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(54) Titre : PROCEDES ET COMPOSITIONS DESTINES A LA PRODUCTION D'ARN A DOUBLE BRIN ACCRUE
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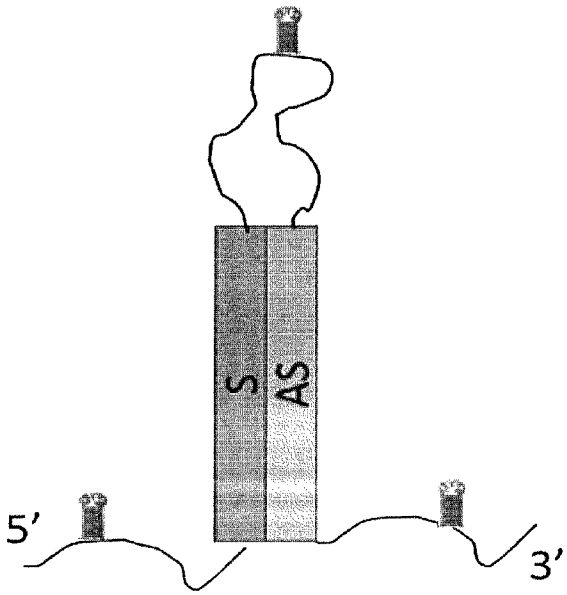

(57) Abrégé/Abstract:
Methods and materials for improved in vivo production of dsRNA are presented. Yields of dsRNA are significantly increased in the presence of capsid protein. The improved yield of dsRNA is not dependent on the presence of specific cognate binding sites for capsid protein associated with the dsRNA, but is dependent on capsid protein.

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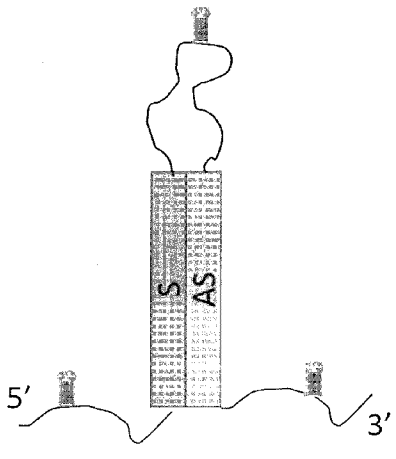
Figure 1



(57) Abstract: Methods and materials for improved in vivo production of dsRNA are presented. Yields of dsRNA are significantly increased in the presence of capsid protein. The improved yield of dsRNA is not dependent on the presence of specific cognate binding sites for capsid protein associated with the dsRNA, but is dependent on capsid protein.

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METHODS AND COMPOSITIONS FOR INCREASED DOUBLE STRANDED RNA PRODUCTION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/308,381 filed March 15, 2016.

SEQUENCE LISTING

[0002] A Sequence Listing is provided herewith as a text file entitled "103827-5009_sequences_ST25" created on March 3, 2017 and having a size of 408 KB.

FIELD OF THE INVENTION

[0003] The present invention relates to methods and compositions for increasing in vivo production of double-stranded RNA.

BACKGROUND OF THE INVENTION

[0004] The ability to suppress gene expression with RNA homologous to targeted gene sequences has greatly increased demand for large scale production of such RNA. However, the chemical fragility of RNA limits commercial development of many of these techniques. Large scale production of purified RNA is constrained by the high costs associated with in vitro synthesis methods and by the low yields and complex processing requirements associated with in vivo methods.

[0005] The susceptibility of RNA to enzymatic and environmental degradation varies widely depending on the nature of the RNA molecule. Single-stranded RNA (ssRNA) is extremely sensitive to degradation and in vivo production of such molecules requires use of production strains lacking endogenous RNAses and benefits by coupling production of the RNA to encapsidation within viral capsid shells to produce Virus-Like Particles (VLPs). Encapsidation reduces degradation of RNA

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during production and allows more aggressive treatment during purification. VLPs effectively preserve such fragile RNA from degradation by sequestering the RNA within a relatively inert protein shell. Double stranded RNA (dsRNA) are somewhat less susceptible to degradation by cellular and environmental RNAses, although the highest in vivo yields of dsRNA also involve production strains lacking RNAses and many dsRNA also benefit from encapsidation. Unfortunately, the semi-rigid nature of the double-stranded stem region of dsRNA limits the range of dsRNA that can be encapsidated since the length of the double-stranded stem structure cannot exceed the interior diameter of the capsid.

[0006] In the course of exploring techniques for increasing the range of dsRNA stems that can be encapsidated, the inventors discovered that under certain conditions a large amount of unencapsidated dsRNA can be recovered directly from cell lysates, but only when the host cells co-express capsid protein or specific portions thereof. The presence of high quantities of intact unencapsidated dsRNA in crude cell lysates represents a significant advance in the ability to generate commercial quantities of such RNA for gene suppression and other activities.

[0007] Dimers of bacteriophage capsid proteins such as those of the leviviruses MS2 or Qβ recognize and bind with affinity to cognate pac sequences. Such pac sequences comprise approximately 19-21 nucleotides comprising an 8 base pair bulged stem and 4 base loop capable of forming a discrete hairpin structure. Such sequences may be referred to herein as pac-sites, pac sequences, pac-site sequences, pac-site hairpins, or pac-site hairpin sequences. The interaction of capsid dimers with their cognate pac site hairpin is well-characterized and is known to play at least two key roles in the bacteriophage life cycle. Binding of capsid dimers to the cognate pac sites is required for programmed translational repression of the phage encoded replicase, which is only expressed early in infection. In addition, capsid protein binding to both to pac-site sequences and multiple dispersed and degenerate RNA sites with cognate coat protein affinity (the packaging signals described by Dykeman et al., *Packaging Signals in Two Single-Stranded RNA Viruses Imply a Conserved Assembly Mechanism and Geometery of the Packaged Genome* J. Mol. Biol. 425:3235-3249 (2013)) are required for proper assembly into progeny bacteriophage.

[0008] The interaction of capsid dimers with cognate pac sites is the subject of a number of different published in vitro and in vivo methods designed to allow encapsidation of heterologous RNAs of various kinds by associating the desired cargo molecule with pac site sequences, either by direct covalent linkage or by various affinity methods. The present invention differs markedly from such approaches in that it comprises co-expression of capsid proteins to produce unencapsidated dsRNA rather than encapsidated RNA. Further, the present invention allows in vivo production of dsRNA entirely lacking pac or any recognized dispersed and degenerate RNA sites with cognate protein affinity. In vivo production of such dsRNA molecules is highly desirable since reducing extraneous sequence reduces the chance of off-target RNAi interactions.

SUMMARY OF THE INVENTION

[0009] The invention described in the following embodiments provides methods and compositions for producing large quantities of unencapsidated dsRNA in vivo. The disclosed methods and compositions represent a significant improvement over current in vivo methods of producing dsRNA.

[0010] In an embodiment the invention comprises a microbial cell containing a gene encoding a self-complementary stretch of sequence separated by non-complementary sequence such that upon hybridization of the complementary sequences a stem-loop structure is formed, wherein the stem portion of the molecule functions as an RNAi precursor when introduced into the target organism. The microbial cell also contains a bacteriophage coat protein gene encoding a capsid protein. Expression of the dsRNA gene and the coat protein gene results in increased accumulation of un-degraded dsRNA and capsid protein. The amount of dsRNA produced in this way greatly exceeds the amount of dsRNA produced in the absence of capsid protein.

[0011] In one embodiment the bacteriophage capsid protein is encoded by the coat protein gene of a species of leviviridae. In a preferred embodiment the coat protein

gene encodes the capsid protein of bacteriophage MS2. In another preferred embodiment the coat protein gene encodes the capsid protein of bacteriophage Qbeta.

[0012] In an embodiment the capsid protein comprises the N-terminus of the MS2 capsid protein. In another embodiment the capsid protein comprises the N-terminal 41, 35, 25, 21 or 12 amino acids of the MS2 capsid protein. In an embodiment the capsid protein comprises the N-terminus of the Qbeta capsid protein. In another embodiment the capsid protein comprises the N-terminal 41, 35, 25, 21 or 12 amino acids of the Qbeta capsid protein.

[0013] In an embodiment the gene encoding the dsRNA may be associated with and expressed from an inducible transcriptional promoter. The coat protein gene may be associated with and expressed from a constitutive or inducible transcriptional promoter. The inducible transcriptional promoter associated with expression of the dsRNA may be the same inducible transcriptional promoter or a different transcriptional promoter from a transcriptional promoter associated with expression of the coat protein gene. In one embodiment the inducible transcriptional promoter associated with expression of the coat protein gene is induced before induction of the inducible transcriptional promoter associated with expression of the dsRNA to allow accumulation of capsid protein prior to production of dsRNA. In another embodiment the transcriptional promoter associated with expression of the coat protein gene is a constitutive transcriptional promoter.

[0014] In an embodiment the gene encoding the dsRNA and the coat protein gene encoding the capsid protein are present on a plasmid or extrachromosomal element. The gene encoding the dsRNA and the coat protein gene may be present on the same plasmid or extrachromosomal element or may be present on separate plasmids or extrachromosomal elements. In another embodiment one or both of the genes encoding the dsRNA and the coat protein may be present on the microbial host cell chromosome or chromosomes.

[0015] In related embodiments, the dsRNA may be purified from the microbial host cell by lysing the cells to produce a lysate and purifying the dsRNA from the

cellular constituents within the lysate prior to processing the purified dsRNA for application. Such processing may include, but is not limited to mixing with excipients, binders or fillers to improve physical handling characteristics, stabilizers to reduce degradation, or other active agents such as chemical pesticides, fungicides, defoliants or other RNAi molecules to broaden the spectrum of application targets, and may include pelletizing, spray drying or dissolving the materials into liquid carriers. In another embodiment the dsRNA is not further purified from the lysate but is processed directly for application. In still another embodiment the microbial host cell is not lysed but is processed directly for application and the dsRNA remains unpurified within the processed cells.

DESCRIPTION OF THE DRAWINGS

[0016] **Figure 1** depicts an RNA stem-loop structure with three pac-site hairpin sequences, one located 5' of the stem-loop structure, one within the loop of the stem-loop structure, and the other 3' of the stem-loop structure.

[0017] **Figure 2** depicts a single strand (sense) sequence flanked on each side by a pac-site hairpin sequence.

[0018] **Figure 3** depicts a single strand (antisense) sequence flanked on each side by a pac-site hairpin sequence.

[0019] **Figure 4** depicts an RNA stem-loop structure with two pac-site hairpin sequences, one located 5' of the stem-loop structure and the other 3' of the stem-loop structure.

[0020] **Figure 5** depicts an RNA stem-loop structure with a single pac-site hairpin sequence located 3' of the stem-loop structure.

[0021] **Figure 6** depicts an RNA stem loop structure lacking any pac site hairpin sequences.

DETAILED DESCRIPTION OF THE INVENTION

[0022] The present invention comprises compositions and methods for producing large quantities of dsRNA in vivo and in some embodiments, recovering such dsRNA directly from cell lysates. In its most basic form, the invention involves co-expressing a bacteriophage capsid protein, or a portion thereof, in conjunction with the desired dsRNA for a period of time sufficient to allow accumulation of the dsRNA in a host cell, lysing the host cell, and optionally recovering intact unencapsidated dsRNA directly from the cell lysate. In the absence of bacteriophage capsid protein intact dsRNA is present in cell lysates in only very small quantities, if at all. In contrast, in the presence of bacteriophage capsid protein a relatively large quantity of unencapsidated dsRNA can be recovered from cell lysates.

