DOI is valuable as a starting material for the benzene-free synthesis of catechol and other benzenoids. Both *rib*C and *par*N were successfully cloned into pUWL201PW shuttle vector producing pUWRC and pUWPN, respectively. The resulted recombinant plasmids were transformed into *S. ribosidificus* for the purpose of gene duplication and studying their influence on ribostamycin production.

Acknowledgement

We acknowledge Department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University for their support and providing equipment facilities.

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Vol. 4 No. 5:4
doi: 10.3823/275

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