Cloning, Expression and Knock-out of *ribE* gene involved in the Biosynthesis of Ribostamycin in *Streptomyces ribosidificus* NRRL B-11466

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ABSTRACT

Streptmyces ribosidificus NRRL B-11466 is a producer of ribostamycin, a 2-deoxystreptamine aminocyclitol aminoglycoside antibiotic (2DOS-ACAGA). Analysis of the ribostamycin and the related 2DOS-ACAGAs such as, paromamycin, lividomycin, butirosin, neomycin, kanamycin, gentamicin and tobramycin biosynthetic gene clusters showed a conserved ribE gene (1.023 kb) and its homologous. The ribE gene or its homologous, were anticipated to encode 2-deoxy-scyllo-inosamine 1-dehydrogenase required for the biosynthesis of 2DOS, the basic aglycone moiety in all 2DOS-ACAGAs. In order to investigate and prove the biochemistry of regarded gene product, cloning and expression of the ribE gene was carried out. The ribE was amplified via PCR from the chromosomal DNA of Streptomyces ribosidificus NRRL B-11466 using appropriate primers. The PCR product was cloned into cloning pUCPU21 and expression pET16b vectors. Heterologous expressions of RibE protein was performed under the control of the T7 promotor in Escherichia coli JM109 (DE3). The RibE protein (35.42 kDa) was obtained in a soluble His-tagged form as determined by SDS-PAGE and Western blot assay. The ribE⁻ knock-out mutant was created and showed no antibiotic production as compared to the wild-type. The knock-out mutant reproduced ribostamycin after plamid-mediated RibE expression confirming the involvement of RibE protein in the biosynthesis of ribostamycin.

Key words: *Streptmyces ribosidificus NRRL B-11466, ribostamycin biosynthesis, 2-deoxy-scyllo-inosamine 1-dehydrogenase, 2-deoxystreptamine biosynthesis, 2DOS*

INTRODUCTION

Aminocyclitol aminoglycoside antibiotics (ACAGAs) are heterogeneous chemical class of natural products composed of most strongly modified sugar units bound glycosidically (mono- to oligosaccharides) and cyclitol derivatives (aglycone) containing amino nitrogen[(1-5)]. ACAGAs are idiolities synthesized by certain soil bacteria mainly by actinomycetes, bacilli and pseudomonades[(6-8)].

They are considered one of the most prominent classes of broad spectrum bactericidal antibiotics. Their bactericidal activities are evolved principally from their interaction with the 16S rRNA, leading to codon misreading and hence inhibit protein synthesis. The 2-deoxystreptamine (2DOS; 1,2,3-trideoxy-1,3-diamino-*scyllo*-inositol)-containing antibiotics comprises the most imperative subclass of this group. This subclass includes several clinically important members such as, ribostamycin, neomycin, kanamycin, and gentamicins. The 2DOS-ACAGAs include both pseudotetrasaccharidic antibiotics such as neomycin (producers are *Streptomyces fradiae* and *S. albogriseus*), paromomycins (*S. rimosus* ssp. *paromomycinus*), lividomycins (*S. lividus*) and pseudotrisaccharidic antibiotics such as ribostamycin (*S. ribosidificus*) and butirosin (*Bacillus circulans*). In addition, they shared a common pseudodisaccharidic intermediate, paromamine (D-glucosamine-alpha-1,4-2DOS) as well as glycosylation of the 2DOS unit at 4 and 5 positions (Fig. 1; 8).

The biosynthetic gene clusters for the above mentioned members of antibiotics were fully sequenced and analysed[(9-11)]. 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[(9)]. 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[(8,11,12)]. As regards to the biosynthetic pathway to 2DOS, certain enzymatic steps have been proven to be involved in the formation of 2DOS moiety. These enzymatic reactions were carbocyclization (2-deoxy-*scyllo*-inosose synthase; NeoC; 9, 12-16) followed by a transamination reaction (L-glutamine: 2-deoxy-

scyllo-inosose aminotransferase; NeoS; also designated BtrR by Huang *et al*., 2005; Tamegai 2002b; Kharel *et al*., 2005[16-18] to form 2-deoxy-*scyllo*-inosamine. The third step was expected to be encoded by 3-aminocyclitol 1-dehydrogenase; RibE or its homologous) to form 1-keto-2,3-deoxy-3-amino-*scyllo*-inositol, a keto intermediate which would be further transaminated by the same aminotransferase (L-glutamine: 2-deoxy-*scyllo*-inosose aminotransferase; NeoS; bifunctional enzymes) to form the 2DOS[9,14,19,20].