[0023] A number of permutations of RNA structure and coat protein were explored to determine the essential elements of the invention and to optimize the yield of dsRNA produced by the invention. This work is summarized in Table 1 which outlines the various elements of the invention described in detail and in the examples below. The leftmost column of Table 1 refer to individual figures representing cartoon depiction of the predicted RNA structure produced from each of the listed plasmid constructs. In each figure "S" represents the sense strand, "AS" represents antisense strand, and the small hairpin structures represent pac site sequences). The table also lists the coat protein (if any) and the yields of dsRNA (or ssRNA, as indicated) associated with each of the listed plasmid constructs.

Table 1. Production of RNA by *E. coli* HT115(DE3) as a function of variation in RNA structure and the presence or absence of coat protein and coat protein variants (n.a. = not applicable; n.d. = not determined).

RNA Structure as depicted in	Plasmid	Loop size (bases)	Stem size (bp)	Stem sequence	Coat protein	RNA en capsid (mg/L)	RNA ex capsid (mg/L)
Figure 1	pAPSE10180	139	180	ErkA	MS2	<2	75-90
Figure 1	pAPSE10181	139	180	ErkA	none	n.a	<2.
Figure 2	pAPSE10189	n.a.	n.a.	beta actin	MS2	20	<2
Figure 3	pAPSE10190	n.a.	n.a.	beta actin	MS2	20	<2
Figure 2	pAPSE10274	n.a.	n.a.	beta actin	none	n.a.	<2
Figure 3	pAPSE10275	n.a.	n.a.	beta actin	none	n.a.	<2
Figure 1	pAPSE10269	166	294	beta actin	MS2	2-10	200
Figure 1	pAPSE10306	166	294	beta actin	none	n.a.	3

Figure 4	pAPSE10216	166	294	beta actin	MS2	5-20	50-250
Figure 4	pAPSE10305	166	294	beta actin	none	n.a.	4
Figure 5	pAPSE10219	166	294	beta actin	MS2	5-20	30-60
Figure 5	pAPSE10304	166	294	beta actin	none	n.a.	3
Figure 6	pAPSE10279	166	294	beta actin	MS2	4	65
Figure 6	pAPSE10303	166	294	beta actin	none	n.a.	4
Figure 4	pAPSE10270	116	294	beta actin	MS2	2-10	200
Figure 4	pAPSE10271	136	294	beta actin	MS2	2-10	200
Figure 4	pAPSE10272	156	294	beta actin	MS2	2-10	200
Figure 4	pAPSE10292	131	294	beta actin	MS2	2-10	150
Figure 4	pAPSE10291	142	294	beta actin	MS2	2-10	160
Figure 4	pAPSE10276	166	50	beta actin	MS2	5-10	80-120
Figure 4	pAPSE10277	166	75	beta actin	MS2	20-30	200-250
Figure 4	pAPSE10366	166	294	beta actin	none (eGFP)	n.a.	<2
Figure 4	pAPSE10181 and pAPSE10149	139	180	ErkA	MS2 in trans	n.d.	200
Figure 1	pAPSE10359	166	294	beta actin	Qbeta	n.d.	n.d.

Figure 4	pAPSE10357	166	294	beta actin	none (U1A)	n.d.	n.a.
Figure 1	pAPSE10372	139	180	ErkA	none (MS2 N-term fragment)	n.a.	75

A. DEFINITIONS

[0024] As used herein, the term "capsid protein" or "capsid" refers to the coat protein of bacteriophage MS2 or Qβ, capable of binding the bacteriophage RNA pac site with high affinity and assembling into a complex hollow tertiary structure in which the bacteriophage RNA is entirely encapsidated within the hollow tertiary structure. In a VLP, the capsid protein forms a hollow tertiary structure in which the heterologous RNA is entirely encapsidated. The term "capsid" also refers to the hollow tertiary structure formed by assembly of individual capsid proteins.

[0025] As used herein "ssRNA" and "dsRNA" refer to "single-stranded RNA and double stranded RNA, respectively. An ssRNA is comprised of an RNA sequence of any length that lacks sufficient internal homology to form any significant secondary structures such as hairpins or other structures dependent on hybridization of internal complementary sequences with one another via Watson-Crick base pairing of nucleotide bases between the complementary sequences. In contrast, a dsRNA comprises RNA sequences with sufficient internal homology to form significant secondary structures such as hairpins due to hybridization of internal complementary sequences with one another via Watson-Crick base pairing of nucleotide bases within the complementary sequences. Significant secondary structures generally involve stretches of homology greater than approximately nine bases, but the exact length depends to some extent on context and on whether such secondary structures impart any biological function to the molecule.

[0026] As used herein "plasmid" or "extrachromosomal element" refers to any extrachromosomal episome capable of replication or stable maintenance within the host cell. Specifically embraced by this definition are plasmids such as pBR322,

pCG1, and pACYC184 which represent the backbones of the described plasmids. Those of ordinary skill in the art will recognize that other plasmids or stably maintained viral episomes can provide the same required functions of maintenance, expression and selection and that alternatives to the basic plasmids described herein may be generated from such other plasmids or stably maintained viral episomes without undue experimentation. A key feature of the present invention is the ability to express the genes encoding a dsRNA and a capsid protein, not specific modes of replication, expression or the selective markers found on episomes containing the genes encoding the dsRNA and capsid protein.

[0027] "Substantially similar sequence" refers to sequence variants of the claimed capsid proteins that retain the ability to facilitate accumulation of dsRNA in a microbial host cell as described herein. Such substantially similar sequences include sequences with at least 26% identity and 47% similarity as shown by the differences between MS2 and Qbeta capsid protein sequences (as determined by blastp). Consequentially, substantially similar sequences encompass conserved and homologous substitutions allowing sequence variants with as little as 95%, 90%, 80%, 70%, 60%, 50%, 40%, 30% or 25% identity to, and 95%, 90%, 80%, 70%, 60%, 50% or 40% similarity to, MS2 or Qbeta capsid protein sequences to facilitate accumulation of dsRNA in a microbial host.

B. COMMON MATERIALS, AND METHODS

[0028] Routine microbial and molecular cloning methods and tools, including those for generating and purifying DNA, RNA, and proteins, and for transforming host organisms and expressing recombinant proteins and nucleic acids as described herein, are fully within the capabilities of a person of ordinary skill in the art and are well described in the literature. See, e.g., Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Davis, et al., Basic Methods in Molecular Biology, Elsevier Science Publishing Co., Inc., N.Y. (1986); and Ausubel, et al, Current Protocols in Molecular Biology, Greene Publ. Assoc., Wiley-Interscience, NY (1995).

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[0029] Each of the recombinant DNA constructs described in further detail below are based on a common plasmid vector series derived from plasmid pBR322. The first of this plasmid vector series contains a custom synthetic DNA fragment (produced by PCR GenScript, Piscataway, NJ) comprising a T7 promoter sequence capable of driving transcription of a single copy of the bacteriophage MS2 capsid gene followed by a T7 terminator. This synthetic sequence was inserted as a BamHI-SphI restriction fragment into the corresponding sites of pBR322 to form plasmid pAPSE10118. A second synthetic sequence comprising a T7 promoter sequence followed by an MS2 pac site sequence, a multi-cloning site containing, in order (5' to 3') AsiSI-PmeI-AscI-RsrII-NotI-PacI restriction sites, a second high affinity variant MS2 pac type sequence (C-pac), a T7 terminator and an SphI restriction site was synthesized (PCR Genscript, Piscataway, NJ) and inserted into the EcoRV site of pAPSE10118 to form pAPSE10136. The two are oriented such that the T7 promoters direct transcription of the same strand of pAPSE10136 (clockwise on the standard pBR322 map) but are separated from one another by a single T7 terminator.

[0030] A 180 nucleotide fragment of the ErkA gene of *Drosophila melanogaster* (corresponding to the sequence of GenBank Accession NM_001300706 between nucleotides 156-335) was amplified by PCR incorporating AsiSI and PmeI restriction sites on the 5' and 3' sides, respectively. Insertion of this ErkA gene fragment into the corresponding sites of pAPSE10136 produced pAPSE10169. A second, complimentary copy of the ErkA gene fragment sequence was generated by PCR amplification incorporating a PmeI restriction site on the 5' end, followed by a synthetic loop sequence containing an additional MS2 pac sequence, followed by a NotI restriction site, followed by the complementary (anti-sense) ErkA gene fragment sequence and a PacI restriction site on the 3' end of the PCR fragment. The synthetic loop sequence comprises random sequence incapable of hybridizing with the ErkA gene fragment sequences. This complementary (anti-sense) copy of the ErkA gene fragment is inserted into the PmeI and PacI restriction sites of pAPSE10136 to form pAPSE10180 (SEQ ID NO: 1). A second series of plasmid vectors, lacking the MS2 capsid protein is derived from pAPSE10180 by deleting the MS2 capsid expression sequences by SphI restriction digestion and re-ligation to produce pAPSE 10181 (SEQ ID NO: 2).

[0031] Plasmids pAPSE10180 and pAPSE10181 represent the basic platform for expression of the RNA constructs discussed herein. Transcription of the ErkA cassette in these plasmids is predicted to produce an RNA transcript capable of forming a large stem-loop structure comprising a 180 base pair stem and a 139 base loop with 3 individual MS2 pac sequences located 5' and 3' of the stem and within the loop itself. One of ordinary skill in the art will understand that substitution of the ErkA gene fragment sequences by other sequences can be easily accomplished by standard cloning and sub-cloning methods.

[0032] Transformation of plasmids pAPSE10180 or pAPSE10181, or any of their derivatives, into host cells capable of inducible expression of T7 polymerase produces cell lines capable of expressing RNA transcripts. All such strains inducibly producing RNA transcripts are referred to generally herein as "expression strains". Unless otherwise indicated, each of the plasmids described herein was electroporated into *E. coli* strain HT115(DE3) with genotype F$^-$, *mcrA*, *mcrB*, IN (*rrnD-rrnE*)1, *rnc*14::Tn*10* (Lambda DE3 lysogen: *lac*UV5 promoter-T7 polymerase)) and the resulting recombinant transformants were selected on LB agar plates containing 12 μg/ml tetracycline and/or 100 μg/ml ampicillin. Single colonies were isolated, the presence of intact plasmid confirmed by restriction enzyme analysis and the confirmed transformed cells archived for future use.

[0033] Standard expression studies comprised inoculating transformed cells into 100 ml of Super Broth containing 0.1% glucose, 0.4% lactose, 100 μg/ml ampicillin and/or 12.5 μg/ml tetracycline and incubating the cultures with vigorous shaking at 37°C. Expression of the T7 polymerase was achieved by auto-induction by depletion of the available glucose and the presence of the lactose inducer. This ensures that all cultures are induced at the same growth stage. Cells were harvested twelve to eighteen hours post-induction (late stationary phase) by centrifugation at 3,000 g at 4°C for 30 minutes and stored on ice until lysis.

