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(54) Title: NUCLEASE-MEDIATED GENOME EDITING

(57) Abstract: The invention relates to the field of genetic engineering tools, methods and techniques for gene or genome editing. Specifically, the invention concerns isolated polypeptides having nuclease activity, host cells and expression vectors comprising nucleic acids encoding said polypeptides as well as methods of cleaving and editing target nucleic acids in a sequence-specific manner. The polypeptides, nucleic acids, expression vectors, host cells and methods of the present invention have application in many fields of biotechnology, including, for example, synthetic biology and gene therapy.

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NUCLEASE-MEDIATED GENOME EDITING

Field of the Invention

The invention relates to the field of genetic engineering tools, methods and techniques for genome or gene editing. Such editing or manipulation of polynucleotide sequences, including structural or control gene sequences has application in many fields of health and biotechnology, for example gene therapy treatments of humans or animals, plant and animal breeding, and improvement of industrial organisms, e.g. by altering enzymes and metabolic pathways, particularly microorganisms; also in the areas of synthetic biology and algal biofuel production for example. Also the invention further relates to research tools and methods for use in basic scientific research involving molecular genetics.

Background to the Invention

Reference to any prior art in the specification is not an acknowledgement or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be combined with any other piece of prior art by a skilled person in the art.

Site-specific nucleases can permit the generation of double strand breaks (DSBs) at selected positions along a strand of DNA. In an organism of interest, this enables DSBs to be made at pre-determined positions in the genome. The creation of such breaks by site-specific nucleases prompts the endogenous cellular repair machinery to be repurposed in order to insert, delete or modify DNA at desired positions in the genome of interest. Targeted DNA cleavage mediated by site-specific nucleases is therefore an important basic research tool which has facilitated the functional determination and annotation of specific genes but amongst other things has also enabled the targeted mutation, addition, replacement or modification of genes in organisms of agricultural, industrial or commercial significance. As the genetic basis of both desirable and undesirable organismal phenotypes is uncovered through DNA sequencing, the ability to generate targeted alterations at specific genomic loci is fundamental to the genetic engineering of useful traits and in the development of clinical treatments for diseases with a genetic basis.

Other site specific nuclease approaches involve single strand target nucleic acid breaks, whether singly or in combination.

During the past decade, a range of molecular tools have been developed to allow for specific genetic engineering in general, and for dedicated editing of eukaryotic genomes in particular. Initially Zinc-Finger Nucleases (ZFNs) were developed, followed by Transcription Activator-Like Effector Nucleases (TALENs). Recently, a revolution has been caused by the development of the CRISPR-associated Cas9 nuclease, as a very efficient, generic and cheap alternative for dedicated genome surgery in a range of eukaryotic cells (from yeast and plant to zebrafish and human) (reviewed by Van der Oost 2013, Science 339: 768-770, and Charpentier and Doudna, 2013, Nature 495: 50-51).

Many useful site-specific nucleases have been discovered in and isolated from prokaryotes. Just like eukaryotes, prokaryotic organisms possess a variable set of defence systems to protect themselves against viruses. The defence strategies that protect their microbial host against invading DNA mainly rely on general (innate) immunity systems, such as the well-known restriction enzymes.

A major recent discovery in this area has been the demonstration of a specific (adaptive) immunity system in bacteria and archaea. This adaptive immune system consists of clustered regularly interspaced palindromic repeats (CRISPR), and CRISPR-associated Cas genes that encode the Cas proteins. The CRISPR-Cas system uses small CRISPR RNAs that guide effector Cas proteins to complementary invading nucleic acids, eventually neutralizing the invasion. Two classes of Cas effector complexes are distinguished: multi-subunit complexes (e.g. *E.coli* Cascade) and single-protein systems (e.g. *Streptococcus pyogenes* Cas9) (Van der Oost et al., 2014, Nature Rev. Microbiol. 12: 479-492).

