Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2837143 C 2016/01/05

(11)(21) 2 837 143

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2012/06/07
(87) Date publication PCT/PCT Publication Date: 2012/12/13
(45) Date de délivrance/Issue Date: 2016/01/05
(85) Entrée phase nationale/National Entry: 2013/11/22
(86) N° demande PCT/PCT Application No.: PL 2012/050020
(87) N° publication PCT/PCT Publication No.: 2012/169917
(30) Priorités/Priorities: 2011/06/08 (US61/494,574);
2011/06/08 (PL P.395178)

(51) Cl.Int./Int.Cl. *C12N 9/22* (2006.01)

(72) Inventeurs/Inventors:
BUJNICKI, JANUSZ MAREK, PL;
SKOWRONEK, KRZYSZTOF JERZY, PL;
PIANKA, DARIUSZ, PL;
SULEJ, AGATA AGNIESZKA, PL

(73) Propriétaire/Owner:
MIEDZYNARODOWY INSTYTUT BIOLOGII MOLEKULARNEJ I KOMORKOWEJ, PL

(74) Agent: BORDEN LADNER GERVAIS LLP

(54) Titre : ENDORIBONUCLEASES A ARN DOUBLE BRIN
(54) Title: DSRNA ENDORIBONUCLEASES

(57) Abrégé/Abstract:
The invention relates to a new double-stranded RNA endoribonuclease, its derivative and / or variant, which has a loop locating in and interacting with the major groove of the double-stranded RNA, exhibiting sequence specific properties in the double-stranded RNA cleavage.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 December 2012 (13.12.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/169917 A1

(51) **International Patent Classification**:
C12N 9/22 (2006.01)

(21) **International Application Number**:
PCT/PL2012/050020

(22) **International Filing Date**:
7 June 2012 (07.06.2012)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:

P.395178	8 June 2011 (08.06.2011)	PL
61/494,574	8 June 2011 (08.06.2011)	US

(71) **Applicant** *(for all designated States except US)*: **MIĘDZYNARODOWY INSTYTUT BIOLOGII MOLEKULARNEJ I KOMÓRKOWEJ** [PL/PL]; ul. Ks. Trojdena 4, PL-02-109 Warszawa (PL).

(72) **Inventors; and**

(75) **Inventors/Applicants** *(for US only)*: **BUJNICKI, Janusz Marek** [PL/PL]; ul. Stanisława Żaryna 7/100, PL-02-593 Warszawa (PL). **SKOWRONEK, Krzysztof Jerzy** [PL/PL]; ul. Piętkiewicza 39, PL-05-080 Hornówek (PL). **PIANKA, Dariusz** [PL/PL]; ul. Małego Franka 3a/15, PL-01-115 Warszawa (PL). **SULEJ, Agata Agnieszka** [PL/PL]; ul. Przy Agorze 17/85, PL-01-960 Warszawa (PL).

(74) **Agent**: **GRZELAK, Anna**; WTS Patent Attorneys, Witek Sniezko & Partners, ul. Rudolfa Weigla 12, PL-53-114 Wroclaw (PL).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2012/169917 A1

(54) **Title**: DSRNA ENDORIBONUCLEASES

(57) **Abstract**: The invention relates to a new double-stranded RNA endoribonuclease, its derivative and / or variant, which has a loop locating in and interacting with the major groove of the double-stranded RNA, exhibiting sequence specific properties in the double-stranded RNA cleavage.

dsRNA endoribonucleases

DESCRIPTION

TECHNICAL FIELD

The subject of the invention is a double-stranded RNA (dsRNA) endoribonuclease exhibiting dsRNA sequence specific cleavage activity, the method of obtaining dsRNA endoribonuclease, the method of obtaining dsRNA endoribonuclease derivative and / or a variant with altered sequence selectivity in dsRNA cleavage, the genetic construct, the host cell, the use of the gene encoding the dsRNA endoribonuclease to its creation, the kit and the enzyme exhibiting dsRNA endoribonucleolytic activity.

BACKGROUND ART

One of the basic tools of molecular biology are proteins with a clearly defined activity, used for example in genetic engineering, diagnostics, medicine and industry in the manufacturing and processing of various products.

DNA restriction endonucleases are sequence dependent enzymes that recognize and cleave specific sequence of double-stranded DNA. There are also known enzymes that cleave RNA in a given sequence, however, such enzymes act on single-stranded sites in RNA. Examples of these enzymes include a phage protein RegB, which cleaves the single-stranded RNA in the middle of the sequence GGAG and Ribonuclease Y, which cleaves the single-stranded RNA in A or AU rich sequences. These enzymes require additional determinants for efficient cleavage, such as RNA secondary structure and in case of RegB the interaction with the ribosomal protein S1 (Lebars, I., et. al., J Biol Chem (2001) 276, 13264-13272, Saida, F. et. al., (2003) Nucleic Acids Res, 31, 2751-2758 and Shahbabian, K. et. al., The EMBO Journal (2009) 28, 3523 - 3533). There were also attempts to change the specificity of Ribonuclease T1 and Ribonuclease MC1 (Hoschler, K. et al. J Mol Biol, (1999) 294, 1231-1238, Numata, T. et. al., Biochemistry, (2003) 42, 5270 - 5278). In these two cases the enzyme variants were created in which their specificity has increased, from one to two nucleotides (Numata, T. et. Al., Biochemistry, (2003) 42, 5270-5278, Czaja, R. et. al., Biochemistry, (2004) 43, 2854-2862; Struhalla, M. et. al. Chembiochem, (2004) 5, 200-205). However, all these Ribonucleases still have a very limited sequence specificity which makes them unsuitable as molecular biology tools in applications similar to those of DNA restriction enzymes.

Ribonuclease III is an archetype of nucleases that cleave double-stranded RNA (dsRNA) and a founding member of the Ribonuclease III superfamily of proteins, which share an evolutionarily conserved catalytic domain. They are divided into four classes based on the occurrence of additional domains. Class 1, i.e. orthodox Ribonuclease III enzymes, have a double-stranded RNA binding domain (dsRBD) and a single Ribonuclease III domain. Class 2 and 3 enzymes are represented by Drosha and Dicer, respectively, which both comprise two Ribonuclease III domains along with a single dsRBD. In addition, enzymes belonging to class 2 possess additional domains, such as a polyproline domain and to class 3 a DExD helicase, DUF283 and PAZ domains. Class 4, called Mini III, includes enzymes that consist solely of the Ribonuclease III domain.

The natural substrate for the Mini III protein from *Bacillus subtilis* is 23S pre-rRNA, in which the 3′ and 5′ ends of the molecule are removed to yield the mature 23S rRNA. The cleavage site for this enzyme is known, however close to the cleavage site of double-stranded pre-rRNA one fragment of 23S pre-rRNA forms an irregular helix, which was speculated to be necessary for substrate recognition (Redko, Y. et. al., Molecular Microbiology, (2008) 68 (5), 1096-1106). In addition, *in vitro* endoribonucleolytic activity of Mini III was shown to be stimulated by the ribosomal protein L3 bound to the 3′ end of the 23S rRNA. There is indirect evidence that protein L3 enhances the cleavage of the substrate by changing the conformation of the RNA (Redko, Y. et. al., Molecular Microbiology, (2009) 71 (5), 1145-1154).

There are no known enzymes for specific and defined dsRNA fragmentation with properties similar to the DNA restriction endonucleases or DNA nickases (JP 54059392 – A, 12.05.1979). The dsRNA can be cleaved by endoribonucleases from Ribonuclease III family, but no details of Ribonuclease III-dsRNA interactions are known (Herskovitz, M.A. et. al., Molecular Microbiology, (2000) 38 (5), 1027-1033). The criteria for site-specific binding and selective processing remain unclear (Dasgupta S. et. al., Molecular Microbiology, (1998) 28 (3), 629-640). However, unspecific dsRNA endonucleases are used for generating short double-stranded RNA fragments (US 2006057590 – A1, 16.03.2006, NOVARTIS). Obtaining enzymes exhibiting sequence specificity in dsRNA cleavage will allow to develop all areas of RNA manipulation techniques, but also to develop new research methods, new applications of such enzymes and new technologies derived from them.

DISCLOSURE OF INVENTION

In the light of the described state-of-the-art, the object of the presented invention is to overcome the indicated disadvantages and to deliver the dsRNA endoribonuclease with high sequence specificity recognition and cleavage. The aim of the present invention is also to deliver

methods of determining, isolation, selection, obtaining and preparation of such sequence-specific dsRNA endoribonucleases, and their improved variants.

The inventors have unexpectedly found out that an enzyme from the Ribonuclease III superfamily, which according to *in silico* modeling contains a loop that locates in and interacts with the major groove of the dsRNA helix, may have a preference for cleaving a particular dsRNA nucleotide sequence. The inventors have found that such preference depends only on the dsRNA sequence and is independent of the presence of irregular dsRNA helix structure and/or interaction with other proteins. The inventors have found out that the enzyme that belongs to the Ribonuclease III superfamily, which contains fragment of polypeptide chain that in *in silico* modeling forms a loop that locates in and interacts with dsRNA major groove, is able to perform specific and defined fragmentation of dsRNA with properties similar to the restriction endonucleases for DNA.

In one aspect the invention provides the dsRNA endoribonuclease exhibiting sequence specific properties in dsRNA cleavage, which has a loop that is locating in and interacting with a major groove of dsRNA and / or its derivative and / or variant exhibiting sequence specificity in dsRNA cleavage. In the preferred dsRNA endoribonuclease, its derivative and / or variant, the loop that is locating in and interacting with a major groove of dsRNA, has the amino acid sequence of dsRNA endoribonuclease, which corresponds to the loop locating in and interacting with a major groove of dsRNA in the model of structure of endoribonuclease Mini III in complex with dsRNA.

In the preferred dsRNA endoribonuclease, its derivative and / or variant the loop that is locating in and interacting with a major groove of dsRNA corresponds to the loop that is locating in and interacting with a major groove of dsRNA formed by a fragment of endoribonuclease FNU from *Fusobacterium nucleatum* as shown in SEQ ID NO3 and / or by fragment of endoribonuclease BSU from *Bacillus subtilis* as shown in SEQ ID NO4 and / or by fragment of endoribonuclease BCE from *Bacillus cereus* as shown in SEQ ID NO5.

The dsRNA endoribonuclease, its derivative and / or variant preferably comprises the sequence or a fragment of the amino acid sequence of dsRNA endoribonuclease BSU from *Bacillus subtilis* of SEQ ID NO1, which shows sequence specificity in dsRNA cleavage and preferably contains an amino acid substitution D94R.

The dsRNA endoribonuclease, its derivative and / or variant also preferably comprises the endoribonuclease FNU from *Fusobacterium nucleatum* or a fragment of endoribonuclease FNU from *Fusobacterium nucleatum*, which exhibits sequence specificity in dsRNA cleavage.

The dsRNA endoribonuclease, its derivative and / or variant also preferably comprises the endoribonuclease BCE from *Bacillus cereus* or a fragment of endoribonuclease BCE from *Bacillus cereus*, which exhibits sequence specificity in dsRNA cleavage.

In next aspect the invention relates to the method of obtaining dsRNA endoribonuclease exhibiting sequence specificity in dsRNA cleavage, which comprises the following steps:

a) selection of dsRNA endoribonuclease, its functional variant and / or derivative that comprises an amino acid sequence forming a loop that is locating in and interacting with a major groove of dsRNA, wherein the loop is formed by the amino acid sequence of dsRNA endoribonuclease, which corresponds to the model of the loop locating in and interacting with a major groove of dsRNA determined by the three-dimensional model of the complex of Mini III endoribonuclease with dsRNA;

b) cloning the gene or fragment thereof which encodes for a dsRNA endoribonuclease, its functional variant and / or derivative, comprising the sequence forming a loop that is locating in and interacting with a major groove of dsRNA.

The method of obtaining dsRNA endoribonuclease additionally preferably includes after step b) the next step c) expressing of the protein encoded by the gene or fragment thereof obtained in step b), and preferably after step c) it is also followed by step d) in which the sequence specificity of the isolated dsRNA endoribonuclease is determined.

In a preferred method of obtaining dsRNA endoribonuclease the loop that is locating in and interacting with a major groove of dsRNA corresponds to the amino acid sequence forming a loop that is locating in and interacting with a major groove of dsRNA formed by a fragment of dsRNA endoribonuclease FNU from *Fusobacterium nucleatum* as shown in SEQ ID NO3 and / or by fragment of endoribonuclease BSU from *Bacillus subtilis* as shown in SEQ ID NO4 and / or by fragment of endoribonuclease BCE from *Bacillus cereus* as shown in SEQ ID NO5.

Moreover, in the next aspect the invention relates to the method of obtaining dsRNA endoribonuclease derivative and / or variant with altered sequence selectivity for sequence specific cleavage of dsRNA, which comprises the following steps:

a) introducing the change(s) in nucleotide sequence encoding the amino acid sequence corresponding to the loop located in the major groove of dsRNA for dsRNA endoribonuclease obtained in the method of obtaining dsRNA endoribonuclease of the invention, exhibiting sequence specificity in dsRNA cleavage;

b) expression of the derivative and / or variant of dsRNA endoribonuclease from the nucleotide sequence obtained in the step a), and

c) identification of the altered sequence specificity of derivative and / or variant of dsRNA endoribonuclease.

In such a preferred method the change in the selectivity of the derivative and / or variant of dsRNA endoribonuclease leads to a derivative and / or a variant with increased selectivity for nucleotide sequence in dsRNA cleavage.