The biosynthesis of paromamine was predicated to be via glycosylation of the 2DOS moiety at 4 position with a nucleoside activated D-glucosamine where both D-glucosamine-1-phosphate nucleotidyltransferase (RibD or its homologous) and UDP-D-glucosamine: 2-deoxystreptamine 4-glycosyltransferase (RibM or its homologous) would be involved[10,21]. Amination of 6'-hydroxyl group would be the next enzymatic step during the course of ribostamycin biosynthesis in order to form neamine (6'-amino-6'-deoxyparomaine; also known as neomycin A), an intermediate isolated from both bulk fermentation of *S. fradiae* and *S. ribosidifcus*[17].

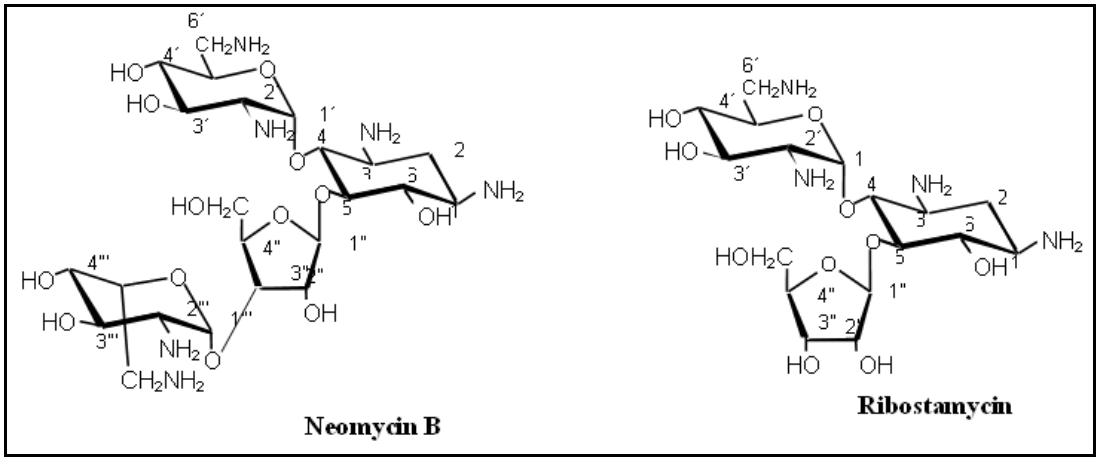

Fig. 1 Chemical structures of ribostamycin and neomycin B. (represent examples of 4,5-diglycosylated ACAGAs[5, 9].

Several studies using labelled precursors and blocked mutants had identified that 2DOS, paromamine, neamine and ribostamycin are intermediates in neomycin biosynthesis[22-25]. Homologous to RibE were also found to be conserved in the other 2DOS-ACAGAs gene clusters of paromomycin (*par*-cluster; ParE), neomycin (*neo*-cluster; NeoE), lividomycin (*liv*-cluster; LivE) and butirosin (*btr*-cluster; BtrE) where similar biosynthetic functions were required (Fig. 2; 9, 10, 11).

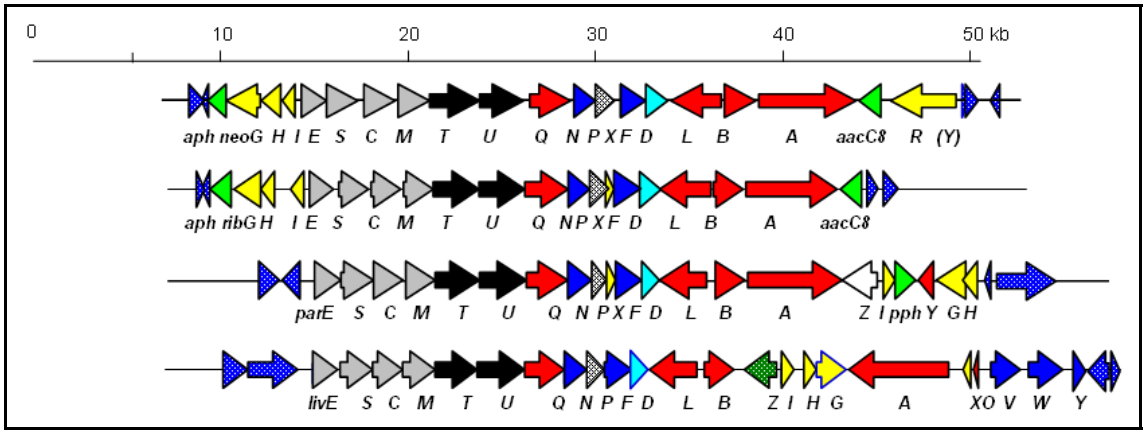

Fig. 2: Organization of open reading frams (ORFs) of the biosynthetic gene clusters of the 4,5-diglycosylated 2DOS-ACAGAs[9].

In the present study, isolation of *rib*E gene from *S. ribosidificus* NRRL B-11466 via PCR was carried out. The *rib*E was cloned and heterologously expressed in *E. coli* JM109 (DE3) as a prerequisite step for the biochemical characterisation of the respective gene product. The *rib*E knock-out mutant was also created and tested for ribostamycin production.