Molecular analyses of CRISPR-Cas have provided the foundation for the development of genome engineering tools. Cas9 is a relatively simple CRISPR-Cas effector complex that can be functionally expressed in a wide range of prokaryotic and eukaryotic cells. Importantly, the RNA guide of Cas9 can easily be manipulated to specifically target any sequence of interest. Although adjusting the specificity for a certain target gene is also possible with the TALEN system, a drawback of this system is that this requires laborious protein engineering. In case of Cas9, only a

short oligonucleotide has to be generated and cloned, saving time and money. Applications of the Cas9 system include general genetic engineering (disruption, repair and integration of genes), control of gene expression (stimulation and silencing) and gene labelling (imaging). Co-expression of Cas9 with different guides allows for multiplexing, for instance generating multiple knockouts simultaneously.

The CRISPR-Cas system allows target-specific cleavage of genomic DNA guided by Cas9 nuclease in complex with a guide RNA (gRNA) that complementarily binds to a 20 nucleotide targeted sequence. Alteration of the sequence of the gRNA therefore allows the Cas9 endonuclease to be programmed to cut double-stranded DNA at sites complementary to the 20-base-pair guide RNA. The Cas9 system has been used to modify genomes in multiple cells and organisms.

Compared with alternative genome editing systems (Zinc Finger Nucleases, TALEN), engineering by Cas9 is very efficient, cheap, and fast.

Despite these developments, the Cas9 system still has some practical draw-backs. Firstly, based on an intrinsic self/non-self-discrimination mechanism, Cas9 requires a sequence motif (protospacer adjacent motif, PAM) in the flanking region adjacent to the target sequence. The PAM-requirement imposes a significant design limitation on the endonuclease system, excluding potential target sites.

Secondly, although RNA-guided nucleases such as Cas9 incorporate guide RNAs which direct cleavage of specific target sites and therefore exhibit a reduction in the significant off-target activity observed in most other available nucleases, a certain level of off-target cleavage still occurs (Pattanayak *et al.,* 2013, Nat. Biotechnol. 31: 839–843), that is, cleavage of genomic sequences that differ from the intended target sequence by one or more nucleotides. Generally, 15-17 nucleotides are required for base pairing with a 20 nucleotide complementary target; the tolerance for mismatches having been hypothesized to explain reported off-target problems. The imperfect specificity of engineered site-specific binding can lead to unintended insertion, modification or deletion of genomic loci during a gene targeting event, which has been associated with cellular toxicity. The consequences of such off

target cleavage events resulting in undesired alterations of genomic loci other than the desired target can be extremely serious in a clinical context.

The sequence-specific cleavage of the intended nuclease target site in the absence of, or with only minimal background off-target cleavage activity is a prerequisite for high-efficiency genomic manipulation in basic research applications and especially in avoiding the cleavage of unintended genes during targeted genomic modifications associated with clinical applications of the site-specific endonuclease technologies, particularly since the resulting double-stranded breaks result in stable, heritable genome modifications.

Despite a great deal of attention being focussed on addressing these undesired features of the Cas9 system, to date they remain largely unresolved.

Imprecise specificity in particular continues to remain a difficulty and has only partially been addressed by expanding the to-be-recognised target sequence by dimers of catalytically inactivated Cas9 fused to the nuclease domain of FokI (dCas9-FokI) (Guilinger *et al.*, 2014, Nat. Biotechnol. 32: 577-582). In addition, engineered nickase variants of Cas9 (in which one of the two nuclease sites is disrupted) have been demonstrated to facilitate homology directed repair in eukaryotic genomes with increased specificity and reduced off-target activity (Ran *et al.*, 2013, Cell 154: 1380–1389. Also, Mali *et al.*, 2013, Nat. Biotechnol. 31: 833–838).

WO 2015/035139 describes compositions, methods, systems, and kits for controlling the activity and/or improving the specificity of RNA-programmable endonucleases, such as Cas9. For example, guide RNAs (gRNAs) are engineered to exist in an "on" or "off" state, which control the binding and hence cleavage activity of RNA-programmable endonucleases. Also described are mRNA-sensing gRNAs that modulate the activity of RNA-programmable endonucleases, based on the presence or absence of a target mRNA. Some gRNAs are described that modulate the activity of an RNA-programmable endonuclease based on the presence or absence of an extended DNA (xDNA).