The invention further relates to a method for producing dsRNA endoribonuclease, which includes the step of expressing dsRNA endoribonuclease, its derivative and / or a variant of the invention exhibiting sequence specificity in dsRNA cleavage.

The invention also relates to a genetic construct which comprises a nucleotide sequence encoding a dsRNA endoribonuclease, a derivative thereof and / or a variant of the invention exhibiting sequence specificity in dsRNA cleavage.

A host cell comprising a genetic construct of the invention is also subject to the invention.

In the next aspect the invention relates to the use of the gene encoding the dsRNA endoribonuclease FNU from *Fusobacterium nucleatum* or fragment thereof and / or its functional variant and / or derivative to produce dsRNA endoribonucleases exhibiting sequence specific dsRNA cleavage. In a beneficial application the dsRNA endoribonuclease FNU from *Fusobacterium nucleatum*, its derivative and / or variant comprises the amino acid sequence shown in SEQ ID NO3.

The invention also relates to the use of the gene encoding the dsRNA endoribonuclease BCE from *Bacillus cereus* or fragment thereof and / or its functional variant and / or derivative to produce dsRNA endoribonucleases exhibiting sequence specific dsRNA cleavage. Preferably the dsRNA endoribonuclease BCE from *Bacillus cereus*, its derivative and / or variant comprises the amino acid sequence of SEQ ID NO5.

In next aspect the invention relates to the use of the gene encoding dsRNA endoribonuclease BSU from *Bacillus subtilis* shown in SEQ ID NO1 or a fragment thereof and / or its functional variant and / or derivative to produce dsRNA endoribonuclease exhibiting sequence-specific dsRNA cleavage. Preferably the gene encoding dsRNA endoribonuclease BSU from *Bacillus subtilis*, its derivative and / or variant comprises the amino acid sequence shown in SEQ ID NO1, even more preferably the gene encoding dsRNA endoribonuclease BSU from *Bacillus subtilis* comprises D94R substitution.

The invention also relates to the kit that comprises dsRNA endoribonuclease, derivative and / or a variant thereof of the invention exhibiting sequence specificity in dsRNA cleavage. It comprises dsRNA endoribonuclease FNU from *Fusobacterium nucleatum* and / or dsRNA endoribonuclease BCE from *Bacillus cereus* and / or dsRNA endoribonuclease BSU from *Bacillus subtilis* or a variant thereof comprising substitution D94R and / or their derivatives and variants exhibiting sequence specificity in dsRNA cleavage.

inted: 19/04/2013 DESCPAMD PCT/PL 2012/050 020 06-04-2013 PL2012050020

The invention also relates to enzyme of dsRNA endoribonuclease activity which comprises a sequence or a fragment of the amino acid sequence from *Bacillus subtilis* shown in SEQ ID NO1, which exhibits sequence specificity and cleaves the dsRNA within the consensus sequence

5' DACCUHD 3'
3' HUGGADH 5'

where H=A or C or U; D= A or G or U and its derivatives and / or variants that retain sequence specificity. The preferred enzyme and its derivatives and / or variants that retain sequence specificity in dsRNA cleavage and cut dsRNA within the consensus sequence

5' YGACCUCGNNG 3'
3' RCUGGAGCNNC 5'

where Y=C or U; R=A or G; N=G or A or U or C.

In the preferred enzyme and its derivatives and / or variants retaining the sequence specificity, the amino acid sequence comprises substitution D94R of amino acid residue 94 presented in SEQ ID NO1, its derivative and / or variant that retain sequence specificity.

The sequence specificity of dsRNA cleavage means the ability of dsRNA endoribonuclease to recognize and cut dsRNA depending only on its sequence and not on the existence of irregular helix structure in one or both strands dsRNA and / or interaction of other assisting proteins.

The term dsRNA endoribonuclease derivative and / or a variant as described herein, means proteins, polypeptides, peptides or recombinant proteins, polypeptides and peptides comprising the amino acid sequence identical or highly similar to the amino acid sequence of dsRNA endoribonuclease exhibiting sequence specific dsRNA cleavage, which has a loop that is locating in and is interacting with the major groove of dsRNA retaining the characteristic activity and sequence preference of dsRNA endoribonuclease. Such examples of derivatives and variants in the model of the structure will have a loop that corresponds to the loop locating in and interacting with the major groove of dsRNA in the structural model of the complex of endoribonuclease Mini III with dsRNA. In dsRNA endoribonuclease derivatives and / or variants exhibiting sequence specificity in dsRNA cleavage, the encoding sequences may be amended by the substitution, replacement, deletion or insertion, or other means in relation to the initial sequence. Such term should by analogy be likewise understood for the gene and/or derivative and / or a variant of the gene coding for dsRNA endoribonuclease with such characteristic.

The dsRNA endoribonucleases exhibiting sequence specificity, their derivatives and / or variants of the invention and their use permits the development of a whole new field of

06/04/2013

techniques for manipulating RNA, as well as to develop new research methods, new uses of such enzymes and new technologies derived from them. The dsRNA sequence-specific endoribonucleases, their derivatives and / or variants, for example, will be used in structural studies of RNA in order to understand the structure of RNA molecules and / or their modifications, in the generation of RNAi molecules, in particular siRNA, in diagnosis and treatment of viral diseases of plants and animals as well as in nanotechnology applications based on the so-called 'RNA tectonics'.

New sequence-specific endoribonucleases of the dsRNA, their derivatives and / or variants of the invention will be used for new biotechnological applications. There are known enzymes that cut single-stranded RNA in a sequence-dependent manner, but their activity depends not only on the sequence of the substrate, but also on its secondary structure, so in practice they are not very useful. New sequence-specific dsRNA endoribonucleases of dsRNA, their derivatives and / or variants of the invention do not have these drawbacks and can be used as common laboratory reagents such as restriction endonucleases used in molecular biology. In addition sequence-specific dsRNA endoribonucleases, their derivatives and / or variants also can be used in medicine, diagnostics and nanotechnology. For example, currently the direct sequencing of RNA in reverse transcription reaction or mass spectrometry is used most often to identify modifications in RNA structural studies, but in both cases analysis of large molecules (eg. rRNA or mRNA) is difficult. In these methods, RNA is fragmented to short RNA products or ribonucleotides by unspecific ribonucleases and the multitude of products makes the interpretation of the results difficult or even impossible. The application of new sequence-specific dsRNA endoribonucleases, their derivatives and / or variants allows the controlled cleavage of RNA molecule into recurring smaller fragments. Molecular weight and properties could be determined independently, allowing for the analysis of chemical modifications of ribonucleotides and RNA structural studies previously impossible or very difficult. Such studies of modifications and structures of RNA molecules will provide information on potential therapeutic targets, for example mechanisms of bacterial resistance to antibiotics. Application of new sequence-specific dsRNA endoribonucleases, their derivatives and / or variants allows for the development of technologies based on RNAi, short interfering dsRNA molecules. Sequence-specific dsRNA endoribonucleases, their derivatives and / or variants will be used in the siRNA methods and applications for gene silencing, for example, in medicine to treat cancer, metabolic diseases and neurodegenerative disorders. Currently, one of the strategies leading to obtain a short dsRNA is to treat the long dsRNA produced from a particular segment of DNA with Ribonuclease III from *Escherichia coli*. This enzyme cuts dsRNA nonspecifically, producing 18 to 25 base pairs fragments. Short fragments are used for gene silencing. A completely new and

unknown possibilities for the production of specific siRNA can be used by sequence-specific dsRNA endoribonucleases, their derivatives and / or variants of the invention enabling the defined pool of dsRNA fragments generation that efficiently silence expression of a particular gene without off target effects.

Sequence-specific dsRNA endoribonucleases, their derivatives and / or variants can be applied in the diagnosis and treatment of diseases caused by dsRNA viruses. Such viruses belong to *Reoviridae* family in which three groups are pathogenic for humans. Currently to detect and identify those groups the reverse transcription reaction is used followed by PCR. Availability of sequence-specific dsRNA endoribonucleases, their derivatives and / or variants of the invention allows manipulation of dsRNA which significantly speeds up the diagnostics. Currently, treatment for rotavirus is highly ineffective. Sequence-specific dsRNA endoribonucleases, their derivatives and / or variants will be used as drugs for the treatment of rotavirus diseases by cleaving a specific viral genome, thereby preventing their further replication.

Sequence-specific dsRNA endoribonucleases, their derivatives and / or variants will also be used in nanotechnology, in particular in the "RNA tectonics" and the creation of nanostructures based on a given RNA sequence and structure.

BRIEF DESCRIPTION OF DRAWINGS

For a better understanding of the invention, it has been illustrated with the examples and attached figures, in which:

Fig.1. A structural model of the complex of endoribonuclease Mini III from *Fusobacterium nucleatum* and the dsRNA. Loop (L) located in major groove of dsRNA marked by an arrow.

Fig. 2. Shows the *in vitro* cleavage by endoribonuclease BSU from *Bacillus subtilis* performed to determine the optimal reaction conditions. A - effect of pH on the cleavage of 234 bp dsRNA.
1 - pH 6.8, 2 - pH 7.0, 3 - pH 7.5, 4 - pH 7.8, 5 - pH 8.0, 6 - pH 8.2, 7 - pH 8.5, 8 - pH 8.8,
9 - dsRNA marker (New England Biolabs No: N0363S). B - the effect of temperature on the cleavage of 234 bp dsRNA. 1 - 15°C, 2 - 25°C 3 - 30°C, 4 - 35°C, 5 - 40°C, 6 - 45°C,
7 - 50°C, 8 - 55°C. C - the effect of NaCl concentration on the cleavage of 234 bp dsRNA.
1 - 5 mM, 2 - 20 mM, 3 - 40 mM, 4 - 60 mM, 5 - 80 mM, 6 - 100 mM, 7 – non cleaved substrate, 8 - marker dsRNA (New England Biolabs No: N0363S); D - the effect of Mg 2+ ion concentration in mM in the cleavage of 234 bp dsRNA. 1 - 0.03, 2 - 0.05, 3 - 0.08, 4 - 0.1,
5 - 0.25, 6 - 0.5, 7 – 1, 8 - 2.5, 9 – 5, 10 - 7.5, 11 – 10, 12 - 12.5, 13 – 15, 14 - 17.5 mM Mg 2+.

Fig. 3. Presents the sensitivity of endoribonuclease BSU to ribose methylation at guanosines near the cleavage site. (A) The sequences of two substrates: the cleavage site marked with arrows,

ribose methylation marked with an asterisk (B) cleavage of substrates with and without ribose methylation. 1 - 30 bp non cleaved substrate without methylation, 2 - 30 bp non cleaved substrate with ribose methylation of the guanosine adjacent to the cleavage site, 3 - 30 bp non cleaved substrate with ribose methylation of guanosine in the second nucleotide adjacent to the cleavage site, 4 - marker dsRNA (New England Biolabs No: N0363S), 5 - 30 bp substrate without methylation treated with endoribonuclease BSU, 6 - 30 bp substrate with ribose methylation of the guanosine residue adjacent to the cleavage site treated with endoribonuclease BSU , 7 - 30 bp substrate with ribose methylation of guanosine in the second nucleotide adjacent to the cleavage site.

Fig. 4. Determination of minimum substrate length for endoribonuclease BSU. 1 - 18 bp substrate treated with endoribonuclease BSU, 2 - untreated 18 bp substrate, 3 - 20 bp substrate treated with endoribonuclease BSU, 4 - untreated 20 bp substrate, 5 - 22 bp substrate treated with endoribonuclease BSU, 6 - untreated 22-bp substrate, 6 - DNA marker (Ultra Low Range, Fermentas no: SM1211).

Fig. 5. Comparison of sequence preferences of endoribonuclease BSU wild-type (endoribonuclease BSU^{WT}) and D95R variant (endoribonuclease BSU^{D95R}). 1 - bacteriophage Φ6 dsRNA genome, 2 - bacteriophage Φ6 dsRNA genome cleaved with endoribonuclease BSU^{WT}, 3 - bacteriophage Φ6 dsRNA genome cleaved with endoribonuclease BSU^{D94R}, 4 - 234 bp dsRNA, 5 – 234 bp dsRNA cleaved with endoribonuclease BSU^{WT}, 6 - 234 bp dsRNA cleaved with D94R variant.

Fig. 6. (A): Identification of the endoribonuclease BSU cleavage site on the top strand of the 234 bp dsRNA. 1 - mapping of the cleavage site on the top strand, 2 - chain termination with ddCTP, 3 – chain termination with ddTTP, 4 - chain termination with ddATP, 5 - chain termination with ddGTP. **(B):** Identification of the endoribonuclease BSU cleavage site on the bottom strand of the 234 bp dsRNA. 1 - mapping of the cleavage site on the bottom strand, 2 - chain termination with ddGTP, 3 – chain termination with ddATP, 4 - chain termination with ddTTP, 5 - chain termination with ddCTP.

Fig. 7. (A): Identification of the cleavage site in 30 bp dsRNA substrate with the sequence surrounding cleavage site in the 234 bp dsRNA. S - substrate, P - product, M - marker, (B): Shows the geometry of the dsRNA cleavage by endoribonuclease BSU.