MATERIALS & METHODS

Bacterial strains, culture media and vectors

S. ribosidificus NRRL B-11466 (ribostamycin producer) was cultured in tryptic soy broth (TSB; 26), or on soy bean mannit agar (SMA; 27) at 28°C. *Escherichia* (*E.*) *coli* DH5α (28) and *E. coli* JM109 (DE3) (Novagen, Germany) were used as hosts for cloning and heterologous protein expression, respectively. All *E. coli* strains were grown on Luria Bertani (LB; 29) liquid or agar medium at 37°C and selected with 100 μgml^{-1} ampicillin and/or X-Gal as a selective medium whenever necessary. The plasmids pUCPU21 (2.725 kb, Wehmeier U., Wuppertal), pET16b (5.711 kb, Novgen, Germany), and pUWL201PW (6.4 kb, Wehmeier U., Wuppertal) were used for gene cloning, expression in *E. coli*, shuttle plasmid, respectively.

Cloning and DNA sequencing of *dpr*A gene

DNA cloning and further manipulations were carried out according to methods described by Hopwood (2003), Kieser et al. (2000) and Sambrook and Russell (2001)[26,30.31]. Preparation, transformation and regeneration of competent *E. coli* cells were performed by standard protocols of Hanahan (1983)[28]. Isolation of chromosomal DNA of *S. ribosidificus* NRRL B-11466 was achieved according to the method of Pospiech and Neumann (1995)[32]. PCR was performed in a Nyx-Technik Inc. Personal Cycler (ATC401, USA). Each assay (50 µl) consisted of 200 ng chromosomal DNA, 100 pmole of each appropriate primer, 0.2 mM dNTPs (Invitrogen, Karlsruhe, Germany), 3 mM $MgCl_2$, 10% DMSO to improve the denaturation of the template DNA and 2 U *Taq* DNA polymerase (Sigma, USA). The two primers were: REK-F (*Nde*I): 5'-TCGGCCATATGAAGGCCCTGGTG-3'and REK-R (*Eco*RI): 5'-ATCGGGGCGAATTCGGCC -3' were designed for amplification of *rib*E gene (1.023 kb). PCR was performed under the following conditions: (98°C, 5 min, then 30 cycles [95°C, 45 sec, 58°C, 45 sec, 72°C for 1 min] and 72°C for 5 min. The PCR products were subjected to agarose gel electrophoresis (0.8%) and the expected DNA fragments were extracted and purified using QIA quick Gel extraction kit (Qiagen, Hilden, Germany). The *rib*E gene was digested with *Nde*I/*Eco*RI and inserted into pUCPU21 (*Nde*I/*Eco*RI) and pET16b (*Nde*I/*Eco*RI) to form pURE3/pETRE3 recombinant plasmids, respectively. Plasmid DNA extraction from *E. coli* strains was carried out according method described by Birnboim and Doly (1979)[33]. The nucleotide sequences of *rib*E was verified by DNA sequencing according to Sanger et al. (1977) using the A.L.F. DNA sequencer (Amersham-Pharmacia Biotech, Freiburg, Germany) and the Thermo-Sequenase sequencing kit (Amersham-Pharmacia Biotech, Freiburg, Germany) before being transformed[34].

Heterologous expression of DHDPS in *E coli* BL21 (DE3)

Expression of *rib*E in *E. coli* was carried out as described by Studier et al. (1990)[35]. Expression was performed under the control of the *T7* promotor using *E. coli* JM109 (DE3) strains. Single colony harbouring the plasmid pETRE3 (RibE), or pET16 (empty vector as a control) were grown overnight in 3 ml LB medium containing 100 μgml^{-1} ampicillin at 37°C on a shaker incubator 250 rpm. 200 µl of these precultures were used to inoculate 20 ml fresh LB medium in 250 ml normal flasks and let them grow at 30°C on shaker incubator 120 rpm to an OD_{600} of 0.5 - 0.6. Induction of *T7*-RNA polymerase was achieved by the addition of 0.5 mM IPTG. Cells 1, 2 and 4 hs after IPTG induction were harvested by centrifugation (13,000 rpm/3 min), washed twice with ice cold 25 mM Tris-HCl, pH 7.5. Cell pellets were then suspended in the appropriate amounts of cell lyses buffer (50 mM Tris-HCl, pH 7.5; 1.0 mM dithiothreitol; 10.0 mM $MgCl_2$; 3.0 mM β-mercaptoethanol) and lysed by sonification. Protein concentrations were quantified according to process described by Bradford (1976)[36]. Electrophoresis of proteins was performed using SDS-PAGE according to Laemmli (1970) where the final concentration of acrylamide in the focus and separating gels was 5.5% and 12%, respectively[37]. Gels were stained with 10 - 15 ml gel staining solution (Coomassie Brilliant Blue R250 $1.5gl^{-1}$; methanol 450.0 ml^{-1}; acetic acid 100.0 ml^{-1}) for 2 - 3 h or overnight with gentle shaking at 55°C. The staining solution was discarded and the gels were then destained using gel destaining solution (methanol 250.0 ml^{-1}; acetic acid 100.0 ml^{-1}; distilled water 650 ml^{-1}) for 1 - 2 h at