Another approach to mitigate off-target activity has centred on the development of software packages to aid in the guide RNA design process by undertaking exhaustive target sequence searches against genomic reference sequences, allowing the selection of target sequences with minimal off-target cleavage effects (Naito *et al.*, 2015, Bioinformatics 31: 1120-1123). However, this merely enables efficient exploration of the target sequence space available for guide sequence design rather than directly addressing the inherent limitations of CRISPR-Cas9 as a genome editing tool.

Thus, currently available nucleases, including CRISPR-Cas9 systems, are not in their current state of development necessarily suitable for the majority of clinical applications or indeed many other target-sensitive genome editing applications. There is a continuing need for genome editing tools with greater inherent specificity and reliability than is currently available in the art.

Schunder *et al.* provided the first indication of a functional CRISPR/Cas system in *Francisella tularensis* (Schunder *et al.*, 2013, *International Journal of Medical Microbiology* 303: 51-60). However, until now the structure and functionality of the system has remained unclear.

Subsequently, a classification of all known CRISPR adaptive immune systems of Archaea based primarily on their concatenated Cas protein sequences was provided by Vestergaard *et al.* in which Cas_Cpf1 was identified as a single protein interference system lacking Cas3, Cas5, Cas7 and Cas8, reminiscent of Cas9 in bacterial Type II systems despite not appearing to share any structural domains (Vestergaard *et al.*, 2014, *RNA biology* 11.2 (2014): 156-167).

Summary of the Invention

In seeking to overcome certain practical disadvantages associated with the Cas9 systems, the inventors provide a novel nuclease (Cpf1) unrelated to Cas9 for application as a gene editing tool. Cpf1 has been found to have uniquely advantageous mechanistic features such as a single nuclease domain and an upstream PAM motif and finds application as an improved tool for dedicated genome

editing in general, and for repairing genetic disorders of human stem cells. Additionally, the Cpf1 nuclease can function as part of a multiplex engineering system for micro-organisms.

Accordingly, the present invention provides an isolated polypeptide or fragment thereof, comprising the amino acid sequence SEQ ID NO: 1 or a sequence of at least 60% identity therewith, and having a nuclease activity.

In preferred aspects, the polypeptide or fragment comprises an amino acid sequence of at least 75%; preferably at least 85%; more preferably at least 90%; even more preferably at least 95% of SEQ ID NO:1.

The invention is based on reference SEQ ID NO:1 but includes any variant sequence having the defined percentage identity therewith. Such percentage identities include any of the following: a reference nucleic or amino acid sequence and sequences of at least a certain percentage identity are disclosed, e.g. at least 60%, then optionally the percentage identity may be different. For example: a percentage identity which is selected from one of the following: at least 60%, at least 61%, at least 62%, at least 63%, at least 64%, at least 65%, at least 66%, at least 67%, at least 68%, at least 69%, at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5% or at least 99.8%. Such sequence identity with a SEQ ID NO: 1 amino acid sequence is a function of the number of identical positions shared by the sequences in a selected comparison window, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

In all aforementioned aspects of the present invention, amino acid residues may be substituted conservatively or non-conservatively. Conservative amino acid substitutions refer to those where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity)

and therefore do not alter the functional properties of the resulting polypeptide. Similarly it will be appreciated by the skilled reader that nucleic acid sequences may be substituted conservatively or non-conservatively without affecting the function of the polypeptide. Conservatively modified nucleic acids are those substituted for nucleic acids which encode identical or functionally identical variants of the amino acid sequences. It will be appreciated by the skilled reader that each codon in a nucleic acid (except AUG and UGG; typically the only codons for methionine or tryptophan, respectively) can be modified to yield a functionally identical molecule. Accordingly, each silent variation (i.e. synonymous codon) of a polynucleotide or polypeptide, which encodes a polypeptide of the present invention, is implicit in each described polypeptide sequence.

The present invention provides a polypeptide or fragment having nuclease activity and comprising the amino acid sequence motif: FQIYN. This corresponds to residues 786 – 790 of SEQ ID NO:1.