Fig. 8. Cleavage of 30 bp dsRNA substrates. 1- 30 bp substrate with preferred sequence, 2- 30 bp substrate with preferred sequence treated with endoribonuclease BSU for 15 minutes, 3 - 30 bp substrate with preferred sequence treated with endoribonuclease BSU for 30 minutes, 4 - 30 bp substrate with preferred sequence treated with endoribonuclease BSU for 60 minutes, 5 - DNA marker (Ultra Low Range, Fermentas no: SM1211), 6 – 30 bp substrate N1, 7 –30 bp substrate

N1 treated with endoribonuclease BSU for 15 minutes, 8- 30 bp substrate N1 treated with endoribonuclease BSU for 30 minutes, 9- 30 bp substrate N1 treated with endoribonuclease BSU for 60 minutes, 10 - 30 bp substrate N2, 11 - 30 bp substrate N2 treated with endoribonuclease BSU for 15 minutes, 12 –30 bp substrate N2 treated with endoribonuclease BSU for 30 minutes, 13 - 30 bp substrate treated N2 with endoribonuclease BSU for 60 minutes, 14 - 30 bp substrate N3, 15 –30 bp substrate N3 treated with endoribonuclease BSU for 15 minutes, 16 - 30 bp substrate N3 treated with endoribonuclease BSU for 30 minutes, 17 - 30 bp substrate N3 treated with endoribonuclease BSU for 60 minutes.

DESCRIPTION OF EMBODIMENTS

The following examples are included to illustrate the invention and to explain its various aspects, and not to limit it and should not be equated with its entire range, which is defined in the appended claims.

In the following examples, unless otherwise indicated, standard materials and methods described in Sambrook J. et al., "Molecular Cloning: A Laboratory Manual, 2nd edition. 1989. Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory Press are employed, or proceded in accordance with manufacturers' recommendations for specific materials and methods.
Herein, unless otherwise indicated standard abbreviations for amino acids and nucleotides or ribonucleotides are used.

Examples

Example 1

In-silico indentification of genes encoding proteins with sequence-dependent dsRNA cleavage activity

Double-stranded RNA (dsRNA) cutting enzymes contain a ribonuclease III domain. This group includes Dicer and Drosha, which contain additional domains necessary for the functioning of these enzymes. Here are also classified bacterial dsRNA endoribonucleases, with additional dsRNA-binding domain and enzymes without any additional dsRNA-binding domains.

The records with numbers 2EZ6, 2GSL, 1U61 which are available in the PDB database (PDB database, present the spatial coordinates of experimentally solved structures of proteins and nucleic acids and their complexes: http://www.pdb.org), and were used to select proteins with a desired substrate specificity. 2EZ6 presents the structure of ribonuclease III from *Aquifex aeolicus* having a dsRNA binding domain, together with the dsRNA substrate. 2GSL and 1U61 present structures of Mini III endoribonucleases (not possessing dsRNA binding domain) from *Fusobacterium nucleatum* and *Bacillus cereus* respectively. Using Swiss-PdbViewer (Guex, N.,

et. al., Electrophoresis, (1997) 18, 2714-2723), the structure of the 2GSL protein was superimposed on the catalytic centre of the 2EZ6 protein in the complex with the RNA substrate. We found that the matched 2GSL endoribonuclease has a loop which locates in the major groove of dsRNA (see Figure 1). The fragment of the polypeptide chain, which locates in the major groove, was identified after the removal of the original enzyme from the 2EZ6 complex and the creation of a new complex with the protein derived from 2GSL and RNA from 2EZ6. A structural model of the Mini III protein-RNA complex indicates that the loop encompasses the sequence AKNSNIKTFPRSCT for *Fusobacterium nucleatum* Mini III protein (FNU), and the alignment of amino acid sequences of proteins similar to the Mini III protein indicates that the loop from *Bacillus subtilis* Mini III protein (BSU) has an amino acid sequence GRNAKSGTTPKNTD. The loop of each member of the Mini III family of proteins has a different amino acid sequence, however it is able to locate in the major groove of dsRNA and provides a basis for sequence-specific interactions of Mini III with dsRNA. The interactions of this loop in Mini III protein with the RNA may lead to sequence preferences of Mini III in the course of dsRNA cleavage.

This means that in particular the enzyme having a loop L in Figure 1 located in the major groove of the dsRNA, Mini III, its functional variants, and other proteins with similar sequences, collectively described as "Mini III family of proteins" may have a nucleotide preference for dsRNA processing independent on the irregular helix dsRNA structure, which has been proven in the further described examples of the invention.

Therefore, for cloning and further enzyme engineering the genes were selected with open reading frames identified by sequencing of bacterial genomes of organisms *Bacillus subtilis*, *Fusobacterium nucleatum* and *Bacillus cereus*.

In the PDB database solved structures for FNU and BCE are available, therefore genes that encode them are also identified. As a result of the amino acid sequence alignment of proteins belonging to the Mini III family another enzyme BSU has also been selected for experimental studies. All proteins that belong to the Mini III family may have a preference for cleavage of particular sequences in dsRNA.

Example 2

Cloning of the genes designated in Example 1 from *Bacillus subtilis*, *Fusobacterium nucleatum* and *Bacillus cereus*

a) Preparation of template DNA

Freeze-dried cells obtained from the ATCC strain collection were suspended in 500 µl LB, and then 1µl of such suspension was added to the PCR reaction. Template DNA was obtained from strains of *Bacillus subtilis* available as ATCC 23857, *Fusobacterium nucleatum* available as

ATCC 25586 and *Bacillus cereus* available as ATCC 1457

b) Vector preparation

500 ng of vector pET28 (Novagen) was cleaved to completion with restriction enzymes NdeI and XhoI and products were separated on agarose gel. Product of size 5289 bp was recovered from the gel using a Gel Out kit (A & A Biotechnology) according to the manufacturer's protocol.

c) Isolation of PCR products for cloning of genes encoding proteins with dsRNA sequence-dependent activity

PCR with 1 µl of DNA template obtained from an appropriate strain in point. a) was performed in Biorad thermocycler in 50 µl reaction mixture: 5 µl reaction buffer, 200 µM dNTP mix, 1 U Pfu polymerase (Fermentas) and 50 pmol of each primer: Bsu28f and Bsu28 for reaction with DNA from *B. subtilis*, Fnu28f and Fnu28r for reaction with DNA from *F. nucleatum*, Bce28f and Bce28r for reaction with the DNA of *B. cereus* (corresponding primer sequences are shown in Table 1). Control reactions were performed without a DNA template.

Table 1.

Organism	Primer name	Primer sequence	SEQ ID NO
Bacillus subtilis	Bsu28f	TACCCATATGCTTGAATTTGATACG	6
	Bsu28r	TACTCGAGTCATGTTGCTGACTCATTTG	7
Fusobacterium nucleatum	Fnu28f	CCGCATATGGACAATGTAGATTTTTCAAAG	8
	Fnu28r	GTGCTCGAGTCATCATTCTCCCTTTATAACTATATTTATAATTTTTTTTATTTC	9
Bacillus cereus	Bce28f	CCGCATATGGTCGATGCAAAGCAATTAAACAG	10
	Bce28r	TACTCGAGTCATGATGATGTGCCCCCTTC	11

PCR reaction was performed in standard conditions. The reaction mixture was separated on agarose gel and the fragments corresponding to the expected sizes 447 bp, 408 bp and 422 bp were isolated from the gel using a Gel Out kit (A & A Biotechnology) and were cleaved with NdeI and XhoI. Cleavage product was purified using the Clean Up kit (A & A Biotechnology) and ligated with the vector obtained in point. b). Ligation reaction was carried out with T4 DNA ligase (Fermentas). 100 µof chemocompetent bacteria *E. coli* strain Top10 (Invitrogen) was transformed with 10 µl of ligation mixture and the resulting transformants were selected on LB solid medium with kanamycin 50 µg ml. Plasmid DNA was isolated from selected colonies grown on 3 ml LB medium with kanamycin (50 µg/ml) using Plasmid Mini kit (A & A Biotechnology). The selection of transformants containing the recombinant plasmids was based

on analysis of restriction maps, and then the samples were sequenced to confirm the correctness of the constructs (DNA Sequencing and Synthesis Service at the IBB PAS).

In this way the following plasmids were obtained:

pET28Bsu encoding wild-type sequence-specific dsRNA endoribonuclease from yazC gene of *B. subtilis* (BSU endoribonuclease amino acid sequence is presented in SEQ ID NO2);

pET28Fnu encoding wild-type sequence-specific dsRNA endoribonuclease from *F. nucleatum* (endoribonuclease FNU);

pET28Bce encoding wild-type sequence-specific dsRNA endoribonuclease from *B. cereus* (endoribonuclease BCE).

Example 3

Expression and purification of the protein from the pET28Bsu vector encoding wild-type enzyme from *Bacillus subtilis*

Escherichia coli strain ER2566 (New England Biolabs) was transformed with the pET28Bsu plasmid obtained in Example 2, transformations were performed as described in Example 2. Strains were selected on LB solid medium with 50 μg/ml kanamycin and 1% glucose. 25 ml of liquid LB medium with 50 μg/ml kanamycin and 1% glucose were inoculated with selected transformants and incubated for 16 hours at 37°C. Then 500 ml of liquid LB supplemented with 50 μg / ml kanamycin, was inoculated with 25 ml culture and incubated with shaking at 37°C to OD_{600} ~0.6 and then protein expression induced by adding IPTG to a 1 mM final concentration. Induction was carried out for 3 hours at 37°C with shaking. Cultures were centrifuged at 5000 g for 10 min at 4°C, resuspended in STE buffer and centrifuged again. Pellet was suspended in 20 ml of lysis solution (50 mM $NaPO_4$ pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM PMSF, 10 mM BME, 0.1% Triton™ X-100), and then the bacterial cells were disintegrated using single pass through the Cell Disruptor (Constant Systems LTD) at pressure of 1360 atmospheres. Lysates were clarified by centrifugation in the ultracentrifuge at 20 000 g at 4°C for 20 min. Protein was purified by affinity chromatography using polyhistidine tag present in the peptide chain.

Cell lysate was applied to a 7 x 1.5 cm column containing 5 ml Ni-NTA agarose (Sigma-Aldrich) equilibrated with four volumes of lysis buffer. The column was washed sequentially with the following buffers: lysis (50 ml), lysis supplemented with 2 M NaCl (50 ml), lysis supplemented with imidazole to a concentration of 20 mM (50 ml). The protein was eluted with lysis buffer supplemented with 250 mM imidazole and 1.5 ml fractions were collected. Flow rate was 0.9 ml / min and temperature 4°C. Fractions containing protein were combined, diluted to total volume of 50 ml in buffer R: 30 mM $NaPO_4$ pH 8.0, 30 mM NaCl, 10% glycerol, 10 mM BME.

In order to cut off polyhistidine tag 4 U thrombin (Sigma-Aldrich no. Catalog T4648) was added and the mixture was incubated at 4°C overnight. To purify the protein from the thrombin and polyhistidine tag ion-exchange chromatography using SP Sepharose™ column (GE Healthcare) was used. Protein was eluted with a linear gradient of NaCl concentration from 30 mM to 1 M in buffer R, 1.5 ml fractions were collected. Fractions with protein were combined, diluted and frozen at -70°C.

Example 4

Preparation of dsRNA substrates

Following substrates were used for determinations of endoribonuclease activity of expressed proteins:

a) bacteriophage Φ6 genome consisting of three segments:2948 bp (S), 4063 bp (M) and 6374 bp (L). This substrate contains 46 consensus cleavage sequences, but does not contain any preferred cleavage sequence. The dsRNA of bacteriophage Φ6 was purchased from Finnzymes.

b) *in vitro* synthesized dsRNA substrate, length 234 bp. This substrate contains single preferred cleavage site.

For the synthesis of 234 bp dsRNA pKSII plasmid with the modified DNA sequence downstream the T7 promoter site (sequence of modified pKSII is presented in SEQ ID NO2) and primers:
bsuRNAf 5'GCGCGTAATACGACTCACTATAGGG 3' (SEQ ID NO12), and
bsuRNAr 5'GGAAAAAAATCCGGCTCGTATGTTGTG 3' (SEQ ID NO13) were used. Synthesis was performed with the Replicator RNAi Kit (Finnzymes, according to the manufacturer's protocol).

c) short 18, 20, 22 and 30 bp dsRNAs.