55°C. Western blotting was applied to detect His-tagged DHDPS and the method of immuno-detection of proteins was adapted as recommended by the provider of BM Chromagenic Western Blotting Kit (Roche-Mannheim) using an anti-His-tag antibody.

Construction of *rib*E knock-out mutant

The pURE3 plasmid was restricted with *Sma*I and ligated into the apramycin resistances cassette (*aacC*4; 38) restricted with *Sma*I at both ends to form the construct pUREAP5. In addition, the plasmid pURE3 was restricted with *Nde*I/*Eco*RI and the resulted passenger DNA fragment (1.0 kb) was cloned into pUWL201PW shuttel plasmid (Wehmeier U., Wuppertal) restricted with *Nde*I/*Eco*RI to form pERW3 recombinant plasmid. Both pUREAP5 and pERW3 were prepared from methylase deficient *E. coli* ET12456 (MacNeil et al. 1992) and were used for transformation of the wild and *ribE* mutant *S. ribosidificus* protoplasts according to protocol described by Babcock and Kendrick (1988), respectively[39]. The plates were incubated overnight at 28°C and thereafter were overlaid with apramycin 50 $\mu g ml^{-1}$ and reincubated for 3-7 days. The apramycin resistant transformants were selected and cultivated on SMA agar for 5 days. Testing antibiotic production by the *ribE* mutant and the wild-type *S. ribosidificus* were carried out. Each stain was cultivated in TSB broth and incubated for 72 hs at 28°C. About 1 ml from each culture was inoculated onto SMA plate and incubated for 5 days at 28°C. Agar plug from each plate was taken and loaded onto the surface of the *E. coli*-seeded LB agar plate. Incubation was made overnight at 37°C and formation of antibiotic inhibition zones was recorded.

Nucleotide sequence accession numbers

The protein sequences of RibE protein reported in this study is available in the EMBL/GenBank database with the accession code CAG34039.1. EMBL/nucleotide sequences accession code AJ744850.

Computer-assisted analysis of DNA and protein sequences

The programs used for computer-assisted analysis of nucleotide and protein sequences were Staden package[40]. Restriction analysis was carried out using: Restriction Enzyme Site Mapper version 3 (http://www.restrictionmapper.org/). PCR amplification temperatures were computed using pDRAW32 (http://www.acaclone.com). Protein molecular weight was calculated using The Sequence Manipulation Suite: Protein Molecular Weight (http://www.bioinformatics.org/sms/prot_mw.html).

RESULTS

Isolation and cloning of both *ribE* gene

Agarose gel electrophoresis of the PCR reaction using chromosomal DNA of *S. ribosidificus* as a template and both REK-F/ REK-R primers showed a DNA band corresponded to the expected size (about 1.0 kb) of the *ribE* gene (Fig. 3A). The PCR product was extracted from the agarose, restricted with *Nde*I and *Eco*RI and ligated into pUCPU21 (*Nde*I/*Eco*RI). About 10 µl of the ligation reaction was transformed into cells of competent *E. coli* DH5α. Six transformants were selected and their plasmids were digested with *Nde*I/*Eco*RI endonucleases. As shown in Fig.3B, the six isolated plasmids (lanes 1-6) except number 2 and 5 contained passenger DNA fragments correspond to the size of *ribE* PCR product (1.1 kb; Fig. 3B). Morover, plasmid analysis was performed where the selected six plasmids were futher digested with *Nde*I/*Sac*II endonucleases. As shown in Fig. 3B (lanes 7-12), two DNA bands of 0.55 and 3.3 kb were only detected with the isolated plasmids from transformants number 3 and 6. The results obtained together with DNA sequencing confirmed that transformants number 3 and 6 were the right clones that harboured the entire *rib*E gene. The recombinant plasmid of the transformant number 3 was selected and named pURE3 (*rib*E; Fig. 4).