The present invention also provides a polypeptide or fragment having nuclease activity and comprising the amino acid sequence motif: FQIYNK. This corresponds to residues 786 – 791 of SEQ ID NO:1.

The present invention also provides a polypeptide or fragment having nuclease activity and comprising the amino acid sequence motif: FQIYNKD. This corresponds to residues 786 – 792 of SEQ ID NO:1.

The present invention also provides a polypeptide or fragment having nuclease activity and comprising the amino acid sequence motif: $X^1X^2X^3$ X^4X^5FQIYNKDX^6X^7, corresponding to residues 781 – 794 of SEQ ID NO:1, wherein X^1 is one of G or K, X^2 is one of K,S or D, X^3 is one of L or I, X^4 is one of Y or F, X^5 is one of L or M, X^6 is one of F or Y and X^7 is one of S, A or V.

In another aspect the present invention provides a polypeptide or fragment having nuclease activity and comprising the amino acid sequence motif: GKLYLFQIYNKDFS. This corresponds to residues 781 – 794 of SEQ ID NO:1.

The amino acid sequence motif may instead comprise residues selected from 784 – 794, 785 – 794, 786 – 794, 787 – 794, 788 – 794 or 789 – 794 of SEQ ID NO: 1. The motif may be selected from residues 783 – 793, 783 – 792, 783 – 791, 783 – 790, 783 – 789 or 783 – 788 of SEQ ID NO:1. Also, the motif may be selected from residues 784 – 793, 785 – 792 or 786 – 790 of SEQ ID NO:1.

Alternatively, in aspects of the invention where a catalytically inactive version of Cpf1 is provided, the RuvC domain may comprise a Glu (E) residue, and short motif Glu-Ile-Asp (GID).

Alternatively, in aspects of the invention where a catalytically inactive version of Cpf1 is provided, the RuvC domain may comprise a Glu (E) residue, and short motif Gly-Ile-Asp (GID).

In aspects of the invention where a catalytically inactive version of Cpf1 is provided, the RuvC domain may comprise a Glu (E) residue, and short motif Glu-Ile-Asp (EID).

In aspects of the invention where a catalytically inactive version of Cpf1 is provided, the RuvC domain may comprise a Glu (E) residue, and short motif Ser-Ile-Asp (SID).

In aspects of the invention where a catalytically inactive version of Cpf1 is provided, the RuvC domain may comprise the amino acid sequence motif: X^8IDRGER wherein X^8 is one of G or S.

In aspects of the invention where a catalytically inactive version of Cpf1 is provided, the RuvC domain may comprise the amino acid sequence motif: DANGAY.

In aspects of the invention where a catalytically inactive version of Cpf1 is provided, the RuvC domain may comprise the amino acid sequence motif: EX^9LN wherein X^9 is one of D, N or E.

In aspects of the invention where a catalytically inactive version of Cpf1 is provided, the RuvC domain may comprise the amino acid sequence motif: EDLN.

A polypeptide or fragment of the invention may be defined both in terms of the reference sequence SEQ ID NO:1 and any percentage variant thereof, in combination with any of the aforementioned amino acid motifs as essential features.

In any aspect of the invention herein, the protein or polypeptide may have an RuvC (nuclease) domain.

In accordance with the invention, the RuvC domain may comprise a short motif GID.

In accordance with the invention, the RuvC domain may comprise a short motif SID.

In accordance with the invention, the RuvC domain may comprise a Glu (E) residue, and short motif GID.

The RuvC domain may comprise a Glu (E) residue, and short motif SID.

Where the RuvC domain comprises a Glu (E) residue, and short motif GID or SID, the D (aspartate) residue of the motif may be a catalytic residue.

The RuvC domain may comprise the amino acid sequence motif X^8IDRGER wherein X^8 is one of G or S. For example, the protein or polypeptide may have an RuvC (nuclease) domain, wherein the RuvC domain comprises the amino acid sequence motif SIDRGER.

Where the RuvC domain comprises an amino acid sequence motif GIDRGER or SIDRGER, the D (aspartate) residue of the motif may be a catalytic residue.

The protein or polypeptide may have an RuvC (nuclease) domain, wherein the RuvC domain may comprise the amino acid sequence motif DANGAY.