Single-stranded RNA oligonucleotides were synthesized at Metabion. Complementary oligonucleotides (1.5 nmol each) were mixed in a 1:1 ratio. 30 µl mixture was heated to 95°C, then cooled for 2 hours to room temperature. Oligonucleotide sequences are listed below:

- 18F – 5'ACCGUCGACCUCGAGGGG 3' (SEQ ID NO14)
- 18R – 5'CCCCUCGAGGUCGACGGU 3' (SEQ ID NO15)
- 20F – 5'AUACCGUCGACCUCGAGGGG 3' (SEQ ID NO16)
- 20R – 5'CCCCUCGAGGUCGACGGUAU 3' (SEQ ID NO17)
- 22F – 5'AUACCGUCGACCUCGAGGGGGG 3' (SEQ ID NO18)
- 22R – 5'CCCCCCUCGAGGUCGACGGUAU 3' (SEQ ID NO19)
- 30F – 5'CGAUACCGUCGACCUCGAGGGGGGGCCCGG 3' (SEQ ID NO20)
- 30R – 5'CCGGGCCCCCCCCUCGUGGUCGACGGUAUCG 3' (SEQ ID NO21)
- 30N1F – 5'UCGAGUUGCCGGUUGCUGUGAUGGCCGUUC 3' (SEQ ID NO22)

- 30N1R – 5'GAACGGCCAUCACAGCAACCGGCAACUCGA 3' (SEQ ID NO23)
- 30N2F – 5'CCACUCUUAGAUACCCGAUUCCCCUGUUUC 3' (SEQ ID NO24)
- 30N2R – 5'GAAACAGGGGAAUCGGGUAUCUAAGAGUGG 3' (SEQ ID NO25)
- 30N3F – 5'UCUGAUGGGCGCUACCGGUUCCGGUAAGUC 3' (SEQ ID NO26)
- 30N3R – 5'GACUUACCGGAACCGGUAGCGCCCAUCAGA 3' (SEQ ID NO27)

Example 5

Cleavage of the dsRNA substrates by the produced enzymes

The reactions of substrate cleavage by the enzyme were carried out at 37°C for 1 hour. 15 µl reaction mixtures contained 4 pmol of corresponding enzyme prepared according to example 3, 2 pmol of a substrate obtained according to Example 4, 1.5 µl reaction buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM MgCl 2, 1 mg/ml BSA). Products were separated in a standard agarose gel electrophoresis or polyacrylamide gel electrophoresis (6% polyacrylamide, TBE: 135 mM Tris-HCl, 45 mM boric acid, 2.5 mM EDTA). After electrophoresis the gels were stained with ethidium bromide for 10 minutes and the products were visualized using UV light.

Example 6

Determination of cleavage sites in dsRNA

a) Primer labeling

RTr primer with sequence 5'GAAACAGCTATGACCATGA 3' (SEQ ID NO28) and RTf primer with sequence 5'GATCCCCCACAATCCTGTC 3' (SEQ ID NO29) were radioactively labeled using [γ-33 P] ATP. Reactions (10 µl volume) containing 10 pmol of primer, 1 µl reaction buffer, 10 µCi [γ-33 P] ATP and 1 U T4 polynucleotide kinase (Fermentas) were carried out at 37°C for 30 minutes.

b) Identification of the cleavage site on 234 bp dsRNA

It was shown that in the 234 bp dsRNA substrate obtained in Example 4b, there is only one cleavage site for endoribonuclease BSU^{WT}. 234 bp dsRNA was cleaved as described in Example 5 using the endoribonuclease BSU^{WT}. Then, the 90 bp and 144 bp products were isolated from the gel. Cleavage site was located using the reverse transcription reaction. 0.1µg of each product was mixed with 1 pmol of radioactively labeled primers from Example 6a. These 12.5 µl mixtures were incubated at 95^0C for 5 minutes. Then the mixtures were supplemented with 4 µl reaction buffer (Fermentas), 20 U Ribonuclease inhibitor RiboLock (Fermentas), 2 µl 10 mM dNTP, 10 U AMV reverse transcriptase (Fermentas) and reactions were carried out at 45°C for 60 min. In parallel sequencing was performed with the same primers and template using a reverse transcription reaction described above, except that in addition to the reaction mixture ddATP, ddCTP, ddGTP, or ddUTP in a ratio of 1:100 to the dNTP were added (to separate

reactions). The reaction products were separated on 6% denaturing polyacrylamide gel (6% polyacrylamide, TBE: 135 mM Tris-HCl, 45 mM boric acid, 2.5 mM EDTA, 8M urea) and subjected to 16 hours exposure to Storage Phosphor Screen (GE Healthcare), and visualized using a Storm scanner (GE Healthcare). The cleavage site between nucleotide position 90 and 91 on the top strand of the dsRNA (see Fig. 6A, Table 2) and between 146 and 147 on the bottom strand was located (see Fig. 6B).

c) Identification of cleavage sites on both strands of dsRNA

To determine the cleavage sites on both strands 30 bp dsRNA substrate was prepared by annealing RNA oligonucleotides 30F and 30R. The 5′ end of one strand was labeled with [γ-33P]ATP and T4 polynucleotide kinase and annealed with a non-labeled complementary oligonucleotide. Single-stranded RNA molecules with a cleavage site sequence derived from 234 bp dsRNA were synthesized (Metabion). Substrates were cleaved with endoribonuclease BSU^{WT}. Products were separated on 15% denaturing polyacrylamide gel (15% polyacrylamide, TBE: 135 mM Tris-HCl, 45 mM boric acid, 2.5 mM EDTA, 8M urea). Visualization of the products was done as in Example 6b. Results are shown in Figure 7. Exact cleavage site and geometry of the generated ends were determined. Endoribonuclease BSU^{WT} generates 2 nucleotide 3′overhangs (Fig. 7b).

Table 2. Identified cleavage site - sequence recognized and cut in the 234 bp dsRNA substrate by endoribonuclease BSU^{WT}

Nucleotide position in the 234 bp dsRNA substrate	-	-	83	84	85	86	87	88	89	90	91	92	93	94	95	96	-	-	
Nucleotide sequence 234 bp dsRNA substrate			**C**	**G**	**U**	**C**	**G**	**A**	**C**	**C**	**U**	**C**	**G**	**A**	**G**	**G**			SEQ ID NO30

It was shown that endoribonuclease BSU^{WT} from *Bacillus subtilis* specifically recognizes and cuts single site in 234 bp dsRNA.

Example 7

Determination of the optimal reaction conditions for *in vitro* cleavage of dsRNA substrates by the produced enzymes

a) Impact of the condition changes on the enzymatic activity of endoribonuclease BSU^{WT}

The influence of various factors on the enzymatic activity of the wild-type endoribonuclease BSU was examined. Optimum conditions were determined in *in vitro* cleavage, in various

temperatures, pH, NaCl concentrations and Mg^{2+} ion concentrations. Cleavage reaction was carried out as in Example 5, by changing only the parameter tested. Effect of pH on the cleavage of substrate was tested at pH values: 6.8, 7.0, 7.5, 7.8, 8.0, 8.2, 8.5, 8.8. It is shown that the best cleavage of 234 bp dsRNA substrate is obtained at pH 6.8, 7.0, 7.5, 7.8 (Fig. 3A). Effect of temperature on activity was tested at temperatures: 15°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C. The optimal temperature for the cleavage was between 35°C and 45°C (Fig. 2B). Outside this temperature range the substrate is cut at a slower rate. Effect of ion concentration was studied at NaCl concentrations of 5, 20, 40, 60, 80, 100 mM. Optimum for enzyme activity ranges from 5 to 60 mM of sodium chloride (Fig. 2C). Cutting efficiency of the substrate decreases at higher concentrations of salt. Effect of Mg^{2+} ion concentration was tested at the values: 0.03, 0.05, 0.08, 0.1, 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 12.5; 15, and 17.5 mM. Optimum Mg^{2+} ions concentration in the reaction mixture is 1 to 2.5 mM (Fig. 2D). Outside this range the substrate is cut at a slower rate.

b) The effect of ribose methylation in the vicinity of the endoribonuclease BSU^{WT} cleavage site

Sensitivity of the endoribonuclease BSU^{WT} to ribose methylation in the vicinity of the cleavage site was analyzed. 30 bp dsRNA substrates with ribose methylation of two guanosines were tested (Fig. 3A). Cleavage reaction was carried out as in Example 5. Two substrates with the methylated guanosine ribose are not cut (Fig. 3B). The enzyme is sensitive to ribose methylation of two guanosine residues, which are close to the cleavage site.

c) Idenfication of a minimum length substrate for dsRNA cleavage by the endoribonuclease BSU^{WT}

Minimum dsRNA substrate for endoribonuclease BSU^{WT} was identified. For this purpose 18, 20 and 22 bp substrates were examined. Endoribonuclease BSU^{WT} is able to cut dsRNA with a length of 22 base pairs (Fig. 4). Shorter substrates are not cut.

Example 8

Construction of the substrate libraries with substitutions and production of the dsRNA substrates

a) Construction of the substrate libraries with substitutions

14 single position substitution libraries were constructed in the fragment of nucleic acid comprising cleavage site for the 234 bp substrate (Table 2). In order to introduce substitutions the pairs of primers were designed with mutation at a given position. One of the pair of primers contains the appropriate substitution. The template for PCR was a modified plasmid pKSII shown in SEQ ID NO2. PCR reaction with each primer pair was carried out according to the

inted: 19/04/2013 DESCPAMD PCT/PL 2012/050 020 06-04-2013 PL2012050020

method and conditions described in Example 2. The sequences of primers used to produce substitute libraries are presented in Table 3 below.

Table 3. Shows the number of positions, sequences and the names of primer pairs used in the substitution library creation (where H = A or C or U; D = A or G or U; B = C or G or U; V = A or C or G)

Library number	Position of the nucleotide substitution	Primer name	Primer sequence	SEQ ID NO
1	83	Subf	CTCGAGGGGGGGCCCGGTA	SEQ ID NO 31
		Sub83r	GTCGACHGTATCGATAAGCTTG	SEQ ID NO 32
2	84	Subf	CTCGAGGGGGGGCCCGGTA	SEQ ID NO 31
		Sub84r	GTCGADGGTATCGATAAGCTTG	SEQ ID NO 33
3	85	Subf	CTCGAGGGGGGGCCCGGTA	SEQ ID NO 31
		Sub85r	GTCGBCGGTATCGATAAGCTTG	SEQ ID NO 34
4	86	Subf	CTCGAGGGGGGGCCCGGTA	SEQ ID NO 31
		Sub86r	GTCHACGGTATCGATAAGCTTG	SEQ ID NO 35
5	87	Subf	CTCGAGGGGGGGCCCGGTA	SEQ ID NO 31
		Sub87r	GTDGACGGTATCGATAAGCTTG	SEQ ID NO 36
6	88	Subf	CTCGAGGGGGGGCCCGGTA	SEQ ID NO 31
		Sub88r	GVCGACGGTATCGATAAGCTTG	SEQ ID NO 37
7	89	Subf	CTCGAGGGGGGGCCCGGTA	SEQ ID NO 31
		Sub89r	HTCGACGGTATCGATAAGCTTG	SEQ ID NO 38
8	90	Sub90f	DTCGAGGGGGGGCCCGGTA	SEQ ID NO 40
		Subr	GTCGACGGTATCGATAAGCTTG	SEQ ID NO 39
9	91	Sub91f	CVCGAGGGGGGGCCCGGTA	SEQ ID NO 41
		Subr	GTCGACGGTATCGATAAGCTTG	SEQ ID NO 39
10	92	Sub92f	CTDGAGGGGGGGCCCGGTA	SEQ ID NO 42
		Subr	GTCGACGGTATCGATAAGCTTG	SEQ ID NO 39
11	93	Sub93f	CTCHAGGGGGGGCCCGGTA	SEQ ID NO 43
		Subr	GTCGACGGTATCGATAAGCTTG	SEQ ID NO 39
12	94	Sub94f	CTCGBGGGGGGGCCCGGTA	SEQ ID NO 44
		Subr	GTCGACGGTATCGATAAGCTTG	SEQ ID NO 39
13	95	Sub95f	CTCGAHGGGGGGCCCGGTA	SEQ ID NO 45
		Subr	GTCGACGGTATCGATAAGCTTG	SEQ ID NO 39
14	96	Sub96f	CTCGAGHGGGGGCCCGGTA	SEQ ID NO 46
		Subr	GTCGACGGTATCGATAAGCTTG	SEQ ID NO 39

PCR products were separated on agarose gel and then isolated as described in Example 2. The isolated products were phosphorylated and ligated. The reaction was carried out at 37°C for 1 hour. The 20 µl ligation mixture contained 100 ng of PCR product, 2 µl reaction buffer, 10 mM ATP, 1 U T4 polynucleotide kinase and 1 U T4 DNA ligase. *E. coli* TOP10 cells were transformed with 10 µl of ligation mixture as described in Example 1. Then cells were plated on the LB petri dish with 100 µg / ml ampicillin. To search for clones with introduced substitutions

06/04/2013 AMENDED SHEET

inted: 19/04/2013 DESCPAMD PCT/PL 2012/050 020 06-04-2013 PL2012050020

constructs the sequence was analyzed by sequencing as described in Example 2. Plasmids with appropriate substitution introduced were numbered as library from 1 to 14, which served as templates to synthesize dsRNA.

b) *In vitro* synthesis of dsRNA substrates from substitution library obtained in point a)

Selected constructs from substitution library identified as a number from 1 to 14 were used as templates to synthesize dsRNA using primers bsuRNAf and bsuRNAr described in Example 4b.

Example 9

Determination of the preferred cleavage sequence for the endoribonuclease BSU^{WT}

The preferred sequence was determined using substrates synthesized in Example 8b. The cleavage reaction was carried out as in Example 5 using endoribonuclease BSU^{WT} prepared in accordance with the Example 3. The following Table 4 shows the determined sequence preference for endoribonuclese BSU^{WT}.

Table 4. Cleavage of 234 bp dsRNA substitution libraries at positions from 83 to 96. "uppercase" - dsRNA cleavage efficiency as for the initial substrate, "lowercase"- impared dsRNA cleavage; "-"- no cleavage

No	- - 83	84	85	86	87	88	89	90	91	92	93	94	95	96 - -	SEQ ID NO
Sequence of the initial substrate	C	G	U	C	G	A	C	C	U	C	G	A	G	G	30
G	G	G	G	g	G	-	-	-	-	-	G	G	G	G	from 47 to 88
A	A	A	A	a	a	A	-	-	-	A	a	A	A	A	
U	U	U	U	U	u	-	-	-	U	U	u	U	U	U	
C	C	C	C	C	-	-	C	C	-	C	-	C	C	C	
Preferred sequence	N	N	N	Y	G	A	C	C	U	C	G	N	N	G	

Endoribonuclease BSU^{WT} during the cleavage of dsRNA has shown sequence preference. The preferred cleavage sequence can be written as shown below in Table 5.