[0034] RNA was isolated from harvested cells by resuspending a 5 ml equivalent of cell culture of harvested cells in sonication buffer comprising Tris-HCl pH 7, 10 mM NaCl and sonicating the suspended cells on ice for 3 minutes. Cell debris was

removed by centrifugation at 16,000 g the supernatant (cleared lysate) was immediately processed to recover RNA and VLPs as described. RNA was recovered from half of the cleared lysate using the commercial Purelink RNA Mini Kit method (Ambion Cat. No. 12183018A, Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions.

[0035] VLPs were purified from the remaining half of the cleared lysate which were diluted to a total volume of 1 ml and treated with 100 units of Benzonase® Nuclease (Sigma Aldrich, St. Louis, MO) at 37°C for two hours. Subsequently, 0.15 mg of Proteinase K was added and the enzymatically treated cleared lysate incubated at 37°C for an additional three hours. The VLPS were recovered from the enzymatically treated cleared lysate by fractional precipitation. A saturated ammonium sulfate solution was prepared by adding ammonium sulfate to water until it reached saturation (approximately 4.1 M). Fifty microliters of the saturated ammonium sulfate solution was added to the enzymatically treated cleared lysate and the mixture placed on ice and incubated for two hours. Unwanted precipitate was removed from the mixture by centrifugation at 16,000 g and the aqueous solution transferred to a clean Eppendorf tube. The aqueous solution was then subjected to a second precipitation by the addition of 0.171 g of dry ammonium sulfate directly to the aqueous solution. The aqueous solution was vortexed and incubated on ice for two hours. The precipitate was spun down at 16,000 g the aqueous phase discarded and the solid precipitate representing purified VLPs resuspended in 100 microliters of sonication buffer.

[0036] RNA was recovered from the resuspended purified VLPs by adding 3 volumes of Trizol LS Reagent (Ambion Cat. No. 10296028, Thermo Fisher Scientific Inc.), vigorously vortexing the mixture, adding 1 ml of chloroform, further vortexing the mixture before pulse centrifugation to separate the aqueous and organic phases of the mixture. The aqueous phase was placed in a clean Eppendorf tube and the RNA purified with a commercial RNA Clean & Concentrator™ kit (Cat. No. R1018, Zymo Research, Irvine, CA) according to the manufacturer's instructions.

[0037] RNA from bacterial and VLP samples were dissolved in 50 µl of nuclease-free water. To determine the concentration of dsRNA in a sample, the samples were treated with RNAse A (Invitrogen Cat. No. AM2274, Thermo Fisher Scientific Inc.) to degrade single stranded RNA under the manufacturers recommended conditions, the concentration of dsRNA was determined spectrophotmetrically by measuring OD_{260} and 1 µg loaded onto Novex 6% TBE-urea gels (Invitrogen, Thermo Fisher Scientific Inc.). One lane of each gel was loaded with dsDNA size markers of known concentration and the samples were electrophoresed, the gel was stained with ethidium bromide and each band quantitated by densitometry using the dsDNA markers as a standard curve.

[0038] RNA yields from constructs producing ssRNA were determined by annealing the sense or anti-sense strand recovered from the induced cells or VLPs with an excess of the cognate strand. The annealed mixture was then treated with RNAse A and the amount of dsRNA incorporating the ssRNA of interest measured as described above.

[0039] Little or no differences in final cell densities were observed between any of the cultures from which the samples were harvested and in all cases the cultures appear to have reached stationary phase prior to harvest. To allow direct sample to sample comparison of RNA yields, all dsRNA and ssRNA concentrations are reported as the amount of such RNA present in a 1 L equivalent of culture.

[0040] Northern blot analysis was used to verify the identity of bands containing the dsRNA transcripts using a DNA oligonucleotide probe against the random sequence comprising the loop of each dsRNA construct (5'-GGCCGGCGTCT-ATTAGTAGATGCC-3', SEQ ID NO 3). RNA from the 6% polyacrylamide denaturing Urea-TBE gel was transferred to a positively-charged BrightStar – Plus nylon membrane (Ambion Cat. No. 10102, Thermo Fisher Scientific Inc.) using the semi-dry Trans-Blot SD transfer apparatus (BioRad, Hercules, CA) for 1 hour at constant current of 0.3 A. RNA was fixed on the membrane by the SpectroLinker XL-1500 UV crosslinking apparatus (Spectronics Corporation, Westbury, NY) using the "optimal crosslink" setting. The membrane was briefly rinsed with water and

prehybridized in 50 ml of 5XSSC, 0.1% SDS buffer at 45°C with gentle shaking. Probe hybridization was carried out overnight at 45°C in 3 ml of prehybridization buffer with gentle shaking. The oligonucleotide probe targeting the hairpin RNA loop was conjugated with TAMRA. Three washes (for 2 minutes each) with 100 ml of water were completed at room temperature and the blot with a ChemiDoc MP imaging system (BioRad, Hercules, CA), using the rhodamine channel.

C. PREFERRED EMBODIMENTS

[0041] The following are among the preferred embodiments of the invention.

[0042] One embodiment of the present invention comprises a bacterial host cell containing a plasmid encoding both a gene for the desired dsRNA and a bacteriophage capsid protein gene, such that the dsRNA and the capsid protein genes are transcribed so that the desired dsRNA is produced and the capsid protein gene translated to produce capsid protein and wherein, after a suitable period of time, unencapsidated dsRNA accumulates within the cell to a much higher degree than in the absence of capsid protein. In other embodiments the dsRNA gene and the capsid protein gene may be present on separate compatible plasmids, autonomously maintained phage or other epigenetic elements, or one or both genes may be present within the chromosome of the bacterial host cell.

[0043] In an embodiment the dsRNA gene and the capsid protein gene are each transcribed from a transcriptional promoter. The transcriptional promoter may be inducible. In one embodiment the transcriptional promoters are identical; in other embodiments the promoters are different. In still other embodiments the transcriptional promoters may be differentially induced. In such differentially inducible embodiments it may be preferable to induce expression of the capsid protein prior to inducing expression of the dsRNA.

[0044] In another embodiment the capsid protein and the dsRNA may be transcribed as a single transcript from a single promoter. The promoter may be inducible. In such embodiments the dsRNA is cleaved from the initial RNA transcript containing the capsid protein coding sequence by post transcriptional processing, such

post transcriptional processing may depend on bacterial host cell processes or may be directed by other RNA processing systems such as ribozymes or specific ribonucleases.

[0045] In one embodiment one or both of the dsRNA and the capsid protein genes are inducibly transcribed from a transcriptional promoter and transcription is terminated by a transcriptional terminator. In an embodiment the inducible transcriptional promoter is the bacteriophage T7 gene 1 promoter. In other embodiments the inducible transcriptional promoter may be the bacteriophage Lambda P_L or P_R promoters, the *lac* operon, *trp* operon, or synthetic *tac* promoter, or bacteriophage T5 promoter. Other transcription promoters, both constitutive and inducible, known to those of ordinary skill in the art, may also be used in some embodiments. In an embodiment the transcriptional terminator is the bacteriophage T7 late terminator. Other transcription terminators, both rho-dependent and rho-independent, known to those of ordinary skill in the art may also be used in some embodiments.

[0046] In an embodiment the coat protein gene encodes a leviviral capsid protein. The coat protein gene may be the MS2 coat protein gene encoding the MS2 capsid protein or substantially similar sequences retaining the ability to allow accumulation of dsRNA in a microbial host cell. The coat protein gene may encode the Qbeta coat protein gene encoding the Qbeta capsid protein or substantially similar sequences retaining the ability to allow accumulation of dsRNA in a microbial host cell.

[0047] In an embodiment the dsRNA is recovered from the bacterial host cells co-expressing bacteriophage capsid protein by chemical or mechanical methods to produce a host cell lysate. In an embodiment the dsRNA is further purified from the host cell lysate to remove host cell derived proteins, nucleic acids and membranes including capsid protein. In another embodiment the host cell lysate is directly processed without further purification. In another embodiment bacterial host cells are killed, by chemical or heat or other means without lysis and the intact killed cells processed without further purification.

EXAMPLES

Example 1

Unencapsidated dsRNAs are produced at higher levels in the presence of capsid protein than in the absence of capsid protein.

[0048] Expression strains containing pAPSE10180 and pAPSE10181 were constructed and dsRNA production induced by the standard expression procedure described above. The amount of encapsidated and unencapsidated dsRNA each strain produced was measured as described. The initial impetus for this experiment was to determine whether an RNA molecule with a 180 base pair double-stranded stem structure could be packed within a VLP. A 180 bp dsRNA stem is approximately 60 nm in length, whereas the interior diameter of an MS2 capsid is approximately 20 nm. Based on this geometric limitation, little or no encapsidation was expected and, due to host nuclease activity, little or no unencapsidated dsRNA was expected to be recoverable from the cell lysates. As expected only small amounts of encapsidated dsRNA (en capsid) were recovered (<2 mg/L) from the pAPSE10180 expression cells. In contrast, surprisingly large amounts of unencapsidated dsRNA (ex capsid) were recovered (75-90 mg/L) from the pAPSE10180 expression cells. Even more surprisingly, virtually no unencapsidated dsRNA was recovered from the pAPSE10181 expression cells.

[0049] To determine whether accumulation of RNA is a specific property of the ErkA sequence, or is a more general property of expressing dsRNA in the presence of capsid protein, a series of expression constructs expressing a 294 base sequence from the beta actin gene of the Colorado potato beetle (*Leptinotarsa decemlineata* strain Freeville, GenBank Accession NM_001300706 between nucleotides 156-335) were produced and tested.

[0050] Initially, plasmids expressing the 294 base beta actin sequence from Colorado potato beetle in the sense and the anti-sense orientation were constructed from pAPSE10180 by replacing the ErkA sequences, to produce pAPSE10189 (SEQ ID NO: 4 and pAPSE10190 (SEQ ID NO: 5) respectively. The beta actin sense and antisense strand sequences were amplified by PCR (Accuprime *Pfx*, Invitrogen Cat.

No. 12344040, Thermo Fisher Scientific Inc.) from a gBlock template using primers that introduce the AsiSI and PmeI restriction sites at the 5' and 3' ends respectively (gBlock template DNA and PCR primers were synthesized by Integrated DNA Technologies, Coralville IA; all restriction endonucleases were from New England BioLabs, Beverly, MA). Restriction digest of pAPSE 10180 and the beta actin sense and antisense PCR fragment with AsiSI and PmeI resulted in DNA fragments that could be ligated together in the desired manner. The pAPSE10180 plasmid backbone lacking the ErkA sequence was gel purified and the sense and antisense beta actin sequences were ligated into the gel purified vector to produce pAPSE 10189 and pAPSE 10190, respectively. When transformed into a suitable expression host, such as HT115(DE3) the cells containing pAPSE10189 produces a ssRNA transcript comprising 294 bases of the sense strand of the beta actin gene flanked by pac sequences as well as co-express MS2 capsid protein, when cultured and induced as described above. Likewise, cells containing pAPSE10190 produces a ssRNA transcript comprising 294 bases of the anti-sense strand of the same region of the beta actin gene flanked by pac sequences as well as co-express MS2 capsid protein when transformed into a suitable expression host, cultured and induced as described. A second set of plasmids, lacking the ability to express MS2 capsid protein were also produced by replacing the ErkA sequences of pAPSE10181 with the sense and anti-sense 294 base fragments of the beta actin gene as described above. These plasmids, pASPE10274 (SEQ ID NO: 6) and pAPSE10275 (SEQ ID NO: 7) respectively, were transformed into HT115(DE3) and cultured and induced as described.