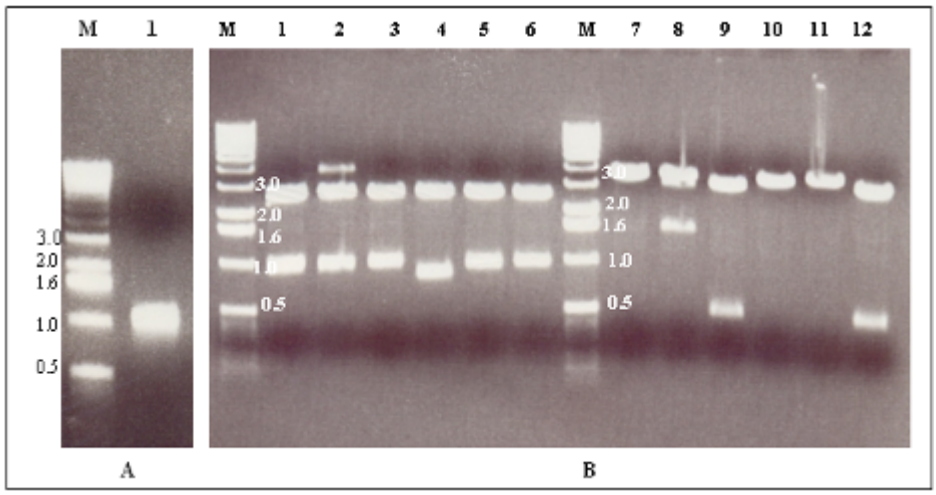

Fig. 3 Agarose gel electrophoresis (0.8%): A: lane 1, PCR product using REK-F and REK-R primers; **B**: lanes 1-6, plasmids extracted from 6 selected transformants digested with *Nde*I/*Eco*RI endonucleases; lanes 7-12, plasmids extracted from the 6 selected transformants (lanes 1-6) digested with *Nde*I/*Sac*II endonucleases. Lane "M" is 1.0 kb DNA ladder (Invitogen, Karlsruhe, Germany).

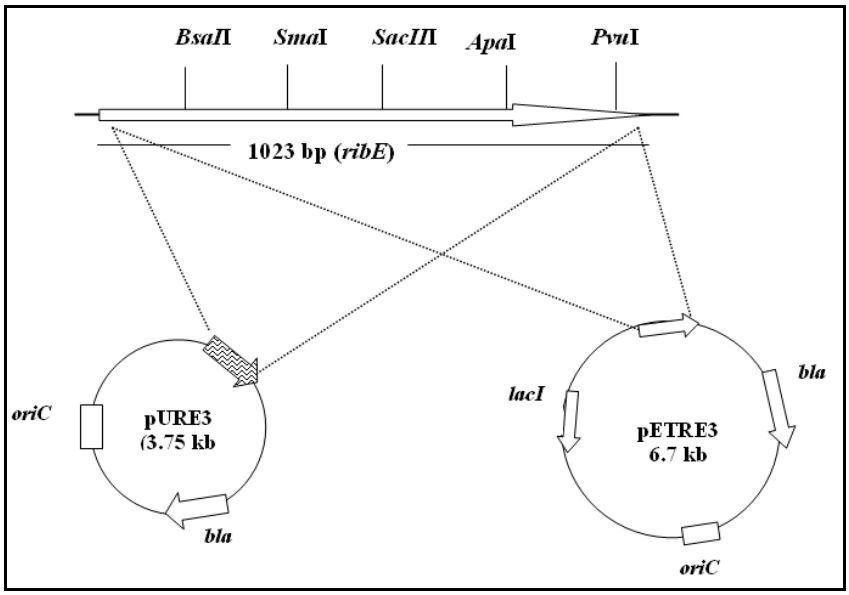

Fig. 4 Restriction analysis of the *rib*E gene and the constructed recombinant plasmids pURE3 and pETRE3. Arrows indicate direction of the ORFs.

The plasmid pURE3 was further digested with *Nde*I/*Eco*RI and the obtained passenger DNA (1.0 kb) was cloned into pET16b also restricted with *Nde*I/*Eco*RI to form the pETRE3 recombinant plasmid (Fig. 4).

Heterologous expression of RibE protein

RibE was overproduced as a soluble N-terminal His-tagged protein in *E. coli* JM109 (DE3) as determined by SDS-PAGE (Fig. 5). Additional band of about 35.4 kDa present in the soluble fraction of the cell-free extracts corresponded in size to the expected molecular masses of the respective His-tagged protein was detected. Result showed that maximal overexpression of the DHDPS was 4 h after IPTG induction. The presence of multiple histadine amino acid residues in the overexpressed N-terminal His-tagged RibE protein was also demonstrated by Western blot assay (Fig. 6)