Table 5. The preferred cleavage sequence of the dsRNA for the endoribonuclease BSU^{WT}. Cleavage site indicated by arrows. (whereY = C or U; R = A or G; N = G or A or U or C)

Printed: 19/04/2013 DESCPAMD PCT/PL 2012/050 020 06-04-2013 PL2012050020

PZ/1243/AGR/PCT

↓
5' YGACCUCGNNG 3'
3' RCUGGAGCNNC 5'
↑

However, endoribonuclease BSU^{WT} is also able to cut the dsRNA substrates that have a consensus sequence as shown in the following Table 6.

Table 6. The consensus sequence of endoribonuclease BSU^{WT} dsRNA substrate. Cleavage site indicated by arrows. (where H = A or C or U; D = A or G or U)

↓
5' DACCUHD 3'
3' HUGGADH 5'
↑

Endribonuclease BSU^{WT} generates sticky ends with 3' 2 nucleotide overhangs in dsRNA.

Example 10

Creation of the endoribonuclease BSU variants

Recombinant coding the wt sequence of endoribonuclease BSU^{WT} (SEQ ID NO1) was subjected to substitution mutagenesis of selected codons coding for the residues situated in the loop which locates in the major groove of dsRNA (Fig. 1). Substitutions in the protein were introduced using properly designed primer pairs. The template for the PCR reaction was plasmid pET28Bsu (Example 2). The PCR reaction to amplify the variants of the coding sequences with the introduced substitutions were performed as described in Example 2 using the primer pair listed in the following Table 7.

Table 7. The sequences and the names of primer pairs used for the introduction of substitutions in order to obtain different endoribonuclease BSU variants in selected amino acid positions. Type of substitution is shown in Table 8.

Position number of the amino acid residue	Primer name	Primer sequence	SEQ ID NOs:
79	K79Af	CCAGAGGCAGAAATGCCAAGTC	SEQ ID NO 89
	K79Ar	CCAGCACCGCTTCCTCTTC	SEQ ID NO 90
80	R80Af	CCGGCAGAAATGCCAAGTCAGG	SEQ ID NO 91

(corrected)

	R80Ar	CCTTCAGCACCGCTTCCTCTTC	SEQ ID NO 92
82	R82Af	CCAATGCCAAGTCAGGGACAAC	SEQ ID NO 93
	R82Ar	CGCCTCTTTTCAGCACCGC	SEQ ID NO 94
83	N83Af	CTGCCAAGTCAGGGACAAC	SEQ ID NO 95
	N83Ar	CTCTGCCTCTTTTCAGCAC	SEQ ID NO 96
85	K85Af	CCTCAGGGACAACACCTAAAAATACAG	SEQ ID NO 97
	K85Ar	CCGCATTTCTGCCTCTTTTCAGC	SEQ ID NO 98
86	S86Af	CTGGGACAACACCTAAAAATAC	SEQ ID NO 99
	S86Ar	CTTTGGCATTTCTGCCTC	SEQ ID NO 100
88	T88Af	CCACACCTAAAAATACAGATGTTC	SEQ ID NO 101
	T88Ar	CGCCTGACTTGGCATTTC	SEQ ID NO 102
91	K91Af	CCAATACAGATGTTCAGACGTACCG	SEQ ID NO 103
	K91Ar	CCGGTGTTGTCCCTGACTTG	SEQ ID NO 104
92	N92Af	CACAGATGTTCAGACGTACCG	SEQ ID NO 105
	N92Ar	GCCTTAGGTGTTGTCCCTG	SEQ ID NO 106
94	D94Af	CCGTTCAGACGTACCGCTAC	SEQ ID NO 107
	D94Ar	CCGTATTTTTAGGTGTTGTCCCTG	SEQ ID NO 108
94	D94Rf	CGTGTTCAGACGTACCGCTACAGTACAG	SEQ ID NO 109
	D94Rr	TGTATTTTTAGGTGTTGTCCCTGACTTG	SEQ ID NO 110

The procedures of phosphorylation, ligation, and constructs transformation were carried out as in Example 8. The extransformants were plated on LB agar with 50 µg/µl kanamycin. The grown colonies were inoculated as in Example 2b, the plasmids were isolated as in Example 2b. The selection of suitable transformants and confirmation of the sequence correctness of the desired substitution was based on the sequencing of the sample (SSIS DNA IBB PAS).

Example 11

Expression and purification of endoribonuclease BSU protein variants and endonucleolytic activity assay

10 variants with substitutions to alanine at positions of the amino acid residues K79, R80, R82, N83, K85, S86, T88, K91, N92, D94 were prepared. Expression and purification of variants were carried out as in Example 3.Then endonucleolytic activities were examined. Results are shown in Table 8. Positions R80, R82, K85, T88, K91, N92, D94 may be involved in sequence specificity of the enzyme. They probably interact with bases in the dsRNA nucleic acid, and

therefore for further substitution mutagenesis the positions were selected in which the substitution to alanine inactivated the enzyme or decreased its activity.

Table 8. Endoribonucleolytic activity of alanine substitution variants of endoribonuclease BSU. "+" - dsRNA cleavage as for the wild type endoribonuclease BSU (BSU^{WT}); "+/-"- impared dsRNA cleavage; "+/--" – impared dsRNA cleavage, "-" - no cleavage

Substitution of amino acid residues in endoribonuclease BSU^{WT}	Endoribonucleolytic activity of variant
K79A	+
R80A	+/-
R82A	+/--
N83A	+
K85A	-
S86A	+
T88A	+/-
K91A	-
N92A	+/--
D94A	+/--

A substitution variant to arginine at the position number 94 (D94R) was created. The protein was purified as in step 3, and its endoribonucleolytic activity was tested on two substrates: bacteriophage Φ6 genome and 234 bp dsRNA. 234 bp dsRNA, which has one preferred cleavage site, was cleaved similarly by the wild type enzyme and D94R variant. Φ6 dsRNA, which has 38 consensus cleavage sites, was not cleaved with the same efficiency by both enzymes. Cleavage by the D94R variant was impaired in comparison to the wild-type enzyme. The results obtained are shown in Figure 5. It is shown that the variant D94R has an increased selectivity to the preferred sequence of dsRNA. Increased selectivity of the enzyme results in the narrowing of the sequence recognition and cleavage of dsRNA.

The above results indicate that the loop locating in the major groove of dsRNA determines the sequence specificity in the dsRNA cleavage determined only by the sequence of dsRNA and independent on the irregular helix structure and/or cooperation with other proteins. The method also demonstrate the selection leading to derivatives and/or variants of dsRNA endoribonucleases exhibiting increased sequence specificity in dsRNA cleavage, preferably in such a method of obtaining derivatives and/or variants with sequence specific cleavage of dsRNA the endoribonuclease derivatives and/or variants are generated with preferably altered, increased selectivity to the specific sequence in dsRNA cleavage.

Example 12

Cleavage of three short 30 bp dsRNAs

30 bp dsRNA substrates were prepared by annealing RNA oligonucleotides 30N1F and 30N1R, 30N2F and 30N2R, 30N3F and 30N3R described in example 4c. 30 bp dsRNA with preferred sequence prepared from oligonucleotides 30F and 30R from example 4c was used as a control. Cleavage reaction was carried out as in Example 5 using endoribonuclease BSU^{WT} prepared in accordance with the Example 3. Prepared substrates were cleaved by endoribonuclease BSU^{WT}. Products were separated on 15% polyacrylamide gel (15% polyacrylamide, TBE: 135 mM Tris-HCl, 45 mM boric acid, 2.5 mM EDTA). Visualization of the products is described in Example 6b. The results are shown in Figure 8. Three tested substrates are not cleaved.

<u>The list of sequences identified in the description:</u>:

SEQ ID NO1 - amino acid sequence of dsRNA endoribonuclease BSU^{WT} from *Bacillus subtilis*

SEQ ID NO2 - a sequence of modified vector pKS II

SEQ ID NO3 – fragment of polypeptide chain of endoribonuclease FNU from *Fusobacterium nucleatum* forming a loop that locates in and interacts with the major groove of the dsRNA

SEQ ID NO4 - fragment of polypeptide chain of endoribonuclease BSU from *Bacillus subtilis* forming a loop that locates in and interacts with the major groove of the dsRNA

SEQ ID NO5 - fragment of polypeptide chain of endoribonuclease BCE from *Bacillus cereus* forming a loop that locates in and interacts with the major groove of the dsRNA

SEQ ID NO6 – nucleotide sequence of primer for endoribonuclease BSU gene amplification

SEQ ID NO7 – nucleotide sequence of primer for endoribonuclease BSU gene amplification

SEQ ID NO8 – nucleotide sequence of primer for endoribonuclease FNU gene amplification

SEQ ID NO9 – nucleotide sequence of primer for endoribonuclease FNU gene amplification

SEQ ID NO10 – nucleotide sequence of primer for endoribonuclease BCE gene amplification

SEQ ID NO11 – nucleotide sequence of primer for endoribonuclease BCE gene amplification

SEQ ID NO12 – nucleotide sequence of bsuRNAf primer for 234 bp dsRNA synthesis

SEQ ID NO13 – nucleotide sequence of bsuRNAr primer for 234 bp dsRNA synthesis

SEQ ID NO14 – nucleotide sequence of 18F oligonucleotide for 18 bp dsRNA preparation

SEQ ID NO15 – nucleotide sequence of 18R oligonucleotide for 18 bp dsRNA preparation

SEQ ID NO16 – nucleotide sequence of 20F oligonucleotide for 20 bp dsRNA preparation

SEQ ID NO17 – nucleotide sequence of 20R oligonucleotide for 20 bp dsRNA preparation

SEQ ID NO18 – nucleotide sequence of 22F oligonucleotide for 22 bp dsRNA preparation

SEQ ID NO19 – nucleotide sequence of 22R oligonucleotide for 22 bp dsRNA preparation

SEQ ID NO20 – nucleotide sequence of 30F oligonucleotide for 30 bp dsRNA preparation

SEQ ID NO21 – nucleotide sequence of 30R oligonucleotide for 30 bp dsRNA preparation

SEQ ID NO22 – nucleotide sequence of 30N1F oligonucleotide for N1 30 bp dsRNA preparation

SEQ ID NO23 – nucleotide sequence of 30N1R oligonucleotide for N1 30 bp dsRNA preparation

SEQ ID NO24 – nucleotide sequence of 30N2F oligonucleotide for N2 30 bp dsRNA preparation

SEQ ID NO25 – nucleotide sequence of 30N2R oligonucleotide for N2 30 bp dsRNA preparation

SEQ ID NO26 – nucleotide sequence of 30N3F oligonucleotide for N3 30 bp dsRNA preparation

SEQ ID NO27 – nucleotide sequence of 30N3R oligonucleotide for N3 30 bp dsRNA preparation

SEQ ID NO28 – nucleotide sequence of RTr primer for reverse transcription reaction

SEQ ID NO29 – nucleotide sequence of RTf primer for reverse transcription reaction

SEQ ID NO30 – nucleotide sequence of 234 bp dsRNA substrate

SEQ ID NO31 – nucleotide sequence of Subf primer for substitution library creation of 234 bp dsRNA

SEQ ID NO32 – nucleotide sequence of Sub83r primer for substitution library creation of 234 bp dsRNA

SEQ ID NO33 – nucleotide sequence of Sub84r primer for substitution library creation of 234 bp dsRNA

SEQ ID NO34 – nucleotide sequence of Sub85r primer for substitution library creation of 234 bp dsRNA

SEQ ID NO35 – nucleotide sequence of Sub86r primer for substitution library creation of 234 bp dsRNA

SEQ ID NO36 – nucleotide sequence of Sub87r primer for substitution library creation of 234 bp dsRNA

SEQ ID NO37 – nucleotide sequence of Sub88r primer for substitution library creation of 234 bp dsRNA

SEQ ID NO38 – nucleotide sequence of Sub89r primer for substitution library creation of 234 bp dsRNA

SEQ ID NO39 – nucleotide sequence of Subr primer for substitution library creation of 234 bp dsRNA

SEQ ID NO40 – nucleotide sequence of Sub90f primer for substitution library creation of 234 bp dsRNA

SEQ ID NO41 – nucleotide sequence of Sub91f primer for substitution library creation of 234 bp dsRNA

SEQ ID NO42 – nucleotide sequence of Sub92f primer for substitution library creation of 234 bp dsRNA

SEQ ID NO43 – nucleotide sequence of Sub93f primer for substitution library creation of 234 bp dsRNA

SEQ ID NO44 – nucleotide sequence of Sub94f primer for substitution library creation of 234 bp dsRNA

SEQ ID NO45 – nucleotide sequence of Sub95f primer for substitution library creation of 234 bp dsRNA

SEQ ID NO46 – nucleotide sequence of Sub96f primer for substitution library creation of 234 bp dsRNA