Where the RuvC domain comprises an amino acid sequence motif DANGAY, the D (aspartate) residue of the motif may be a catalytic residue.

The protein or polypeptide may have an RuvC (nuclease) domain, wherein the RuvC domain may comprise the amino acid sequence motif: EX^9LN wherein X^9 is one of D, N or E. For example, the protein or polypeptide may have an RuvC (nuclease) domain, wherein the RuvC domain comprises the amino acid sequence motif: EDLN. Where the RuvC domain comprises an amino acid sequence motif EDLN, ENLN or EELN, the E (glutamate) residue of the motif may be a catalytic residue.

In accordance with the invention, the polypeptide or fragment may have an RuvC (nuclease) domain comprising a Glu (E) residue, and the amino acid sequence motifs SID and DANGAY.

Optionally, the polypeptide or fragment may have an RuvC (nuclease) domain comprising a Glu (E) residue, and the amino acid sequence motifs SID and EDLN.

Optionally, the polypeptide or fragment may have an RuvC (nuclease) domain comprising a Glu (E) residue, and the amino acid sequence motifs SID, DANGAY and EDLN.

Optionally, the RuvC (nuclease) domain may comprise the amino acid sequence motif: $X^8IDRGER$ wherein X^8 is one of G or S, and the amino acid sequence motif DANGAY.

Optionally, the RuvC (nuclease) domain may comprise the amino acid sequence motif: $X^8IDRGER$ wherein X^8 is one of G or S, and the amino acid sequence motif: EX^9LN wherein X^9 is one of D, N or E.

Optionally, the RuvC (nuclease) domain may comprise the amino acid sequence motif: $X^8IDRGER$ wherein X^8 is one of G or S, and the amino acid sequence motif: EDLN.

Optionally, the RuvC (nuclease) domain may comprise the amino acid sequence motif: $X^8IDRGER$ wherein X^8 is one of G or S, and the amino acid sequence motif: DANGAY and the amino acid sequence motif: EX^9LN wherein X^9 is one of D, N or E.

Optionally, the RuvC (nuclease) domain may comprise the amino acid sequence motif: X^8IDRGER wherein X^8 is one of G or S, and the amino acid sequence motifs DANGAY and EDLN.

Preferably, the RuvC (nuclease) domain will comprise the amino acid sequence motifs: SIDRGER, DANGAY and EDLN.

In other aspects, the polypeptide or fragment may have an Arginine-rich motif.

The Arginine-rich motif may comprise the amino acid sequence motif: $X^{10}YX^{11}X^{12}X^{13}LX^{14}X^{15}X^{16}EX^{17}X^{18}X^{19}X^{20}X^{21}ARX^{22}X^{23}$, wherein X^{10} is one of D or N, X^{11} is one of R, Q or H, X^{12} is one of K, E, S or D, X^{13} is one of A, K or L, X^{14} is one of D, N or A, X^{15} is one of V, N, Q, K or A, X^{16} is one of R, K or I, X^{17} is one of Y, K or I, X^{18} is one of D or E, X^{19} is one of N, R or M, X^{20} is one of K, V, F or D, X^{21} is one of E, A, D or S, X^{22} is one of R, Q or K and X^{23} is one of N, A, S or D.

The Arginine-rich motif may comprise the amino acid sequence motif: DYRKALDVREYDNKEARRN, DYQKKLDNREKERVAARQA, DYREKLNQREIEMKDARQS, DYHSLLDKKEKERFEARQN or NYHDKLAAIEKDRDSARKD.

In accordance with the invention, the polypeptide or fragment may have an RuvC (nuclease) domain comprising a Glu (E) residue, and the amino acid sequence motifs Ser-Ile-Asp (SID), DANGAY and EDLN the amino acid sequence motif. Preferably, the RuvC (nuclease) domain will comprise the amino acid sequence motif: X^8IDRGER wherein X^8 is one of G or S, and the amino acid sequence motifs DANGAY and EDLN. More preferably, the RuvC (nuclease) domain will comprise the amino acid sequence motifs: SIDRGER, DANGAY and EDLN.