[0051] Analysis of un-encapsidated RNA recovered from the cells whether co-expressed with capsid protein (as with pAPSE10189 and pAPSE10190) or not (pAPSE10274 and pAPSE10275) showed that virtually no ssRNA can be recovered. However, VLPs recovered from pAPSE10189 and pAPSE10190 yield at least 20 mg/L of ssRNA of sense or anti-sense sequence respectively. This confirms that the plasmid expression systems are capable of producing ssRNA and capsid protein as expected.

[0052] A dsRNA expression cassette comprising the 294 base Colorado potato beetle beta actin genes was constructed by a process similar to that described for the dsRNA ErkA expression cassette. In this case, the random DNA sequence comprising

the loop between the sense and anti-sense strands of the beta actin sequences comprised 166 bases, including the same internal pac site sequence as found in pAPSE10180 and 10181. This beta actin expression cassette was cloned into pAPSE10180 replacing the ErkA related stem loop sequence to form plasmid pAPSE10269 (SEQ ID NO: 8), and into pAPSE10181 to form plasmid pAPSE10306 (SEQ ID NO: 9). The plasmids were transformed into HT115(DE3), cultured, and induced as described. Analysis of the encapsidated dsRNA produced by the cells containing pAPSE10269 strain showed that 2-10 mg/L dsRNA could be recovered from VLPs. However, much higher levels of the beta actin dsRNA could be recovered from the cells containing pAPSE10269 in unencapsidated form (200 mg/L). Strikingly, analysis of the RNA produced by the pAPSE10306 strain showed that in the absence of co-expressed capsid protein only about 3 mg/L of dsRNA could be recovered.

[0053] Thus, the high levels of unencapsidated dsRNA are consistent with a model in which such dsRNA are not packaged efficiently , but for some reason appear to be present within cells co-expressing capsid protein with the dsRNA at much higher levels than in cells which lack capsid protein. One model to account for this observation is that binding of capsid protein to the pac sites inhibits degradation by host cell nucleases.

Example 2

Specific pac site-capsid protein interaction is not required for high level production of dsRNA.

[0054] To test whether capsid protein bound to pac sites in the dsRNA results in the observed increase in dsRNA production in cells co-expressing capsid protein, perhaps inhibiting endogenous host nuclease degradation of the bound dsRNA, a series of constructs comprising the basic beta actin dsRNA described above were produced with varying numbers and locations of pac sites. Plasmids pAPSE10216 (SEQ ID NO: 10) and pAPSE10305 (SEQ ID NO: 11), are identical to pAPSE10269 and pAPSE10306 respectively, except they lack the internal loop pac site. Plasmids pAPSE10219 (SEQ ID NO: 12) and pAPSE10304 (SEQ ID NO: 13) are identical to pAPSE10217 and pAPSE10306 respectively, except they have only a single pac site

located on the 3' end of the stem of the dsRNA. Plasmids pAPSE10279 (SEQ ID NO: 14) and pAPSE10303 (SEQ ID NO: 15) are identical to pAPSE10216 and pAPSE10306 except they lack all pac site sequences entirely. Each of these plasmids was transformed into *E. coli* HT115(DE3), cultured and induced as described. Analysis of the encapsidated RNA recovered from VLPs of each of pAPSE10216 and pAPSE10219 show that 5-20 mg/L of dsRNA is encapsidated. Strikingly, even the strain containing pAPSE10279 entirely lacking pac sites produced 4 mg/L of encapsidated dsRNA, indicating that this level of encapsidation may represent non-specific entrainment of dsRNA present in the cells at the time the capsids were formed. Furthermore, the strain containing pAPSE10216 produced as much as 250 mg/L of unencapsidated dsRNA in the presence of capsid protein. The strains containing pAPSE10219 and pAPSE10279 produced 30-60 mg/L and 65 mg/L of unencapsidated dsRNA, respectively in the presence of capsid protein. All of the strains containing plasmids comprising the expression cassettes without co-expression of capsid protein produced <4 mg/L of dsRNA.

[0055] Together, these results indicate that the ability of capsid protein to increase the amount of unencapsidated dsRNA that can be recovered from cell lysates is not dependent on the specific binding of capsid protein to its cognate pac site sequence. Although the highest levels of unencapsidated dsRNA are recovered from constructs containing at least 5' and 3' flanking pac sites (approximately 200 mg/L), significant amounts of unencapsidated dsRNA are produced by constructs having only a single 3' flanking pac site, or lacking pac sites entirely. Cells containing plasmids producing dsRNA lacking pac sites altogether produce significantly higher amounts of dsRNA (65 mg/L) when capsid protein is co-expressed with the dsRNA relative to the cell lines lacking capsid protein altogether (3-4 mg/L). The approximately 16X increase in recoverable dsRNA between cells co-expressing capsid protein and those lacking capsid protein (65 mg/L versus 3-4 mg/L) is much more than the approximately 3X-4X increase due to the presence of pac sites (65 mg/L versus 200-250 mg/L). The effect of capsid protein co-expression appears to involve something other than mere binding to cognate pac site sequences that may (or may not) be present on the dsRNA.

Example 3

Loop size and structure are irrelevant to high level production of dsRNA.

[0056] To test what effect, if any, differences in loop sequence might exert on the production of dsRNA in the presence and absence of co-expressed capsid protein, a series of constructs with different lengths of internal non-homologous loop sequences were inserted between each of the 294 base sense and anti-sense beta actin sequences of pAPSE10269.

[0057] Plasmids pAPSE10270 (SEQ ID NO: 16), pAPSE10271(SEQ ID NO: 17), pAPSE10272 (SEQ ID NO: 18) and pAPSE10292 (SEQ ID NO: 19) have non-homologous loop sizes of 116 bases, 136 bases, 156 bases and 166 bases respectively. Each of these loop sequences has very little propensity for any secondary structure as determined by the m-fold structure prediction program (Zucker and Stiegler (1981) *Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information* Nucl. Acids. Res. 9(1):133-48). In addition, the 139 base loop sequence found associated with the ErkA stem sequences in pAPSE10180 and having a slightly higher propensity for structural interactions within the loop was also placed between the sense and anti-sense beta actin sequences of pAPSE10269, to form pAPSE10292. Additionally, pAPSE10291 (SEQ ID NO: 20) comprising a 142 base loop sequence with a high degree of propensity for forming secondary structure based on internal homology was synthesized and constructed as described.

[0058] Each of the plasmids described in this Example were transformed into *E. coli* expression strain HT115(DE3), cultured and induced and the amount of encapsidated and unencapsidated dsRNA determined as described. In each case 2-10 mg/L of dsRNA was recovered from the VLPs produced by inducing expression of the plasmid, indicating that loop size or structure had little or no effect on the ability of VLPs to encapsidate the dsRNA. Likewise, expression from each of the plasmids produced between 100 and 200 mg/L unencapsidated dsRNA, indicating that loop size or structure had little or no effect on overall production of unencapsidated dsRNA in the presence of capsid protein.

Example 4

Stem size is irrelevant to high level production of dsRNA.

[0059] Differences in stem sequence derived from the *Drosophila melanogaster* ErkA gene sequences expressed from pAPSE10180 and the Colorado potato beetle beta actin gene sequences expressed from pAPSE10269 do not make a significant difference in the ability in expression strains to produce large quantities of unencapsidated dsRNA (75-90 mg/L from pAPSE10180 versus 200 mg/L from pAPSE10269). Nor does the length of the dsRNA stem (180 base pairs in the dsRNA produced from pAPSE10180 and 294 base pairs in dsRNA from pAPSE10269). To more systematically test what affect, if any, differences stem sequence length might exert on the production of dsRNA in the presence and absence of co-expressed capsid protein, a series of expression constructs with different lengths of stem sequences were substituted for each of the 294 base stem forming sense and anti-sense beta actin sequences of pAPSE10269.

[0060] Plasmids pAPSE10276 (SEQ ID NO: 21) and pAPSE10277 (SEQ ID NO: 22) encode dsRNA with potential double-stranded stems of 50 and 75 base pairs respectively. The dsRNA expressed by both plasmids comprise 166 bases of non-homologous loop sequence. Although these dsRNA structures are significantly shorter than those in dsRNA from the corresponding ErkA and beta actin constructs, they still exceed the interior diameter of the MS2 VLP.

[0061] When transformed into the *E. coli* expression strain HT115(DE3), cultured and induced as described, pAPSE 10276 produces 5-10 mg/L of encapsidated dsRNA and 80-120 mg/L of unencapsidated dsRNA. Plasmid pAPSE 10277 produces 20-30 mg/L encapsidated dsRNA and 200-250 mg/L unencapsidated dsRNA. These values are similar to those observed for pAPSE10180 and pAPSE10269 described earlier in this Example, indicating that differences in stem length and sequence do not play a major role in producing dsRNA in cells co-expressing capsid protein.

Example 5

Capsid protein is required for high level production of dsRNA.

[0062] To confirm the requirement for capsid protein, plasmid pAPSE10216, which produces a dsRNA product at high levels in the presence of capsid protein, was altered to replace the MS2 coat protein gene with eGFP. A gBlock template comprising the T7 promoter to T7 terminator sequences of pAPSE10216 (spanning the sequences between the unique BamHI and SalI sites of the plasmid) in which the coding sequence of MS2 coat protein was replaced with the coding sequence of eGFP was designed, produced and amplified with primers encompassing the BamHI site on the 5' side and the SalI site on the 3' side. The resulting 1 kb fragment was digested with BamHI and SalI and then ligated into BamHI-SalI digested pAPSE10216 to form pAPSE10366 (SEQ ID NO: 24). Plasmid pAPSE10366 was confirmed by restriction digest and transformed into the *E. coli* expression strain HT115(DE3), cultured and induced as described, pAPSE10366 produces <2 mg/L of unencapsidated dsRNA, in contrast to the 200 mg/L produced by pAPSE10216. In addition, the cells expressed high amounts of eGFP as evidenced by the intense fluorescence produced on induction (data not shown) confirming that the basic dual expression plasmid used throughout these studies performs as expected. This result further demonstrates that capsid protein is necessary for accumulation of unencapsidated dsRNA in cells expressing the target RNA gene that otherwise accumulate unencapsidated dsRNA in the presence of capsid protein.

[0063] To further confirm that the presence of capsid protein is essential to the high levels of unencapsidated dsRNA production a plasmid compatible with pAPSE10181 and capable of inducible expression of the MS2 capsid protein is constructed. pAPSE10149 (SEQ ID NO: 23) is based on pACYC184. This plasmid comprises a P15A origin of replication that is not excluded by the colE1 based origin of replication of pAPSE10181 and a chloramphenicol acetyl transferase antibiotic marker to allow selection of co-transformants containing both pAPSE10181(encoding ampicilin resistance) and pAPSE10149 (encoding chloramphenicol resistance). Plasmid pAPSE10149 also comprises the same T7 promoter sequence capable of driving transcription of a single copy of the bacteriophage MS2 capsid gene followed

by a T7 terminator as found in pAPSE10118 cloned into the BamHI and SphI sites of pACYC184. Plasmid pAPSE10149 is transformed into expression strains already containing pAPSE10181 to produce ampicilin and chloramphenicol resistant double transformants. Expression studies of such double transformants show that co-expression of the capsid protein from pAPSE10149 in conjunction with pAPSE10181 produces 200 mg/L of unencapsidated dsRNA whereas cells containing pAPSE10181 alone produce <2 mg/L of unencapsidated dsRNA (see Example 1). This demonstrates that providing capsid protein in trans is sufficient to facilitate production of high levels of unencapsidated dsRNA to host cells containing a plasmid expressing the dsRNA target that otherwise fail to accumulate unencapsidated dsRNA in the absence of capsid protein.