SEQ ID NO47 – nucleotide sequence of 83G 234 bp dsRNA

SEQ ID NO48 – nucleotide sequence of 83A 234 bp dsRNA

SEQ ID NO49 – nucleotide sequence of 83U 234 bp dsRNA

SEQ ID NO50 – nucleotide sequence of 84A 234 bp dsRNA

SEQ ID NO51 – nucleotide sequence of 84U 234 bp dsRNA

SEQ ID NO52 – nucleotide sequence of 84C 234 bp dsRNA

SEQ ID NO53 – nucleotide sequence of 85G 234 bp dsRNA

SEQ ID NO54 – nucleotide sequence of 85A 234 bp dsRNA

SEQ ID NO55 – nucleotide sequence of 85C 234 bp dsRNA

SEQ ID NO56 – nucleotide sequence of 86G 234 bp dsRNA

SEQ ID NO57 – nucleotide sequence of 86A 234 bp dsRNA

SEQ ID NO58 – nucleotide sequence of 86U 234 bp dsRNA

SEQ ID NO59 – nucleotide sequence of 87A 234 bp dsRNA

SEQ ID NO60 – nucleotide sequence of 87U 234 bp dsRNA

SEQ ID NO61 – nucleotide sequence of 87C 234 bp dsRNA

SEQ ID NO62 – nucleotide sequence of 88G 234 bp dsRNA

SEQ ID NO63 – nucleotide sequence of 88U 234 bp dsRNA

SEQ ID NO64 – nucleotide sequence of 88C 234 bp dsRNA

SEQ ID NO65 – nucleotide sequence of 89G 234 bp dsRNA

SEQ ID NO66 – nucleotide sequence of 89A 234 bp dsRNA

SEQ ID NO67 – nucleotide sequence of 89U 234 bp dsRNA

SEQ ID NO68 – nucleotide sequence of 90G 234 bp dsRNA

SEQ ID NO69 – nucleotide sequence of 90A 234 bp dsRNA

SEQ ID NO70 – nucleotide sequence of 90U 234 bp dsRNA

SEQ ID NO71 – nucleotide sequence of 91G 234 bp dsRNA

SEQ ID NO72 – nucleotide sequence of 91A 234 bp dsRNA

SEQ ID NO73 – nucleotide sequence of 91C 234 bp dsRNA

SEQ ID NO74 – nucleotide sequence of 92G 234 bp dsRNA

SEQ ID NO75 – nucleotide sequence of 92A 234 bp dsRNA

SEQ ID NO76 – nucleotide sequence of 92U 234 bp dsRNA

SEQ ID NO77 – nucleotide sequence of 93A 234 bp dsRNA

SEQ ID NO78 – nucleotide sequence of 93U 234 bp dsRNA

SEQ ID NO79 – nucleotide sequence of 93C 234 bp dsRNA

SEQ ID NO80 – nucleotide sequence of 94G 234 bp dsRNA

SEQ ID NO81 – nucleotide sequence of 94U 234 bp dsRNA

SEQ ID NO82 – nucleotide sequence of 94C 234 bp dsRNA

SEQ ID NO83 – nucleotide sequence of 95A 234 bp dsRNA

SEQ ID NO84 – nucleotide sequence of 95U 234 bp dsRNA

SEQ ID NO85 – nucleotide sequence of 95C 234 bp dsRNA

SEQ ID NO86 – nucleotide sequence of 96A 234 bp dsRNA

SEQ ID NO87 – nucleotide sequence of 96U 234 bp dsRNA

SEQ ID NO88 – nucleotide sequence of 96C 234 bp dsRNA

SEQ ID NO89 – nucleotide sequence of K79Af primer

SEQ ID NO90 – nucleotide sequence of K79Ar primer

SEQ ID NO91 – nucleotide sequence of R80Af primer

SEQ ID NO92 – nucleotide sequence of R82Ar primer

SEQ ID NO93 – nucleotide sequence of R82Af primer

SEQ ID NO94 – nucleotide sequence of R82Ar primer

SEQ ID NO95 – nucleotide sequence of N83Af primer

SEQ ID NO96 – nucleotide sequence of N83Ar primer

SEQ ID NO97 – nucleotide sequence of K85Af primer

SEQ ID NO98 – nucleotide sequence of K85Ar primer

SEQ ID NO99 – nucleotide sequence of S86Af primer

SEQ ID NO100 – nucleotide sequence of S86Ar primer

SEQ ID NO101 – nucleotide sequence of T88Af primer

SEQ ID NO102 – nucleotide sequence of T88Ar primer

SEQ ID NO103 – nucleotide sequence of K91Af primer

SEQ ID NO104 – nucleotide sequence of K91Ar primer

SEQ ID NO105 – nucleotide sequence of N92Af primer

SEQ ID NO106 – nucleotide sequence of N92Ar primer

SEQ ID NO107 – nucleotide sequence of D94Af primer

SEQ ID NO108 – nucleotide sequence of D94Ar primer

SEQ ID NO109 – nucleotide sequence of D94Rf primer

SEQ ID NO110 – nucleotide sequence of D94Rr primer

CLAIMS

1. Use of a dsRNA endoribonuclease for sequence specific cleavage of a dsRNA substrate, wherein the endoribonuclease:

comprises the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:1 with D94R mutation;

and has a loop that is locating in and interacting with a major groove of dsRNA, which corresponds to a loop locating in and interacting with a major groove of dsRNA in a model of structure of endoribonuclease Mini III in complex with dsRNA;

and wherein the dsRNA endoribonuclease exhibits the dsRNA sequence specific activity within the consensus sequence

5' DACCUHD 3'
3' HUGGADH 5'

where H = A or C or U;

D = A or G or U;

and wherein the specific sequence in the dsRNA substrate recognized by the dsRNA endoribonuclease is the consensus sequence

5' DACCUHD 3'
3' HUGGADH 5'

where H = A or C or U;

D = A or G or U;

and wherein the dsRNA substrate comprises and is cleaved within the recognition sequence by the dsRNA endoribonuclease.

2. The use according to claim 1, wherein the dsRNA endoribonuclease exhibits the dsRNA sequence specific activity within the consensus sequence

5' YGACCUCGNNG 3'
3' RCUGGAGCNNC 5'

where Y = C or U;

R = A or G; and

N = G or A or U or C.

3. The use according to claim 1 or 2, wherein the specific sequence in the dsRNA substrate recognized by the dsRNA endoribonuclease is the consensus sequence

5' YGACCUCGNNG 3'
3' RCUGGAGCNNC 5'

where Y = C or U;

R = A or G; and

N = G or A or U or C,

4. Use of a dsRNA endoribonuclease for sequence specific cleavage of a dsRNA substrate,

wherein the dsRNA endoribonuclease:

comprises the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:1 with D94R mutation;

and has a loop that is locating in and interacting with a major groove of dsRNA, which corresponds to a loop locating in and interacting with a major groove of dsRNA in a model of structure of endoribonuclease Mini III in complex with dsRNA;

and wherein the dsRNA endoribonuclease exhibits the dsRNA sequence specific activity within the consensus sequence

5' DACCUHD 3'
3' HUGGADH 5'

where H = A or C or U;

D = A or G or U;

and wherein the specific sequence in the dsRNA substrate recognized by the dsRNA endoribonuclease is the consensus sequence

5' DACCUHD 3'
3' HUGGADH 5'

where H = A or C or U;

D = A or G or U; and

wherein the dsRNA endoribonuclease is combinable with the dsRNA substrate in a mixture to cleave the dsRNA substrate within the recognition sequence by the dsRNA endoribonuclease.

5. The use according to claim 4, wherein the dsRNA endoribonuclease exhibits the dsRNA sequence specific activity within the consensus sequence

5' YGACCUCGNNG 3'
3' RCUGGAGCNNC 5'

where Y = C or U;

R = A or G; and

N = G or A or U or C.

6. The use according to claim 4 or 5, wherein the specific sequence in the dsRNA substrate recognized by the dsRNA endoribonuclease is the consensus sequence

5' YGACCUCGNNG 3'
3' RCUGGAGCNNC 5'

where Y = C or U;

R = A or G; and

N = G or A or U or C.

7. The use according to any one of claims 4-6, wherein the dsRNA endoribonuclease is combinable with the dsRNA substrate in a mixture to cleave the dsRNA substrate

at a temperature from 35°C to 45 °C; and/ or

at a sodium chloride concentration from 5 to 60 mM.

8. The use according to claim 7, wherein the dsRNA endoribonuclease is combinable with the dsRNA substrate in a mixture to cleave the dsRNA substrate at a Mg^{2+} concentration of 1 to 2.5 mM.

9. A dsRNA endoribonuclease, comprising:

the amino acid sequence of SEQ ID NO:1 with D94R mutation;

and having a loop that is locating in and interacting with a major groove of dsRNA, which corresponds to a loop locating in and interacting with a major groove of dsRNA in a model of structure of endoribonuclease Mini III in complex with dsRNA;

wherein the specific sequence in the dsRNA substrate recognized by the dsRNA endoribonuclease is the consensus sequence

5' DACCUHD 3'
3' HUGGADH 5'

where H = A or C or U;

D = A or G or U;

and wherein the dsRNA endoribonuclease exhibits the dsRNA sequence specific activity within the consensus sequence.

10. The dsRNA endoribonuclease according to claim 9, wherein the consensus sequence is

5' YGACCUCGNNG 3'
3' RCUGGAGCNNC 5'

where Y = C or U;

R = A or G; and

N = G or A or U or C.

11. A method for producing dsRNA endoribonuclease, wherein the method comprises the step of *ex vivo* expressing the dsRNA endoribonuclease as defined in claim 9 or 10, employing an *ex vivo* expression system.

12. The method according to claim 11, wherein the *ex vivo* expression system is a cell culture.

13. A genetic construct comprising a nucleotide sequence encoding the dsRNA endoribonuclease as defined in claim 9 or 10.

14. A host cell comprising the genetic construct as defined in claim 13.

15. A kit comprising the dsRNA endoribonuclease, as defined in claim 9 or 10 and instructions for its use in sequence specific cleavage of the dsRNA substrate.