In further embodiments a polypeptide or fragment of any aspect of the invention preferably does not comprise an HNH (nuclease) domain. Additionally or alternatively, in yet a further embodiment, a polypeptide or fragment of any aspect of the invention does not comprise a recognition lobe that is typically present in Cas9.

Certain polypeptides or fragments of the invention may have nuclease activity that is provided by a single site in the polypeptide.

Other polypeptides or fragments of the invention may further comprise a zinc finger-domain, although the metal-binding site (typically 4 amino acids, Cys and/or His) is not complete in all Cpf1 variants.

Polypeptides or fragments of the invention may have a nuclease activity which is single strand cleavage, e.g. nickase activity.

Preferably, two subunits of Cpf1 may be used in a dimeric arrangement where nuclease domains of each of the two subunits cleave individual DNA strands. Preferably, such a dimer may be a homodimer where the RuvC-like domains of each of the two subunits cleave individual DNA strands. Alternatively, Cpf1 polypeptides of the invention may be engineered to contain more than one nuclease domain, native or otherwise, which permit cleavage of both DNA strands.

Polypeptide or fragments of the invention preferably have binding affinity for a guide RNA molecule.

In other aspects, a polypeptide or fragment of the invention may have a guide RNA comprising a sequence substantially complementary to a sequence comprised in a target nucleic acid strand.

In further embodiments, a polypeptide or fragment of the invention preferably has binding affinity for a polynucleotide sequence motif in a target nucleic acid strand. This sequence motif is usually known as a protospacer adjacent motif (PAM) sequence. Preferably the nucleotide sequence motif is at least 3 contiguous nucleic acid residues.

The PAM is located on the target (adjacent to protospacer). Typically, the SEED domain of the guide RNA (the region most likely responsible for initial guide/target base pairing) is complementary to the target nucleic acid sequence. Preferably, the SEED part of the guide does not tolerate mismatches.

In order to further improve the polypeptides or fragments of the invention, additional amino acids may be added, preferably by way of a fusion to the N or C terminus. The additional amino acid sequence may have nucleic acid or chromatin modifying, visualising, transcription activating or transcription repressing activity and is preferably translationally fused through expression in natural or artificial protein expression systems, or covalently linked by a chemical synthesis step to the at least one subunit; preferably the at least one functional moiety is fused or linked to at least the region of the N terminus and/or the region of the C terminus.

The additional amino acid sequence having nucleic acid or chromatin modifying, activating, repressing or visualising activity may be a protein; optionally selected from a helicase, a nuclease, a nuclease-helicase, a DNA methyltransferase (e.g. Dam), or DNA demethylase, a histone methyltransferase, a histone demethylase, an acetylase, a deacetylase, a phosphatase, a kinase, a transcription (co-)activator, an RNA polymerase subunit, a transcription repressor, a DNA binding protein, a DNA structuring protein, a marker protein, a reporter protein, a fluorescent protein, a ligand binding protein (e.g. mCherry or a heavy metal binding protein), a signal peptide (e.g. TAT-signal sequence), a subcellular localisation sequence (e.g. nuclear localisation sequence) or an antibody epitope.

When the protein is a nuclease, it may be one selected from a type II restriction endonuclease such as FokI, or a mutant or an active portion thereof. Preferably, one protein complex of the invention may be fused to the N terminal domain of FokI and another protein complex of the invention may be fused to the C terminal domain of FokI. These two protein complexes may then be used together (in a dimeric configuration) to achieve an advantageous locus specific double stranded cut in a nucleic acid, whereby the location of the cut in the genetic material is at the design and choice of the user, as guided by the RNA component (defined and described below) and due to presence of a so-called "protospacer adjacent motif" (PAM) sequence in the target nucleic acid strand (also described in more detail below).

In a preferred embodiment, a protein or polypeptide of the invention has an additional amino acid sequence which is a modified restriction endonuclease, e.g.