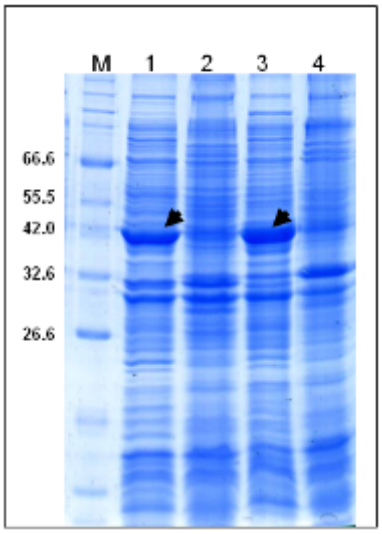

Fig. 5 Laemmli SDS-PAGE gels (10%) of the cell-free extracts of *E. coli* JM109 (DE3) harbouring: lanes 1 & 3, pETRE3 (RibE; 2 & 4 hs after IPTG induction); lane 2 & 4 (pET16b; 2 & 4 hs after IPTG induction). M indicates protein marker (kDa; New England Biolabs). Arrows mark protein bands corresponding to the molecular mass of the expressed RibE protein

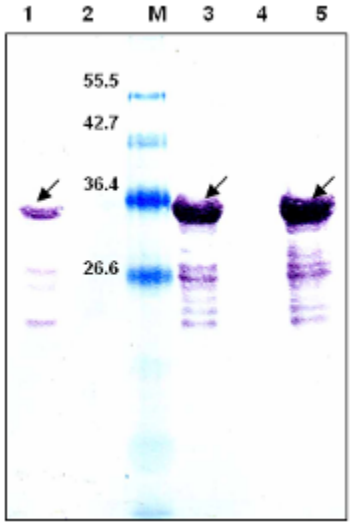

Fig. 6 Detection of His-tagged RibE protein by Western Blot. Lanes 1, 3,5: cell free extracts of *E. coli* JM109 (DE3)/pETRE3 (RibE; 1, 2, 4 hs after IPTG induction); lanes: 2, 4 cell-free extracts of *E. coli* JM109 (DE3)/pET16b (control; 2 and 4 hs after IPTG induction). M indicates protein marker (kDa). Arrows mark protein bands corresponding to the molecular mass of the expressed RibE protein.

Construction of *ribE* knock-out mutant

The *ribE* gene in *S. ribosidificus* was knocked-out via in-frame insertion of the apramycin gene cassette. Results showed that the *ribE* mutant was able to grow on TSB agar containing apramycin (50 μml^{-}) while the wild stain was not able to grow under this condition. Further verification of construction of the *ribE* mutant was carried out using PCR using chromosomal DNA of both *ribE*$^{-}$ mutant and the wild strain as templates and REK-F and REK-R primers (Fig.7). A PCR product of about 1.0 kb corresponding in size to the expected size of the *ribE* gene was only detected in case of using chromosomal DNA of the wild strain. No PCR product was detected upon using chromosomal DNA of the *ribE* mutant strain.

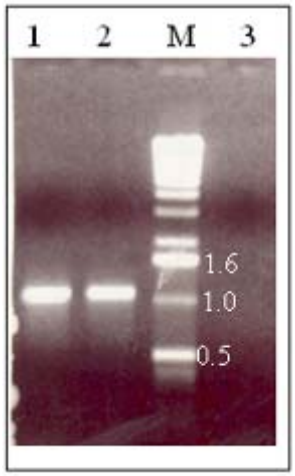

Fig. 7 Agarose gel electrophoresis (0.8%) of PCR products using REK-F and REK-R primers and the following templates: lane 1, chromosomal DNA of *S. ribosidificus* (wild strain); lane 2, plasmid pERW3; lane 3, chromosomal DNA of *ribE* mutant *S. ribosidificus*. Lane "M" is 1.0 kb DNA ladder (Invitogen, Karlsruhe, Germany).

Both wild and *ribE* mutant were tested for antibiotic production. An inhibition zone was only detected upon use of the wild strain however, no inhibition zone was observed with the *ribE* mutant strain (Fig. 8). Morover, the *ribE* mutant regained antibiotic production (presence of inhibition zone) after being transformed with the shuttel pERW3 (Fig. 8; *ribE* gene cloned into pUWL201PW plasmid; Wehmeier U, Wuppertal).

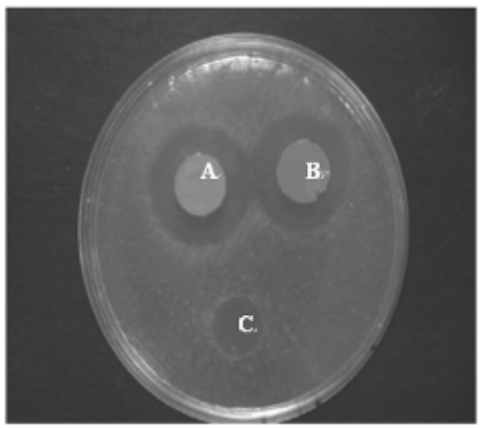

Fig. 8 Detection of antibiotic inhibition zones on an *E. coli*-seeded LB agar plate: **A**: *S. ribosidificus* NRRL B-11466 (wild strain); **B**: *ribE* mutant of *S. ribosidificus* harbouring pERW3; **C**: *ribE* mutant of *S. ribosidificus*.