Fig. 1

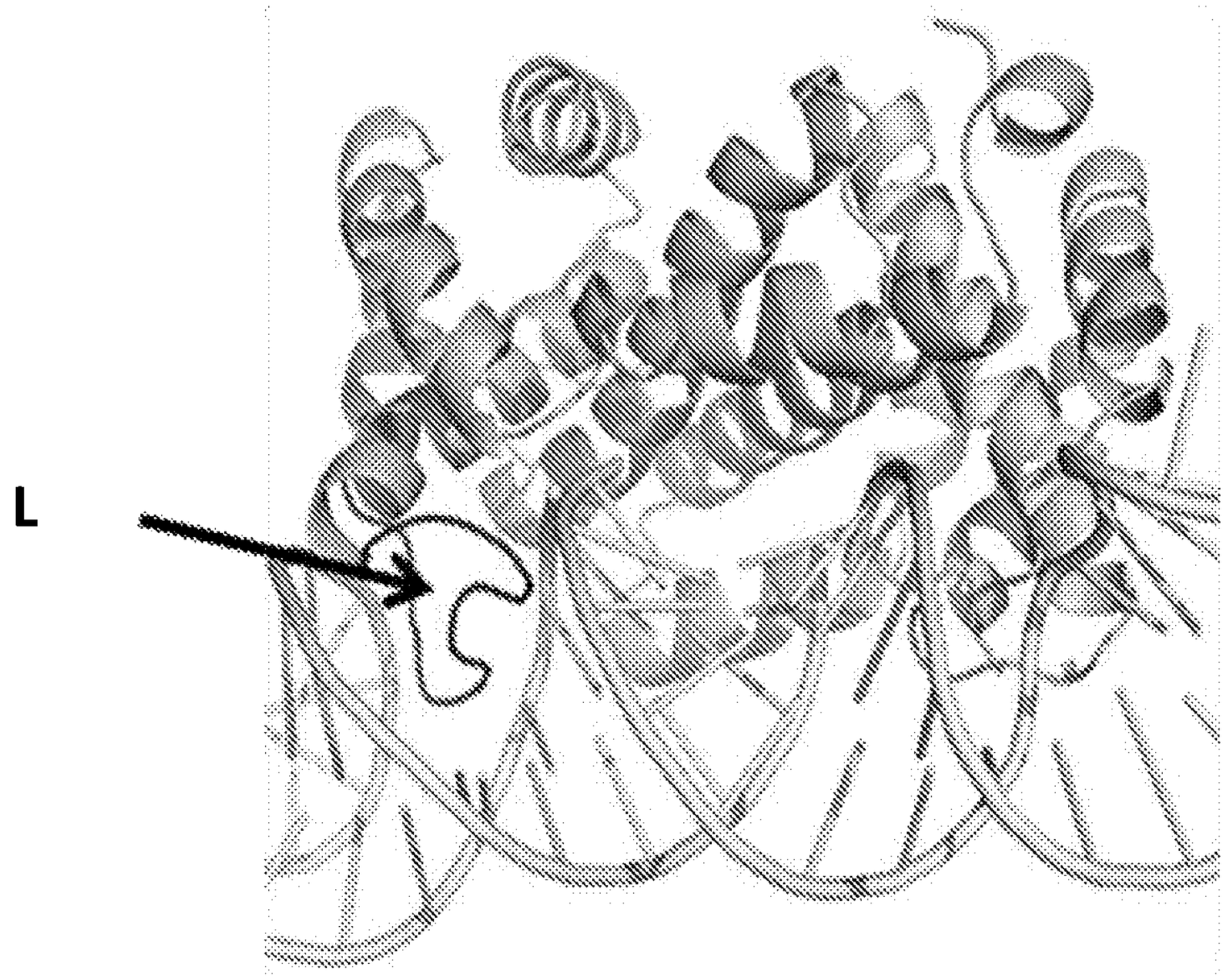
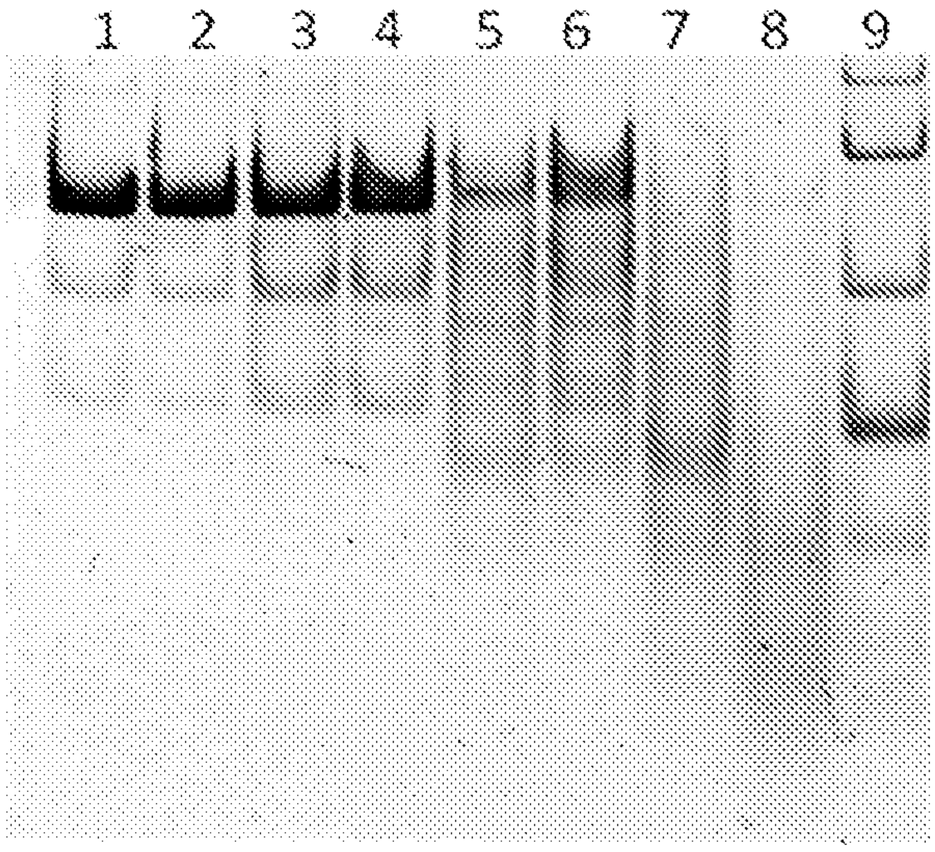
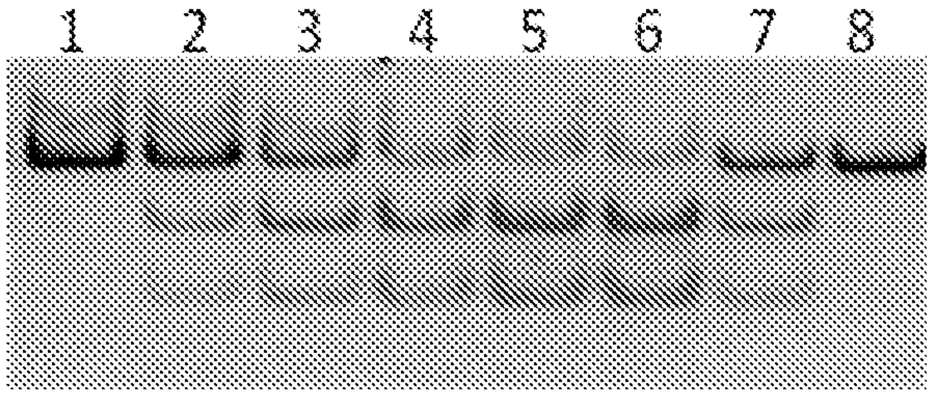


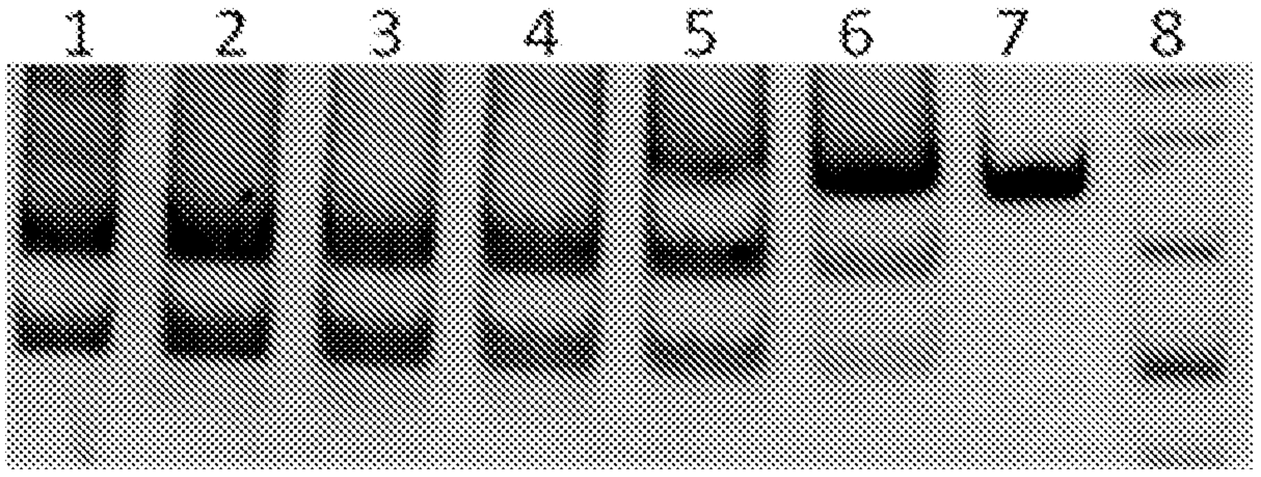
Fig. 2



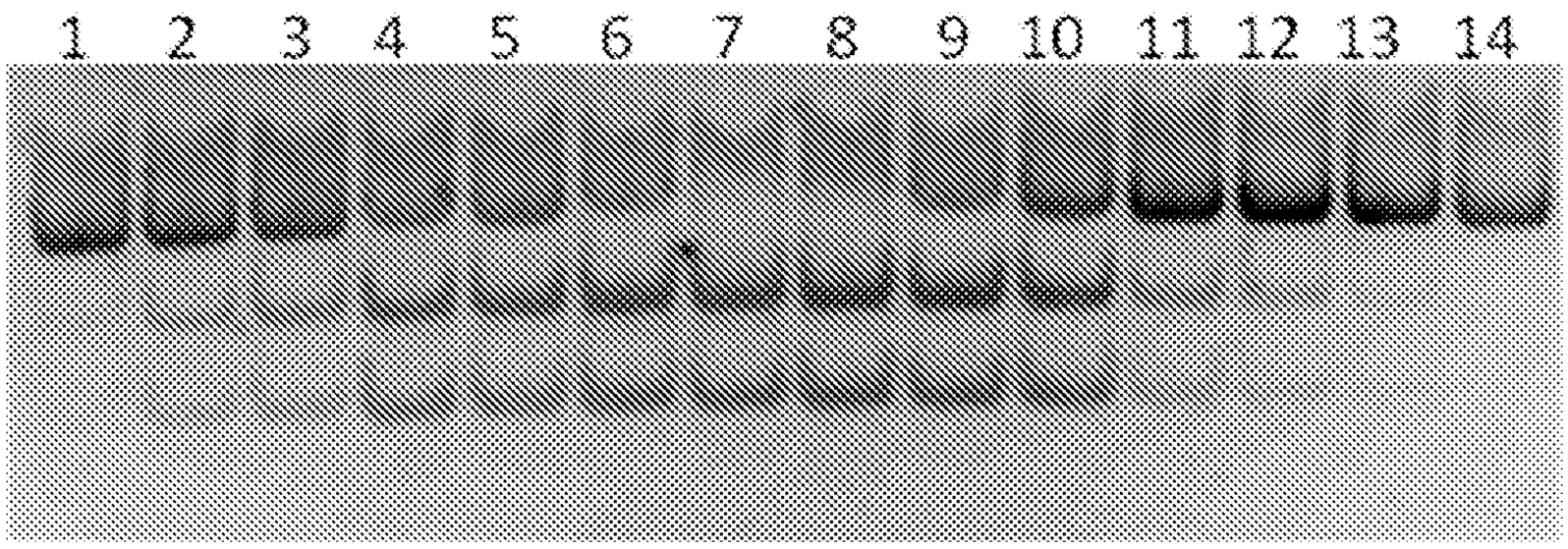
A



B



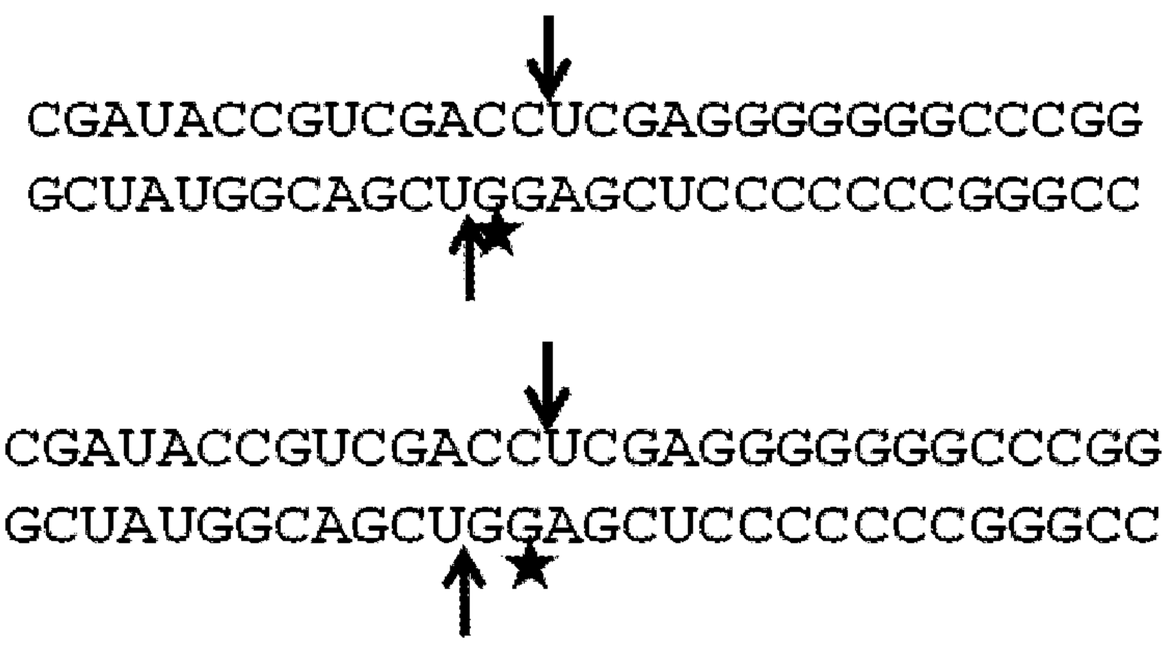
C



D

Fig 3

A


CGAUACCGUCGACCUCGAGGGGGGGCCCGG
GCUAUGGCAGCUGGAGCUCCCCCCCCGGGCC
CGAUACCGUCGACCUCGAGGGGGGGCCCGG
GCUAUGGCAGCUGGAGCUCCCCCCCCGGGCC