Example 6

Other capsid proteins can induce high level production of dsRNA.

[0064] To test whether the accumulation of unencapsidated dsRNA is a unique property of bacteriophage MS2 capsid protein, or whether other capsid proteins share this property, a plasmid expression system analogous to pAPSE10216 was constructed. This plasmid, pAPSE10359 (SEQ ID NO: 25) comprises a Qbeta capsid protein and Qbeta pac sites at the 5' and 3' ends of the beta actin dsRNA expression cassette, but is in all other aspects similar to pAPSE10216.

[0065] Briefly, the Qbeta coat protein gene sequence (Genebank Accession NC_001890 between nucleotides 1343 and 1744) was synthesized as a gBlock fragment by Integrated DNA Technologies, Coralville, IA. The synthetic fragment was amplified with PCR with primers that introduced a BamHI restriction site followed by a T7 promoter sequence upstream of the Qbeta coat protein gene followed by a T7 terminator and a SphI restriction site. The amplified synthetic fragment and plasmid pBR322 were digested with BamHI and SphI and ligated together to form intermediate plasmid pAPSE10358. The beta actin dsRNA sequence of pAPSE10269 was amplified by PCR with primers that introduced an EcoRI restriction site followed by a Qbeta pac sequence followed by the beta actin dsRNA sequence followed by a second copy of the Qbeta pac sequence followed by a BamHI

restriction site. This amplified beta actin containing sequence and plasmid pAPSE10358 were digested with EcoRI and BamHI and ligated together to form pAPSE10374. Plasmids pAPSE10374 and pAPSE10216 were digested with AsiSI and NotI. This cleaves pAPSE10374 into two fragments of 4,713 and 113 base pairs and pAPSE10216 into two fragments of 5,204 and 786 base pairs. The 4,713 and 786 base pair fragments were isolated and ligated together to produce pAPSE10359.

[0066] When transformed into the *E. coli* expression strain HT115(DE3), cultured and induced as described, pAPSE10359 will produce a large amount of unencapsidated dsRNA relative to the amount of dsRNA produced from a similar construct lacking capsid protein (pAPSE10305). This pattern, similar to that observed for pAPSE10216 and pAPSE10305 described in Example 1, will confirm that expression of the Qbeta capsid protein, like the MS2 capsid protein, is sufficient to increase the amount of dsRNA produced in vivo.

Example 7

RNA binding proteins other than capsid proteins are not sufficient for high level production of dsRNA.

[0067] To test whether the accumulation of unencapsidated dsRNA is a function of general RNA binding or is specific to bacteriophage capsid proteins, a plasmid expression system, pAPSE10357 (SEQ ID NO: 26) was constructed comprising the RNA binding domain of the human U1A protein and its hairpin cognate binding site from human U1 snRNA 5' and 3' of the sense and antisense stem loop structure of the beta actin dsRNA. Plasmid pAPSE10357 is similar to pAPSE10216 with the capsid protein replaced by the human U1A RNA binding protein and U1A binding site sequences at the 5' and 3' ends of the beta actin dsRNA expression cassette, but is in all other aspects similar to pAPSE10216.

[0068] The DNA sequence encoding the N-terminal 102 amino acids comprising the RNA binding domain of the human U1A protein was amplified from a cloned copy of the U1A protein (Plasmid pAV105, Professor Kathleen Hall, Washington University, St. Louis, MO) using PCR primers that introduced a BamHI restriction site followed by a T7 promoter sequence upstream of the U1A gene fragment

followed by a T7 terminator and a SphI restriction site. The amplified synthetic fragment and plasmid pBR322 were digested with BamHI and SphI and ligated together to form intermediate plasmid pAPSE10356. The beta actin dsRNA sequence of pAPSE10269 was amplified by PCR with primers that introduced an EcoRI restriction site followed by the hairpin binding site sequence from human U1 snRNA sequence followed by the beta actin dsRNA sequence followed by a second copy of the hairpin binding site sequence from human U1 snRNA sequence followed by a BamHI restriction site. This amplified beta actin containing sequence and plasmid pAPSE10356 were digested with EcoRI and BamHI and ligated together to form pAPSE10373. Plasmids pAPSE10373 and pAPSE10216 were digested with AsiSI and NotI. This cleaves pAPSE10373 into two fragments of 4,627 and 113 base pairs and pAPSE10216 into two fragments of 5,204 and 786 base pairs. The 4,713 and 786 base pair fragments were isolated and ligated together to produce pAPSE10357.

[0069] When transformed into the *E. coli* expression strain HT115(DE3), cultured and induced as described, pAPSE10357 will not produce a significant amount of unencapsidated dsRNA relative to the amount of dsRNA produced from a similar construct lacking capsid protein (pAPSE10305). This will confirm that the mere presence of an RNA binding site and binding protein in conjunction with the dsRNA is not sufficient to increase the amount of dsRNA produced in vivo. Alternatively, production of significant amounts of unencapsidated dsRNA will indicate that the presence of RNA binding sites at the 5' and 3' end and the cognate RNA binding protein is sufficient for increasing in vivo production of dsRNA.

Example 8

The N-terminus of capsid protein is sufficient for high level production of dsRNA.

[0070] To examine whether the increased production of dsRNA from plasmids containing both the dsRNA gene and the coat protein gene requires the intact capsid protein or whether only a portion of the protein is required, a frame-shift mutation was introduced into the coat protein gene sequence of pAPSE10180. Double digestion of pAPSE10180 with the restriction enzymes StuI and PmlI produces two restriction

fragments, a large fragment of 5,485 base pairs and a small thirteen base pair fragment comprising about 4 codons of the capsid protein CDS about 40 codons from the coat protein start codon of pAPSE10180. The restriction enzymes produce blunt-ended termini and the larger fragment was re-ligated to produce plasmid pAPSE10372 (SEQ ID NO: 27), which, in addition to producing an intact inducible dsRNA ErkA-specific sequence, also comprises an inducible frame-shifted protein that includes the N-term 41 codons of the MS2 coat protein followed by 27 codons of frame-shifted sequence before terminating at a stop codon (SEQ ID NO: 28). When pAPSE10372 was transformed into *E. coli* expression strain HTE115(DE3) and cultured and induced as described, 75 mg/L of dsRNA was produced. This indicates that the N terminus of the capsid protein alone is sufficient to increase production of dsRNA as well as the intact capsid protein (compare yields from pAPSE10180 and pAPSE10372 in Table 1).

[0071] The N-terminus of the MS2 capsid protein forms a distinctive three-dimensional structure comprised of four separate beta sheets (D. Peabody, *The RNA binding site of bacteriophage MS2 coat protein,* The EMBO Journal 12(2) 595-600 (1993)). Each of these sheets, βD from amino acids 31-35, βC from amino acids 22-25, βB from amino acids 19-21 and βA amino acids 8-11 may play a role in the ability of the N-terminus capsid protein fragment to improve dsRNA production. Note that the nomenclature is that of Peabody and the numbering includes the N-terminal methionine omitted by Peabody. Progressive deletion of each of these structural motifs can determine the minimum sequence requirement for improving dsRNA production.

Example 9

Fed batch fermentation produces very high level production of dsRNA.

[0072] To determine whether quantities of dsRNA could be increased by improving the microbial growth conditions, glucose fed batch fermentations were conducted. Briefly, fed-batch fermentations were carried out in an Eppendorf BioFlo 115 fermenter at 37 ^{0}C. The pH was controlled by automatic addition of 30% ammonium hydroxide. The dissolved oxygen probe was calibrated to 0% by

unplugging the DO probe and to 100% with air saturation. The vessel was aerated at 2 vvm and dissolved oxygen maintained at 30% by cascade control of agitation. An overnight culture of HT115 (DE3) containing pAPSE10379 was grown in LB containing 100 ug/ul of ampicillin and 12.5 ug/ul of tetracycline at 37 ^{0}C to inoculate the seed medium. The seed media is a defined media consisting of 5.68 g/L Na_2HPO_4, 1.34 g/L KH_2PO_4, 6.6 g/L $(NH_4)_2SO_4$, 10 g/L glucose, 1X trace metal and 1X vitamin solutions maintained at a pH of 7.0. To ensure plasmid stability antibiotics are added at 100 ug/ul ampicillin and 12.5 ug/ul tetracycline. At saturation (OD_{600} 3-5) the seed cultures are used to provide 10% inoculum for the fermenter.

[0073] During fed batch-cultures a 50% (w/v) solution of glucose was added according to a carbon limiting DO stat feeding strategy. The basal medium consists of 6g/L K_2HPO_4, 3 g/L $NaHPO_4$, 10 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4$, 1X trace metal solution with antibiotics added at 100 ug/ul of ampicillin and 12.5 ug/ul of tetracycline. Upon exhaustion of the initial carbon source provided by the glucose the feed solution is added automatically in a manner that maintains the DO level at 30% of saturation.

[0074] Once the cell culture has reached an OD_{600} of 60 the cells are induced with 1 mM IPTG or a feed of 20 g/L of lactose by switching the glucose feed to a lactose feed. After induction 1 mL samples are taken at different times post induction. The samples are lysed by sonication of the cell pellet into 20 mM Tris-HCl at pH 7. Total RNA from the cell pellet is purified using well-known Trizol extraction procedures. Briefly 1 volume of cell lysate is added to 1 volumes of Trizol RNA extraction reagent. Addition of 1 volume of chloroform results in the RNA partitioning to the aqueous layer leaving the protein and DNA contaminants behind.

[0075] To analyze the yield of dsRNA the total RNA sample is diluted to 1 ug/ul and subjected to RNAseA treatment. The reaction is carried out in 20 mM Tris at pH 7.0 and 37 ^{0}C for 40 minutes. Once this is done proteinase K is added to the reaction to remove the nuclease and is allowed to react at 37 ^{0}C for 40 minutes. Upon completion of this step the dsRNA remaining is diluted in half, quarters and eighths in order to determine the concentration of the dsRNA using gel densitometry.

[0076] Quantification of dsRNA yield by gel densitometry was performed by comparing the intensity of dsRNA bands versus dsDNA bands of known mass and weight on a 1.5% agarose gel containing ethidium bromide. The lambda 100 bp quantifiable DNA marker was used and a standard curve was generated to determine the range in which the dsRNA from the fermentation can be reliably quantified. The computer program calculates the amount of dsRNA in the amount of sample loaded on the gel and a back calculation that considers the dilution steps is performed. Yields of dsRNA at levels as high as 3 g/L have been calculated with both IPTG and lactose as inducers under these conditions. These results indicate that further increases in dsRNA production are possible by improving fermentation conditions.

Example 10

Compositions and methods for dsRNA production in gram positive bacteria.