FokI. The modification is preferably in the catalytic domain. In preferred embodiments, the modified FokI is KKR Sharkey or ELD Sharkey, which is fused to the Cpf1 protein. In a preferred application of these complexes of the invention, two of these complexes (KKR Sharkey and ELD Sharkey) may be together in combination. A heterodimer pair of protein complexes employing differently modified FokI has particular advantage in targeted double stranded cutting of nucleic acid. If homodimers are used then it is possible that there is more cleavage at non-target sites due to non-specific activity. A heterodimer approach advantageously increases the fidelity of the cleavage in a sample of material.

Advantageously the above modifications can permit a user to select in a predetermined manner a precise genetic locus which is desired to be cleaved, tagged or otherwise altered in some way, e.g. methylation, using any of the nucleic acid or chromatin modifying, visualising, transcription activating or transcription repressing entities defined herein. The other component part of the system is an RNA molecule which acts as a guide for directing the complexes of the invention to the correct locus on DNA or RNA intending to be modified, cut or tagged.

In further embodiments, a polypeptide or fragment of the invention is preferably bound to a guide RNA and to a target nucleic acid. In this form a complex is formed which provides targeted DNA strand nuclease activity, wherein a desired target locus is cleaved.

In another aspect the present invention provides a polynucleotide comprising a polynucleotide sequence encoding a polypeptide or fragment of the invention as hereinbefore defined.

In further aspect, the present invention provides an expression vector comprising a polynucleotide as aforementioned.

The invention also provides an expression vector as defined above, further comprising a nucleotide sequence encoding a guide RNA which has substantial complementarity to a desired sequence in the target nucleic acid strand. Guide RNA in the native state is a single RNA consisting of a crRNA.

The invention further provides an expression vector of the invention which is preferably a viral vector, e.g. Adenovirus, or Adeno-associated Virus (AAV).

In other aspects, the invention provides a host cell transformed to express a polypeptide or fragment of the invention as hereinbefore described.

Typically, the expression vector DNA can be delivered to the host cell by transformation, electroporation or virus (AAV). Also, RNA can be delivered into a host cell by injection or electroporation. Proteins can be delivered to cells via electroporation, peptide (HIV) tags. In another aspect the present invention provides a host cell as hereinbefore described, additionally transformed to contain a guide RNA comprising a sequence substantially complementary to a sequence comprised in a target nucleic acid strand in the host cell.

The invention includes any host cell transformed with an expression vector as hereinbefore described.

The invention also provides a method of cleaving a target nucleic acid strand at a specific locus, comprising exposing the target nucleic acid to a polypeptide or fragment of the invention, and with a guide RNA molecule which comprises a sequence substantially complementary to a sequence comprised in the target nucleic acid strand.

The invention further provides a method of cleaving a target nucleic acid strand at a specific locus in the genome of a cell of an organism, comprising transforming the cell with an expression vector of the invention as described herein, and transforming the cell with a vector which expresses a guide RNA comprising a sequence substantially complementary to a sequence comprised in a target nucleic acid strand.

In further aspect, the invention provides a method of cleaving a target nucleic acid strand at a specific locus in the genome of a cell of an organism, comprising transforming the cell with an expression vector of the invention as described herein.

In another aspect the present invention provides a method of non-homologous end joining gene editing comprising (a) transforming the cell with an expression vector of the invention, and transforming the cell with a vector which expresses a guide RNA comprising a sequence substantially complementary to a sequence comprised in a target nucleic acid strand; or (b) transforming the cell with an expression vector of the invention. In these aspects of the invention the polypeptides of the invention are modified or used to cause double stranded breaks.

In a further aspect the invention provides a method of homologous end joining gene editing comprising (a) transforming the cell with an expression vector of the invention, and transforming the cell with a vector which expresses a guide RNA comprising a sequence substantially complementary to a sequence comprised in a target nucleic acid strand; or (b) transforming the cell with an expression vector of the invention; so as to create a double strand break at a desired locus in the genetic material, and exposing the genetic material to a polynucleotide sequence which has end regions complementary to the broken end regions of the genetic material.

Detailed Description

The protein of amino acid sequence SEQ ID NO: 1 is a large protein (about 1300 amino acids) that contains an RuvC-like nuclease domain homologous to the respective domains of Cas9 and transposable element ORF-B, along with an arginine-rich region similar to that in Cas9 and a Zinc Finger (absent in Cas9 but shared with ORF-B), but lacks the HNH nuclease domain that is present in all Cas9 proteins.