B

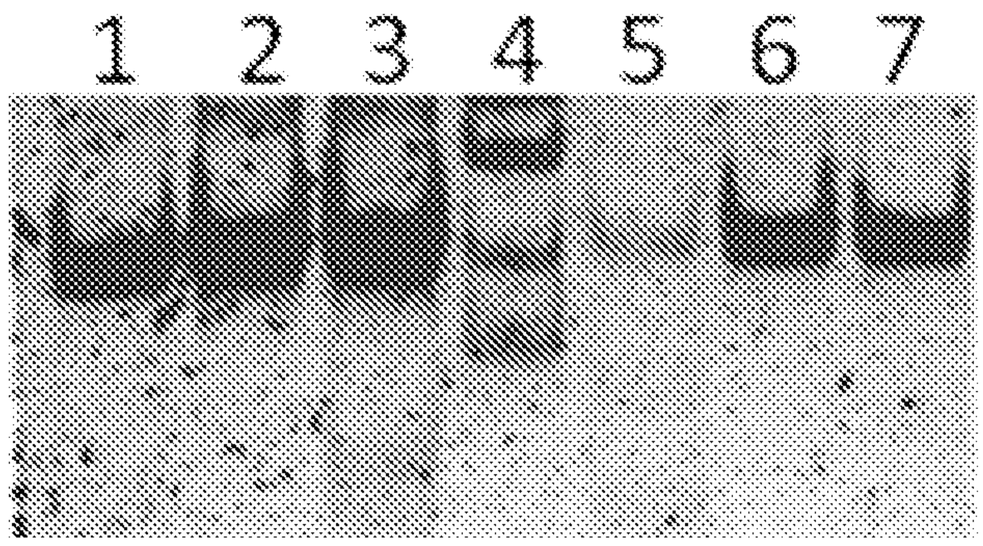

1 2 3 4 5 6 7

Fig 4

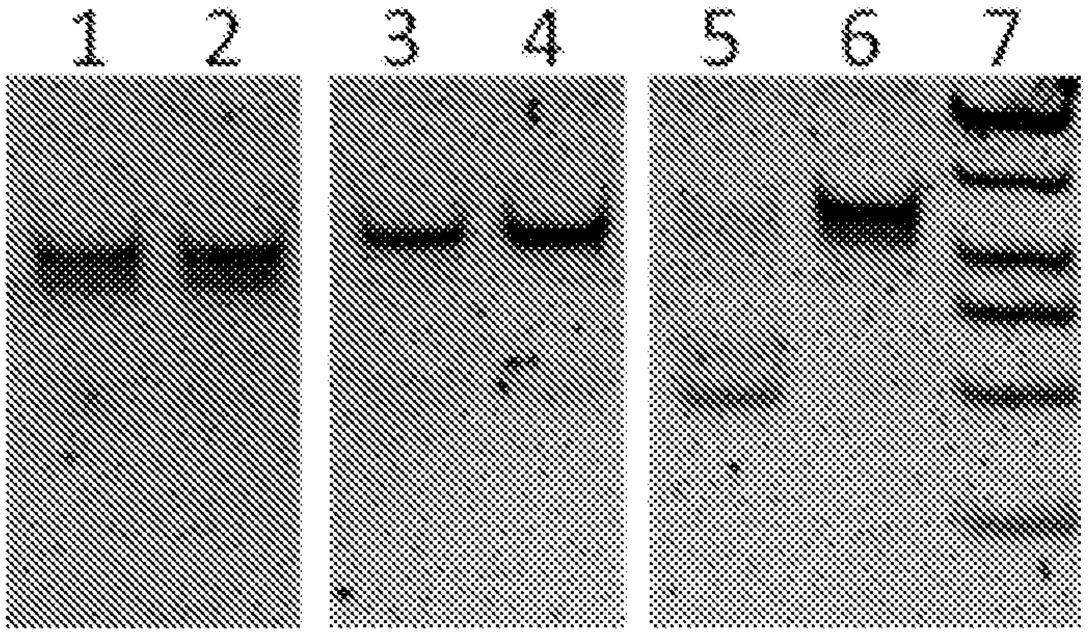

1 2 3 4 5 6 7

Fig. 5

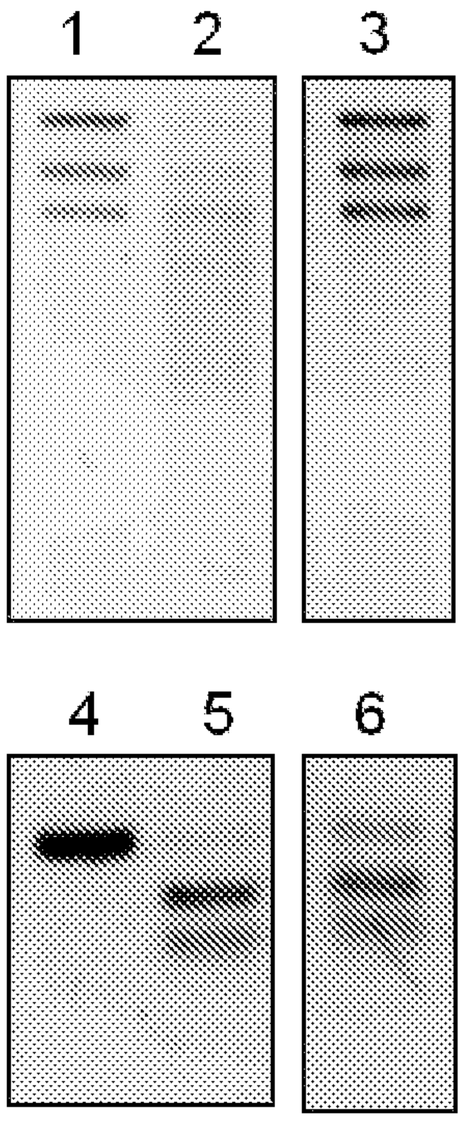

1
2
3
4
5
6

Fig. 6

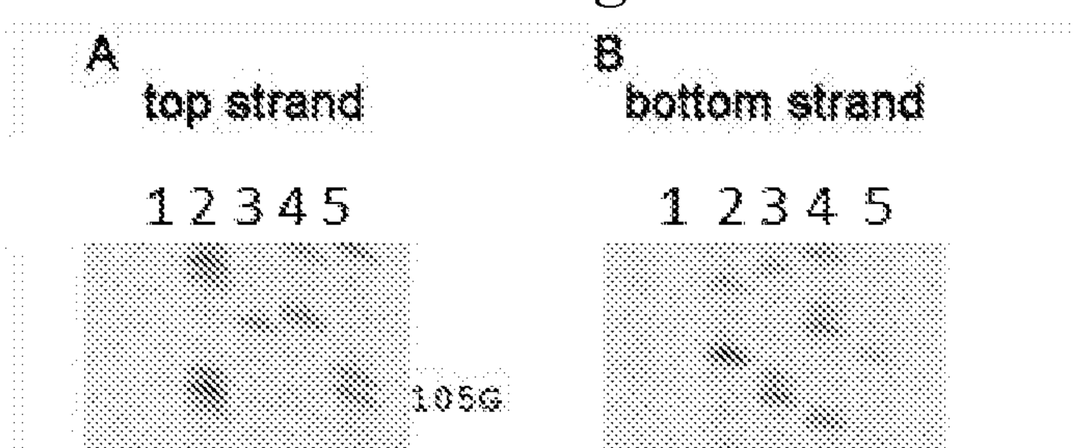

A
top strand
1 2 3 4 5
105G
95G
85T
75A
B
bottom strand
1 2 3 4 5
155A
145G
135C

Fig. 7

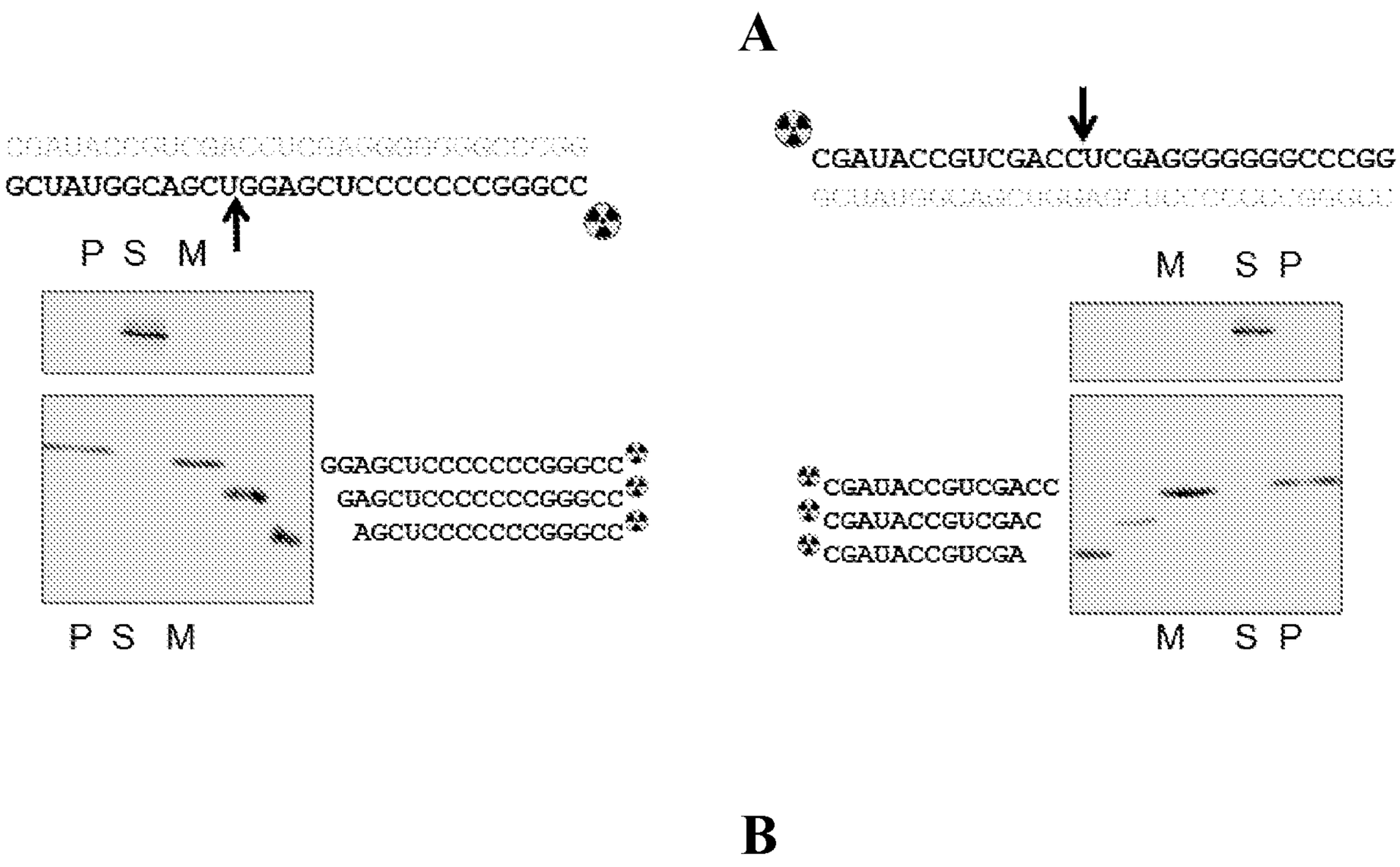
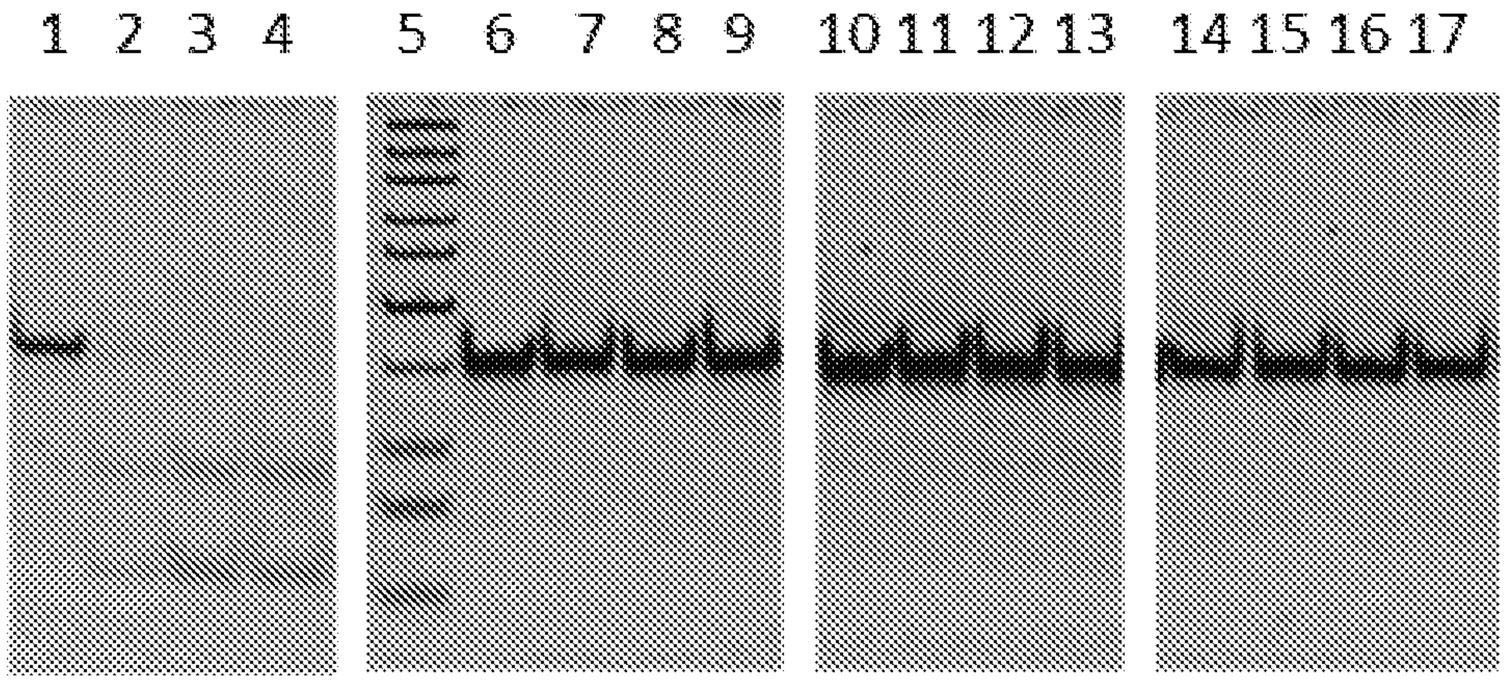

A
CGAUACCGUCGACCUCGAGGGGGGGCCCGG
GCUAUGGCAGCUGGAGCUCCCCCCCCGGGCC
P S M
GGAGCUCCCCCCCCGGGCC
GAGCUCCCCCCCCGGGCC
AGCUCCCCCCCCGGGCC
P S M
CGAUACCGUCGACC
CGAUACCGUCGAC
CGAUACCGUCGA
M S P
M S P
B
CGAUACCGUCGACCUCGAGGGGGGGCCCGG
GCUAUGGCAGCUGGAGCUCCCCCCCCGGGCC

Fig. 8


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17