[0077] The ability of gram-positive bacteria to produce increased levels of dsRNA by co-expression of capsid proteins can be examined in the following manner. *Corynebacterium glutamicum* MB001(DE3) strain DSM 102071, containing an inducible T7 RNA polymerase gene (described in Kortmann, et al., *A chromosomally encoded T7 RNA polymerase-dependent gene expression system for* Corynebacterium glutamicum*; construction and comparative evaluation at the single cell level.* Microb Technol. 8(2):253-65. Mar. 2015) is modified to knockout the *rnc* gene homolog encoding RNAse III. Briefly, PCR primers capable of amplifying a 1.2 kb sequence homologous to the sequence present in *C. glutamicum* strain MB001(DE3) immediately upstream of the *rnc* gene and PCR primers capable of amplifying a 1.5 kb sequence homologous to the sequence immediately downstream of the *rnc* gene are synthesized. A PCR amplification reaction using *C. glutamicum* strain MB001(DE3) genomic DNA and said primers results in a single DNA fragment comprising the 1.2 kb and 1.5 kb target sequences joined together (by standard overlap PCR methods) to produce an approximately 2.7 kb SalI-BamHI synthetic DNA fragment. This SalI-BamHI DNA fragment and plasmid pK18*mobsacB* (ATCC 87097, described by Schafer, et al., *Small mobilizable multi-purpose cloning vectors derived from the* Escherichia coli *plasmids pK18 and pK19: selection of defined deletions in the chromosome of* Corynebacterium glutamicum. Gene 145:69-73) are digested with SalI

and BamHI and the products ligated together to produce plasmid pAPSE10429 (SEQ ID NO: 29). Plasmid pAPSE10429 is transformed into *C. glutamicum* strain MB001 and transformants selected on kanamycin containing solid LB medium to identify chromosomal integrants. Kanamycin resistant clones are transferred to a solid LB medium containing 20% sucrose. Conversion of sucrose by the *sacB* gene product is toxic to *C. glutamicum* strain MB001 so only those chromosomal integrants that subsequently delete the *sacB* gene from the chromosome can survive on such media. Surviving colonies are grown up and screened by PCR to confirm concomitant loss of the *rnc* locus from the chromosome. The desired strain is designated *C. glutamicum* MB001(DE3) *rnc*. This strain possesses an inducible T7 RNA polymerase and lacks the *rnc* gene and is suitable for testing the efficacy of dsRNA production in the presence and absence of capsid protein.

[0078] A shuttle vector capable of expression of capsid coat protein and dsRNA in both *E. coli* and *C. glutamicum* is constructed by synthesizing a DNA comprising the origin of replication of the gram-positive plasmid pCG1 (GeneBank Accession No. AB027714; described by Trautwetter and Blanco, *Structural organization of the* Corynebacterium glutamicum *plasmid pCG100*. J. Gen. Microbiol. 137:2093-101 1991) and the kanamycin resistance gene of pK18*mobsacB*. This synthetic DNA (SEQ ID NO: 30) is ligated into the previously described dsRNA containing plasmids at the unique NruI restriction site to allow testing whether the presence of capsid protein in gram-positive *C. glutamicum* MB001(DE3) *rnc* strain produces dsRNA at high levels as described below.

[0079] Insertion of the synthetic DNA comprising the pCG1 origin of replication and the kanamycin resistance gene is accomplished by digesting pAPSE10279 with NruI and ligating the phosphorylated synthetic DNA into the plasmid to produce plasmid pAPSE10430 (SEQ ID NO: 31). Plasmid pAPSE10430 contains the kanamycin resistance gene, the bacteriophage MS2 coat protein, and the dsRNA construct based on the previously described 294 base sense and antisense sequences homologous to the Colorado potato beetle beta actin gene separated by a 166 base non-homologous loop and entirely lacking any pac sequences. In similar fashion, the synthetic DNA comprising the pCG1 origin of replication and the kanamycin

resistance gene is also ligated into NruI digested pAPSE10303 to produce pAPSE10431 (SEQ ID NO: 32). Plasmid pAPSE10431 contains resistance genes to ampicillin and kanamycin, as well as the same inducible dsRNA construct as pAPSE10430. However, pAPSE10431 lacks the inducible MS2 coat protein gene of pAPSE10430. The relevant features of pAPSE10430 and pAPSE10431 are presented in Table 2 and the relationship between these two plasmids and their parental plasmids, pAPSE10279 and pAPSE10303, respectively, can be determined by comparing Table 2 and Table 1.

[0080] Additional plasmids containing one, two, and three pac sites, with and without MS2 coat protein, are constructed using the same procedure. Plasmid pAPSE10432 (SEQ ID NO: 33) containing a single pac site 3' of the beta actin stem loop structure and encoding the MS2 coat protein gene is produced by ligating the synthetic DNA fragment into the NruI site of pAPSE10219. Plasmid pAPSE10433 (SEQ ID NO: 34) is produced by ligating the synthetic DNA fragment into the NruI site of pAPSE10304. Plasmid pAPSE10433 is identical to pAPSE10432 except it lacks an inducible MS2 coat protein gene. Plasmid pAPSE10434 (SEQ ID NO: 35) containing two pac site sequences located one on either side of the beta actin stem loop and encoding the MS2 coat protein is produced by ligating the synthetic DNA fragment into the NruI site of pAPSE10216. Plasmid pAPSE10435 (SEQ ID NO: 36) is produced by ligating the synthetic DNA fragment into the NruI site of pAPSE10305. Plasmid pAPSE10435 is identical to pAPSE10434 except it lacks an inducible MS2 coat protein gene. Plasmid pAPSE10436 (SEQ ID NO: 37) containing three pac site sequences with one each 5' and 3' of the beta actin stem loop and one within the loop sequence itself (as depicted in Figure 1) and encoding the MS2 coat protein is produced by ligating the synthetic DNA fragment into the NruI site of pAPSE10269. Plasmid pAPSE10437 (SEQ ID NO: 38) is produced by ligating the synthetic DNA fragment into the NruI site of pAPSE10306. Plasmid pAPSE10437 is identical to pAPSE104360 except it lacks an inducible MS2 coat protein gene.

[0081] In each case, following ligation of the synthetic DNA fragment into the NruI site of the target plasmid, transformants the ligation reactions are desalted and transformed in to *C. glutamicum* MB001(DE3) *rnc* and selected for resistance to

kanamycin. The selected clones are subsequently grown at 32 °C in 100 ml of LB media containing kanamycin until the culture reaches OD600 0.8, at which time isopropyl β-D-thiogalactopyranoside is added to a final concentration of 1 mM to induce T7 polymerase directed transcription of the MS2 coat protein and the dsRNA, or just the dsRNA precursor in the plasmids lacking coat protein. The induced cultures are allowed to grow for at least 4 hours post-induction to allow sufficient time for accumulation of the MS2 coat protein and dsRNA target. Cells are collected by centrifugation at 3,000 g at 4 C. Each pellet is stored at 4 °C until processing.

[0082] The dsRNA is purified by re-suspending each pellet in approximately 0.1 volume of 20 mM Tris-HCl, pH 7.0, containing 10 mM NaCl and sonicated to lyse the cells. Cell debris is removed by centrifugation at 16,000 g. The resulting lysate is mixed with 3 volumes of Trizol (Ambion Life Technologies) and the RNA is extracted by adding 1 volume of chloroform. Addition of NaCl to a final concentration of 500 mM to the aqueous layer and subsequent ethanol precipitation results in a pellet containing the 294 bp siRNA precursor and RNA from the *C. glutamicum* host.

[0083] To determine the amount of dsRNA produced by the *C. glutamicum* transformed with plasmids containing various pac site configurations, with and without MS2 coat protein, the ethanol pellets are resuspended and treated with RNAseA for 1 hour at 37 °C followed by Proteinase K digestion for 1 hour at 37 °C. Quantification of the dsRNA is accomplished by gel densitometry using a BioRad ChemiDoc MP Imaging System. Several dilutions of the treated dsRNA are run on a 1.5% agarose gel containing 0.001% ethidium bromide. A 100 bp quantifiable dsDNA ladder (QuantiBP DNA ladder Lambda) is used as the standard curve and the dsRNA is quantified at the concentration that falls within the linear range of the standard curve. Software such as Image Lab 4.1 determines the concentration of the dsRNA loaded on the gel and a final yield of dsRNA is determined by accounting for the dilutions associated with the dsRNA samples present on the gel.

[0084] Table 2 summarizes the predicted results of the dsRNA yield determination of the Colorado potato beetle beta actin dsRNA produced by *C.* glutamicum

MB001(DE3) *rnc* and the various plasmids described above. Such results confirm that gram positive hosts such as *C. glutamicum* produce large quantities of dsRNA by co-expression of the MS2 coat gene and a dsRNA target of interest.

Table 2. Predicted production of dsRNA by *C. glutamicum* MB001(DE3) *rnc* as a function of variation in dsRNA structure and the presence or absence of coat protein.

RNA Structure as depicted in	Plasmid	Loop size (bases)	Stem size (bp)	Stem sequence	Coat protein	dsRNA (mg/L)
Figure 6	pAPSE10430	166	294	beta actin	MS2	~60
Figure 6	pAPSE10431	166	294	beta actin	none	~4
Figure 5	pAPSE10432	166	294	beta actin	MS2	~120
Figure 5	pAPSE10433	166	294	beta actin	none	~4
Figure 4	pAPSE10434	166	294	beta actin	MS2	~250
Figure 4	pAPSE10435	166	294	beta actin	none	~4
Figure 1	pAPSE10436	166	294	beta actin	MS2	~250
Figure 1	pAPSE10437	166	294	beta actin	none	4

Example 11

Compositions and methods for dsRNA production in yeast.

[0085] To create a *Saccharomyces cerevisiae* production host suitable for dsRNA accumulation utilizing the MS2 bacteriophage coat protein, the Rnt1 gene of *S cerevisiae* YPH 500 (ATCC 76626) is knocked out according to the procedure of Gardenr and Jasperson (Gardner, JM and Jaspersen, SL, *Manipulating the yeast*

genome: deletion, mutation and tagging by PCR. Methods Mol Biol. 1205:45-78, 2014). The KanMx4 gene is amplified from pML104-KanMx4 plasmid (Laughery, et al., *New vectors for simple and streamlined CRISPR-Cas9 genome editing in* Saccharomyces cerevisiae. Yeast 32(12):711-20 Sep. 21, 2015) with PCR primers including 60 base pair (bp) upstream (forward primer) and 60 bp downstream (reverse primer) regions of the *S. cerevisiae* Rnt1 gene. The resulting PCR product is introduced into chemically competent *S. cerevisiae* cells following the established *S. cerevisiae* transformation protocol. The transformed cells are incubated overnight without selection marker to allow for homologous recombination to occur, where in the kanMx4 gene carrying 60 bp upstream and downstream regions of Rnt1 replaced the Rnt1 gene. Following overnight incubation, the transformed cells are plated on YPD plates carrying G418 as selection marker. G418 resistant colonies are screened by PCR to confirm presence of kanMx4 gene and deletion of Rnt1 gene in the YPH 500 genome.

[0086] *S. cerevisiae* expression vectors pESC-His, pESC-Leu, pESC-Ura and pESC-Trp are widely used for recombinant protein expression in *S. cerevisiae*. Each of the pESC vectors (Agilent Technologies, Santa Clara CA) contains one of four different yeast-selectable markers (HIS3, TRP1, LEU2, or URA3) in the same vector backbone, which allows expression of two different genes in a single yeast cell. The pESC series vectors are used with *S. cerevisiae* strain YPH 500 (*MATα ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1).* In this example, the pESC-Trp vector is selected for expression of MS2 coat protein and target dsRNA sequence inside *S. cerevisiae*, although any of the other pESC vectors could be employed using similar methods since these vectors can replicate in *S. cerevisiae* as well as *E.coli,* which facilitates molecular manipulations necessary to produce dsRNA.