DISCUSSION

For 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[10,11,21]. 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[10,11,21]. 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[10]. 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 (Fig. 9; 13, 21).

Most of the attempts to elucidate the biosynthetic pathway for 2DOS by *in vivo* labelling and enzymological methods in the crude extracts of their producing strains have been made using the producers of neomycin-type AGAs[25,41,42]. Similar observations were made in gentamicin and butirosin producers, in which 2-deoxy-*scyllo*-inosose and 2-deoxy-*scyllo*-inosamine could be demonstrated to be precursors of 2DOS[25,41,42]. Only for the BtrC-related enzymes KanC/GenC (2-deoxy-*scyllo*-inosose synthases or cyclases) and for the StsC-related aminotransferase enzymes KanS1/GenS1/ForS (L-glutamine:ketocyclitol aminotransferases I and II) a clear assessment of their functions can be postulated on the basis of published enzymological data[6,12,15-17,43,44,45]. Moreover, a relatively save prediction on the involvement in the 2DOS pathway of the oxidoreductases belonging to the "E"-type gene products (RibE and its homologous; putative cyclitol 1-dehydrogenases) could already be made on the basis of their distribution and level of conservation among the clusters for 2DOS-AGAs (Piepersberg et al., 2007). Therefore, to confirm the biochemical function of RibE, the respective gene (*ribE*) was cloned and heterologously expressed. For cloning of the *ribE* gene, two homologous primers (REK-F/ REK-R) were designed to amplify the *ribE* gene using the chromosomal DNA of *S. ribosidificus* NRRL B-11466.

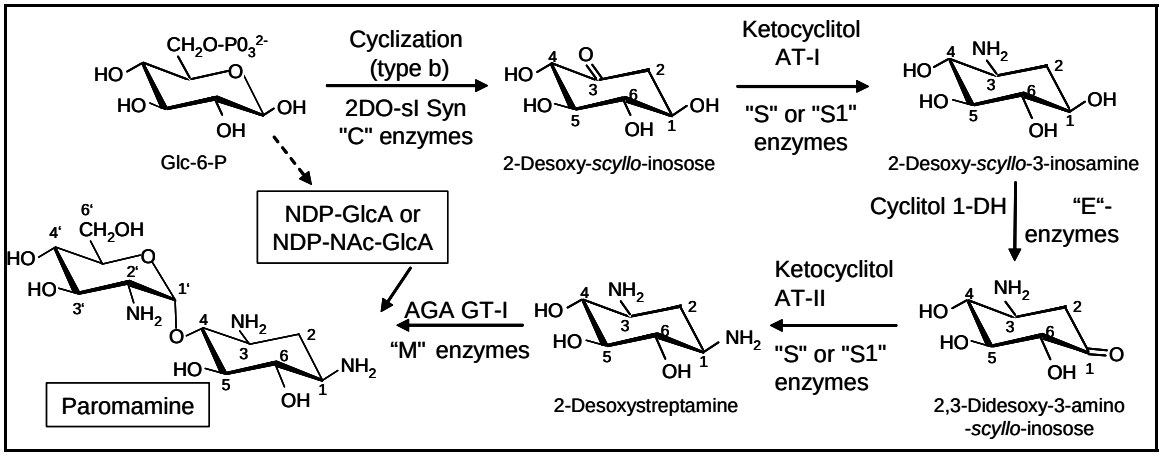

Fig.9 The general paromamine pathway. The enzymes involved are named only by the enzyme specific letters "C", "S" or "S1", "E" and "M" according to the nomenclature given in in the summitted gene clusters[9,21].

The obtained PCR product (1.1 kb) was treated with *Nde*I/*Eco*RI and ligated into pUCPU21 restricted with the same restriction endonucleases. The forward primer was designed for the introduction of an *Nde*I site, changing the sequence at the natural start codon for the ability to create start codon fusion of the *ribE* gene into the promotor/ribosome-binding site cassettes of pET16b expression vector. On the other hand, the reverse primer was designed for the introduction of a *Eco*RI site located immediately downstream of the natural stop codon in order to allow orientated cloning into the pET16b expression vector.

Accordingly, RibE protein of *S. ribosidificus* was overproduced as a soluble N-terminal His-tagged protein in *E. coli* JM109 (DE3) as determined by SDS-PAGE. Additional band of about 35.4 kDa present in the soluble fraction of the cell-free extracts corresponded in size to the expected molecular masses of the respective His-tagged protein was detected. Maximal overexpression of the RibE was 4 hs after IPTG induction. The presence of multiple His residues in the overexpressed N-terminal His-tagged respective protein was also demonstrated by Western blot assays. However, the unavailability of 2-deoxyinosamine, the putative RibE-substrate made us to study the biosynthetic role of the respective enzyme by knocking-out the respective gene (*ribE*). Therefore, the *ribE* was knocked-out via in-frame insertion of *aacC4* cassette coded for apramycin resistance[38]. The formation of *ribE* mutant was verified by its growth on TSB agar in the presence or apramycin (50 μgml^{-1}) as well as by PCR. The PCR was carried out using chromosomal DNA of both wild and mutant strain and both REK-F and REK-R primers. In contrast to the wild strain no PCR (1.1 kb)

product was detected with the mutant strain confirming the disruption of the *ribE* gene via homologous recombination with the apramycin gene cassette.