[0087] The pESC-Trp vector is modified by cloning a 50-base pair multi-cloning site linker containing BamHI, SwaI, AsiSI, NotI, SacII and NheI sites, downstream of the GAL1 promoter into the existing BamHI and NheI sites. Following this, the beta actin stem loop sequence (dsRNA) of pAPSE10279 is excised as an AsiS1/Not1 fragment and ligated into the AsiS1/Not1 sites of the modified pESC-Trp vector.

Expression of the dsRNA in this plasmid is under the control of galactose inducible promoter GAL1. The new vector is named pAPSE10439 (SEQ ID NO: 39). Another plasmid, pAPSE10440 (SEQ ID NO: 40), which is identical to pAPSE10439, but also includes the MS2 coat protein. Plasmid pAPSE10440 is constructed by PCR amplifying the MS2 coat protein expression sequences of pAPSE10279 with a forward primer carrying an EcoRI restriction site on the 5' end and the reverse primer carrying SacI site on the 3' end. The PCR product is digested with EcoRI and SacI and cloned into the cognate sites of pAPSE10439. Thus, pAPSE10439 inducibly expresses the dsRNA from the GAL1 promoter, whereas pAPSE10440 inducibly expresses the dsRNA sequence from the GAL1 promoter and the MS2 coat protein from the GAL10 promoter,

[0088] Similar plasmid pairs are constructed using this technique. Plasmids pAPSE10441 (SEQ ID NO: 41) and pAPSE10442 (SEQ ID NO: 42) are produced by digesting pAPSE10439 and pAPSE10440 with AsiSI and NotI and isolating the vector fragment. Plasmid pAPSE10219 is also digested with AsiSI and NotI and the dsRNA sequence is isolated. The isolated dsRNA sequence is ligated into the pAPSE10439 vector to form pAPSE10441 and the isolated dsRNA sequence is ligated into the pAPSE10440 vector to form pAPSE10442. Plasmids pAPSE10443 (SEQ ID NO: 43) and pAPSE10444 (SEQ ID NO: 44) are produced by digesting pAPSE10439 and pAPSE10440 with AsiSI and NotI and isolating the vector fragment. Plasmid pAPSE10216 is also digested with AsiSI and NotI and the dsRNA sequence is isolated. The isolated dsRNA sequence is ligated into the pAPSE10439 vector to form pAPSE10443 and the isolated dsRNA sequence is ligated into the pAPSE10440 vector to form pAPSE10444. Plasmids pAPSE10445 (SEQ ID NO: 45) and pAPSE10446 (SEQ ID NO: 46) are produced by digesting pAPSE10439 and pAPSE10440 with AsiSI and NotI and isolating the vector fragment. Plasmid pAPSE10269 is also digested with AsiSI and NotI and the dsRNA sequence is isolated. The isolated dsRNA sequence is ligated into the pAPSE10439 vector to form pAPSE10445 and the isolated dsRNA sequence is ligated into the pAPSE10440 vector to form pAPSE10446.

[0089] Chemically competent YPH 500 DRnt1 cells are transformed with each of the above mentioned plasmids (pAPSE10439-46) separately and individual clones selected on synthetic dextrose minimal (SD) tryptophan (trp) drop out plates. After inoculating the 100 ml SD-Trp drop out broth the cultures are grown for 12 to 16 hours. The cells from the culture are then harvested by centrifugation at 3000 g for 5 minutes, the cell pellet is washed once with sterile water and the cells re-suspended in synthetic galactose minimal broth (SG) lacking tryptophan. The cells are grown in the SG-trp drop out broth overnight to induce production and accumulation of dsRNA and MS2 coat protein (where appropriate). Cells are harvested by centrifugation at 3,000 g at 4 C. Each pellet is stored at -20 °C until processing.

[0090] The dsRNA is purified by re-suspending each pellet (10 ml culture) in approximately 1.0 ml of yeast cell lysis buffer (Sigma C4482). The resulting lysate is mixed with 3 volumes of Trizol (Ambion Life Technologies) and the RNA extracted by adding 1 volume of chloroform. Addition of NaCl to a final concentration of 500 mM to the aqueous layer and subsequent ethanol precipitation results in a pellet containing the dsRNA and RNA from the *S. cerevisiae* host. The resulting RNA pellet is dissolved in 20 mM Tris HCl pH 7.0 and RNA concentration of the sample determined. To determine the amount of dsRNA produced by the *S. cerevisiae* strains, a known amount of RNA (10 ug) from each RNA sample from pAPSE10439-pAPSE10446) are digested with RNAseA for 1 hour at 37 °C followed by Proteinase K digestion for 1 hour at 37 °C. The resulting samples contain only the dsRNA target. Quantification of the dsRNA is done by gel densitometry using a BioRad ChemiDoc MP Imaging System. Several dilutions of the RNAse A reaction are run on a gel that contains 1.5% agarose and 0.001% ethidium bromide. A 100 bp quantifiable dsDNA ladder (QuantiBP DNA ladder Lambda) is used as the standard curve and the dsRNA is quantified at the concentration that falls within the linear range of the standard curve. Using Image Lab 4.1 software, the concentration of the dsRNA loaded on the gel is determine and a final yield of dsRNA calculated by accounting for the dilutions of the dsRNA loaded on the gel.

[0091] Table 3 summarizes the predicted results of the dsRNA yield determination of the Colorado potato beetle beta actin dsRNA produced by *S. cerevisiae* YPH-500

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and the various plasmids described above. Such results confirm that yeasts such as *S. cerevisiae* produce large quantities of dsRNA by co-expression of the MS2 coat gene and a dsRNA target of interest.

Table 3 Predicted production of dsRNA by *S. cerevisiae* YPH 500 as a function of variation in dsRNA structure and the presence or absence of coat protein.

RNA Structure as depicted in	Plasmid	Loop size (bases)	Stem size (bp)	Stem sequence	Coat protein	dsRNA (mg/L)
Figure 6	pAPSE10440	166	294	beta actin	MS2	~60
Figure 6	pAPSE10439	166	294	beta actin	none	~4
Figure 5	pAPSE10442	166	294	beta actin	MS2	~120
Figure 5	pAPSE10441	166	294	beta actin	none	~4
Figure 4	pAPSE10444	166	294	beta actin	MS2	~250
Figure 4	pAPSE10443	166	294	beta actin	none	~4
Figure 1	pAPSE10446	166	294	beta actin	MS2	~250
Figure 1	pAPSE10445	166	294	beta actin	none	4

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WE CLAIM:

1. A method for producing unencapsidated dsRNA in a microbial cell, the microbial cell comprising (1) a coat protein gene encoding a capsid protein, wherein the capsid protein is the capsid protein of bacteriophage MS2, the capsid protein of bacteriophage Qβ, or an N-terminal fragment thereof, and (2) a gene encoding a heterologous dsRNA molecule comprising a self-complementary stretch of sequence separated by noncomplementary sequence such that upon hybridization of the complementary sequences a stem-loop structure is formed, the stem structure having a length exceeding the interior diameter of an MS2 capsid, the method comprising co-expressing the dsRNA with the coat protein gene, wherein the amount of unencapsidated dsRNA produced is significantly higher than the amount of unencapsidated dsRNA produced when the coat protein gene is not co-expressed.
2. The method of claim 1, wherein the capsid protein is encoded by the coat protein gene of bacteriophage MS2.
3. The method of claim 1, wherein the capsid protein is encoded by the coat protein gene of bacteriophage Qβ.
4. The method of claim 1, wherein the gene encoding the dsRNA and the coat protein gene encoding the capsid protein are expressed from an inducible promoter.
5. The method of claim 4, wherein the coat protein gene encoding the capsid protein is expressed from a constitutive promoter and the gene encoding the dsRNA is expressed from an inducible promoter.
6. The method of claim 1, wherein the coat protein gene encoding the capsid protein is expressed prior to or concomitant with the gene encoding the dsRNA.

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7. The method of claim 1, wherein the gene encoding the dsRNA and the coat protein gene encoding the capsid protein are present on one plasmid or extrachromosomal element within the microbial cell.

8. The method of claim 1, wherein the gene encoding the dsRNA and the coat protein gene encoding the capsid protein are present on separate plasmids or extrachromosomal elements within the microbial cell.

9. The method of claim 1, wherein one of the genes encoding the dsRNA and the capsid protein are present on a plasmid or extrachromosomal element and the other of the genes encoding the dsRNA and the capsid protein are present on the chromosome of the microbial cell.

10. The method of claim 1, wherein the gene encoding the dsRNA and the coat protein gene encoding the capsid protein are present on the chromosome of the microbial cell.

11. The method of claim 1, wherein the dsRNA is an RNAi precursor.

12. The method of claim 1, comprising a step of recovering the unencapsidated dsRNA from a lysate of the microbial cell.

13. The method of claim 1, wherein a lysate of the microbial cell comprising the unencapsidated dsRNA is processed for application without further purification of the dsRNA.

14. The method of claim 1, wherein after producing the dsRNA the microbial cells are processed for application without lysis or further purification of the dsRNA.

15. The method of claim 1, wherein the capsid protein is an amino-terminal fragment of MS2 or Qβ comprising at least the first 11 amino acids and not more than the first 41 amino acids of the capsid protein.

16. The method of claim 15, wherein the amino-terminal fragment comprises the first 41 amino acids of the capsid protein.

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17. The method of claim 15, wherein the amino-terminal fragment comprises the first 35 amino acids of the capsid protein.

18. The method of claim 15, wherein the amino-terminal fragment comprises the first 25 amino acids of the capsid protein.

19. The method of claim 15, wherein the amino-terminal fragment comprises the first 21 amino acids of the capsid protein.

20. The method of claim 15, wherein the amino-terminal fragment comprises the first 12 amino acids of the capsid protein.

21. The method of claim 1, wherein the microbial cell is a bacterium.

22. The method of claim 1, wherein the microbial cell is a gram-negative bacterium.

23. The method of claim 1, wherein the microbial cell is a strain of Escherichia coli.

24. The method of claim 1, wherein the microbial cell is a gram-positive bacterium.

25. The method of claim 1, wherein the microbial cell is a strain of Corynebacterium glutamicum.

26. The method of claim 1, wherein the microbial cell is a yeast.

27. The method of claim 1, wherein the microbial cell is a strain of Saccharomyces cerevisiae.

28. A method according to claim 1, wherein the microbial cell is cultured under fed batch fermentation conditions, whereby accumulation as high as 3 g/L of recoverable unencapsidated dsRNA in the microbial cell is achievable.

29. A method according to claim 1, wherein the stem structure of the dsRNA is at least 75 base pairs in length.

30. A method according to claim 1, wherein the stem structure of the dsRNA is more than 20 nm in length.

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31. A method for producing unencapsidated dsRNA in a microbial cell, the microbial cell comprising (1) a leviviridae coat protein gene encoding a capsid protein, wherein the capsid protein is the capsid protein of bacteriophage MS2, the capsid protein of bacteriophage Qβ, or an N-terminal fragment thereof, and (2) a dsRNA gene comprising a self-complementary stretch of sequence separated by noncomplementary sequence such that upon hybridization of the complementary sequences a stem-loop structure is formed, the stem structure having a length exceeding the interior diameter of an MS2 capsid, the method comprising co-expressing the dsRNA with the coat protein gene, wherein the amount of unencapsidated dsRNA produced is significantly higher than the amount of unencapsidated dsRNA produced when the coat protein gene is not co-expressed.

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Figure 1

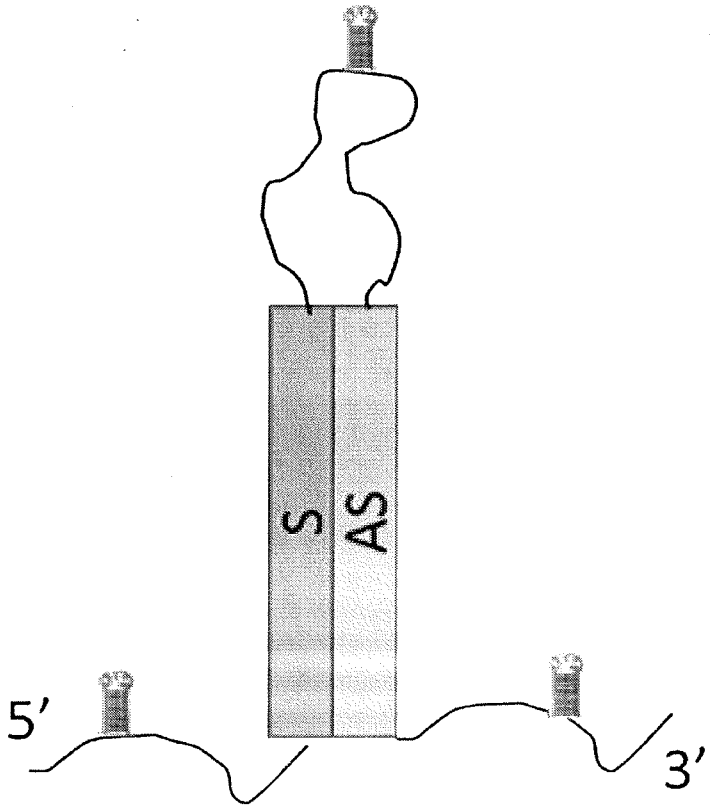


Figure 2

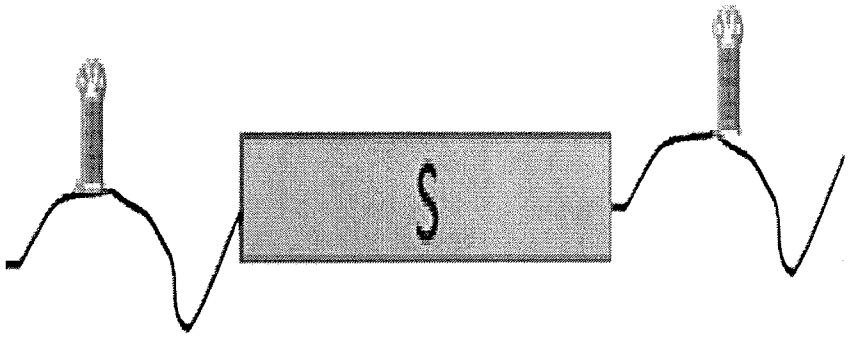


Figure 3

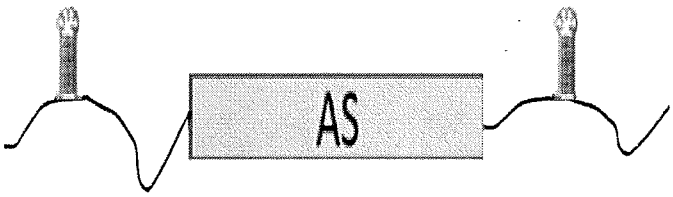


Figure 4

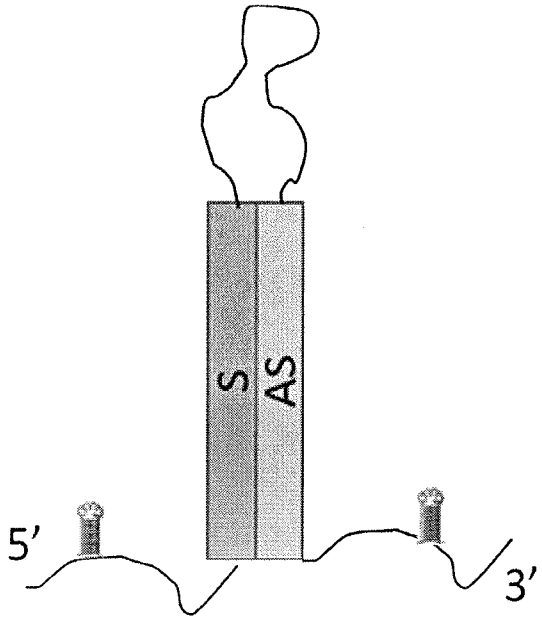


Figure 5

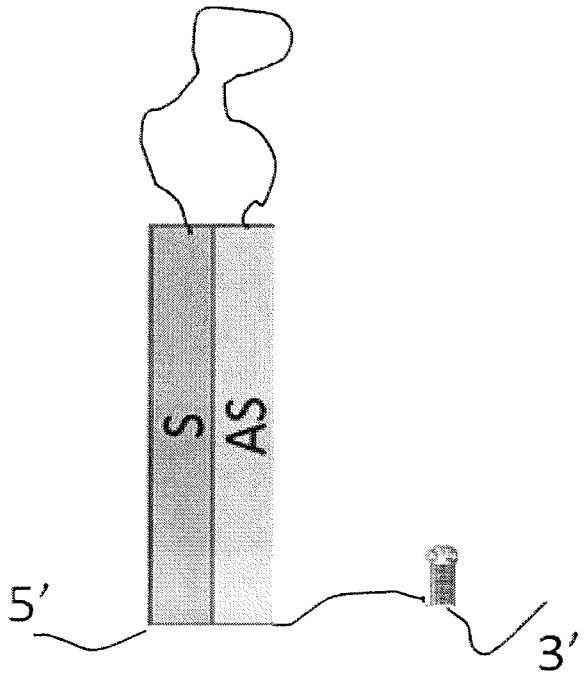
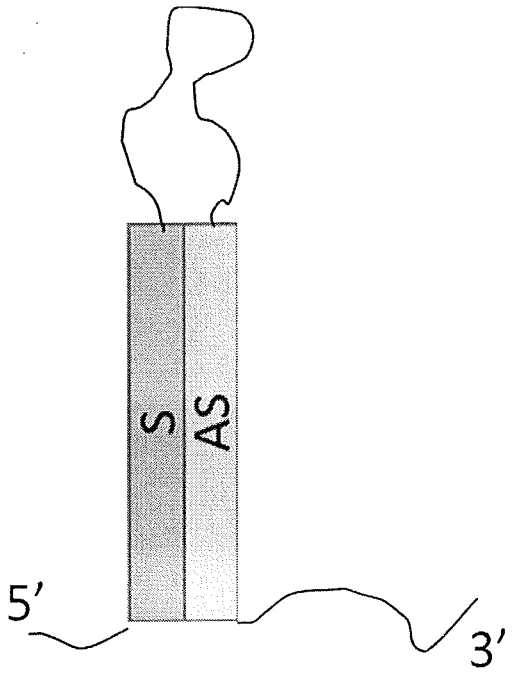
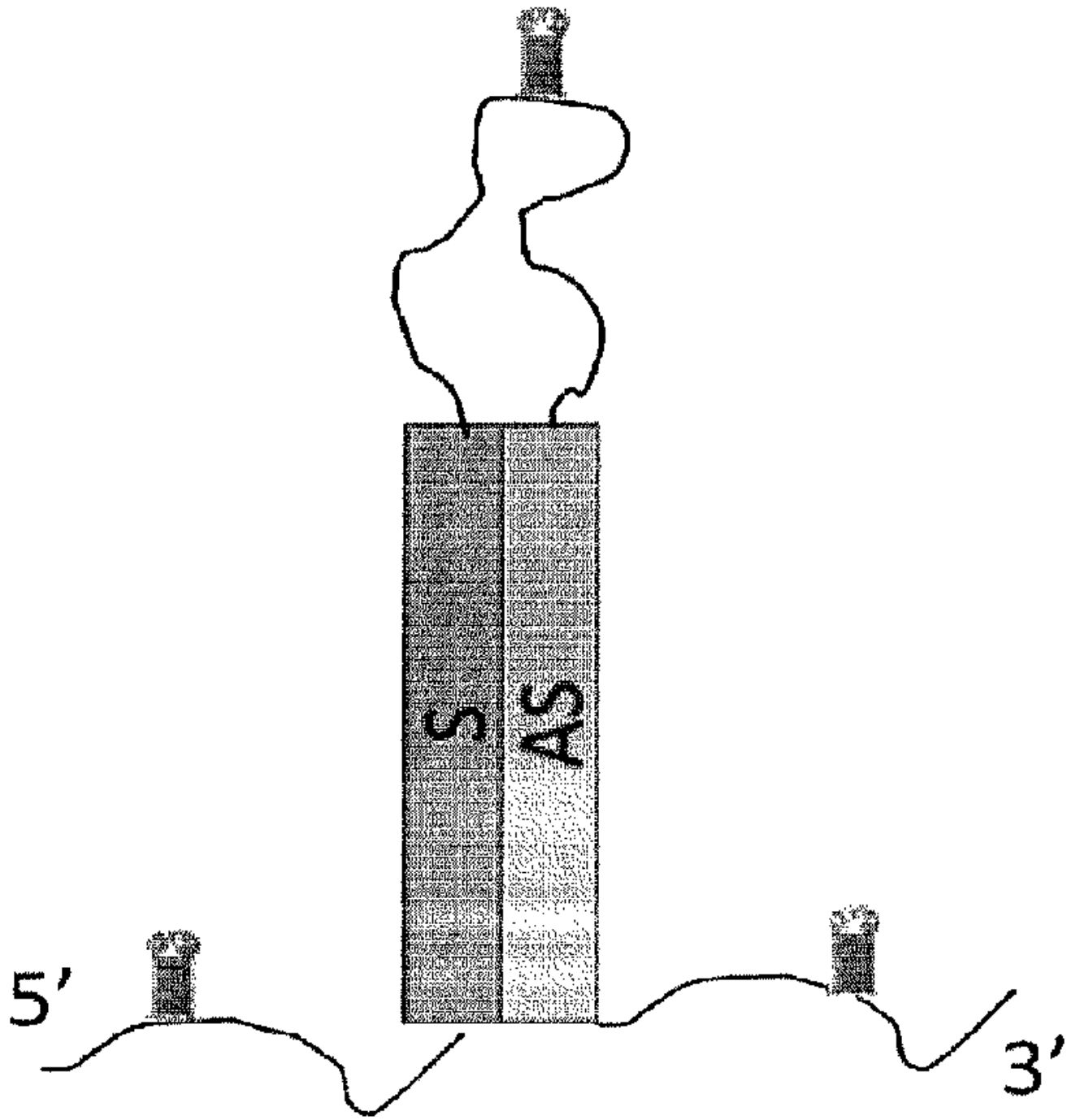


Figure 6

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