Both wild and *ribE* mutant were tested for antibiotic production. An inhibition zone was only detected upon use of the wild strain. No inhibition zone was observed with the *ribE* mutant strain. The obtained result confirmed the involvement of RibE in the biosynthesis of ribostamycin. Since no inhibition zone was detected with the mutant strain. In addition to that, formation of the 2DOS was an essential intermediate required for ribostamycin formation. This could be interpreted as RibE would be involved in the formation of 2DOS moiety, the essential intermediate for exerting the antibiotic activity. Moreover, homologous RibE complementation in the *ribE* mutant via transformation of pERW3, a recombinant shuttle plasmid was undertaken (*ribE* gene cloned into pUWL201PW plasmid; Wehmeier U, Wuppertal). It was found that, the *ribE* mutant regained antibiotic production after being transformed confirming its role in the biosynthesis of ribostamycin. This study gives good basis for the following aspects to be further investigated in future: i) clarifying the complete biosynthetic pathway of ribostamycin in *S. ribosidificus* and the related antibiotics such as neomycin in *S. fradiaes* and butirosins in *Bacillus circulans*; 2) biocombinatorial formation of ribostamycin derivatives which is considered one of the most important approaches for getting new members of these valuable metabolites especially those conferring resistance to the clinically relevant pathogens.

Acknowledgments

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استنساخ و انتاج البروتين وتثبيت فاعلية جين ريب-بى الداخل فى التكوين الحيوى لمضاد الريبوستاميسن داخل سلالة الستربتومايسس ريبوزيديفكس NRRL B-11466

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ينتج الريبوستاميسن بواسطة سلالة الستربتومايسس ريبوزيديفكس NRRL B-11466 و الذى يمثل واحدا من المضادات الحيوية لمجموعة الامينوجليكوزايد والتى تحتوى على وحدة ٢-دى أوكسى-ستربتامين. ولقد أظهرت التحاليل السابقة للمجموعات الجينية المسئولة عن التكوين الحيوى لهذا المضاد و المضادات الحيوية الأخرى التابعة لنفس المجموعة مثل الباروماميسن و الليفدومايسن و البتيروزين و النيومايسن و الكناميسن و الجنتاميسن و التوبراميسن أن هناك جين ريب-إى و أمثالة موجود فى جميع المجموعات الجينية. وأوضحت ايضا التحاليل الجينية توقع مسئولية هذا الجين وكذلك أمثالة عن أكسده مركب٢-دى أوكسى-إنوزامين الضرورى لتكوين مركب ٢- دى أوكسى-ستربتامين ، الوحدة الغير سكرية داخل كل هذه المضادات. و لدراسة الوظيفة الحيوية لجين ريب-إى (١٠٢٣ ثنائى النيكلوتيد) تم عمل إستنساخ وإنتاج البروتين الخاص به. لهذا تناول هذا البحث تكبير و فصل هذا الجين من الحمض النووى (الكروموسوم) لسلالة الستربتومايسس ريبوزيديفكس NRRL B-11466 عن طريق تفاعل البلمره المتسلسل باستخدام البادئات الاماميه و الخلفيه المناسبة و تركيبة على ناقل استنساخ **(pUCPU21)** و آخر للانتاج البروتينى **(pET16b)** و إدخالهم داخل سلالة الايشيريشيا كولى-جى إم ١٠٩ دى-اى٣ تحت تاثير محث تى-٧. وعن طريق الفصل البروتينى باستخدام جيل البولى أكريلاميد و عمل طبعة ويسترن تم التاكد من إنتاج بروتينن ريب-إى (حوالى ٤٢,٣٥ كيلو دالتون) فى صوره ذائبه و متحد بعديد الهيستادين. إضافا الى ذلك، تم عمل طفرة من السلالة المنتتجه لهذا المضاد حيث تم تثبيت عمل هذا الجين وتبين عدم انتاجيتها لهذا المضاد. كذلك استعادت الطفرة قدرتها على انتاجية المضاد الحيوى بعد تركيب جين ريب-إى على ناقل مناسب وإدخالة مره اخرى داخل هذه الطفرة مما يؤكد ضلوع هذا الجين فى التكوين الحيوى لمضاد الريبوستاميسن داخل سلالة الستربتومايسس ريبوزيديفكس NRRL B-11466.