The invention will now be described in detail with reference to the examples and to the drawings in which:

Figure 1 shows the domain structure of the novel CRISPR-Cas nuclease, Cpf1. Three RuvC nuclease domains, a Zinc-finger and an arginine-rich domain that allows for interaction with RNA guide and DNA target are shown.

Figure 2 shows the results of an *in silico* analysis of conserved Protospacer Adjacent Motif (PAM). Panel A shows a Weblogo based on 5' flanks of protospacers depicted in Table 1. Panel B shows a Weblogo based on 3' flanks of protospacers depicted in Table 1.

Figure 3 shows the results of a multiple alignment of the Cpf1 protein family. Each sequence is labelled with GenBank Identifier (GI) number and systematic name of an organism. Predicted secondary structure (SS) is shown by shading. Active site residues of RuvC-like domain(s) are shown as bold and double underlined. Potential bridge helix is shown by shading and with single underline. The amino acid sequence FQIYN is also indicated in bold, by shading and dotted underline.

Example 1 - Novel nucleases for gene editing

Specific examples are (1) CRISPR-associated Cpf1 from the marine bacterium *Francisella novicida* (Fn-Cpf1), and (2) CRISPR-associated Cpf1 from the archaeon *Methanomethylophylus alvus* strain Mx1201 (Mal-Cpf1) that resides in the human gut.

Without the inventors wishing to be bound by any particular theory, Cpf1 recognises the crRNA in a sequence-specific manner, after which cleavage occurs of the double stranded RNA segment, and eventually formation of an effector complex consisting of Cpf1 and a single crRNA guide. Cpf1 may operate as a dimer, with the RuvC-like domains of each of the two subunits cleaving individual DNA strands. Alternatively, Cpf1 may contain more than one nuclease domain which permits cleavage of both DNA strands. Alternatively, one or more RuvC domains of Cpf1 may exhibit unusual flexibility that allows for cleavage of both strands.

The following examples were performed in parallel for the bacterial Fno-Cpf1 and archaeal Mal-Cpf1 protein variants:

Cloning is carried out of the entire CRISPR locus, including *cas* operon (cpf1-cas4-cas1-cas2), leader region, CRISPR array, and flanking regions (approximately 10 kb) in low-copy vector (*e.g.* pACYC184) in an *E. coli* K12 strain; no details are known

about the maturation of the guide, which may be similar to that of Cas9 (tracrRNA/RNaseIII), or may be similar to that of Cascade (Cas6-like ribonuclease, although that is not part of cpf1 operons), or may be unique. Further detailed materials and methods are provided in Sapranauskas *et al.,* 2011, Nucleic Acids Res. 39: 9275–9282.

Standard procedures were used to optimize chances for functional protein production of the selected Cpf1 proteins in *E. coli*: (i) by performing codon harmonization design to adjust *cpf*1 nucleotide sequences (see Angov *et al.*, 2008, PLoS One 3, e2189); (ii) by including N-terminal or C-terminal strepII tag, that will allow for affinity purification; (iii) by cloning synthetic gene in T7 expression vector (*e.g.* pET24d) and transform plasmid to non-production strain of *E. coli* (*e.g.* JM109, lacking T7 RNA polymerase gene), (iv) transferring plasmid via second transformation to production strain of *E. coli* (*e.g.*, BL21(DE3), containing T7 RNA polymerase gene under control of rhamnose promoter, that allows for accurate tuning of expression, (v) varying expression conditions (medium, inducer concentration, induction time), (vi) using optimal conditions for liter-scale cultivation, after which cells are harvested and mechanically disrupted to obtain cell-free extract (small volumes by sonication; large volumes by French Press), (vii) separating membrane and soluble fractions, and perform affinity purification using streptactin resin, (viii) testing relevant fractions by SDS-PAGE, and storing the pure protein for subsequent analyses.

As well as the above, additionally, the predicted crRNA gene is sequenced, or a single-guide RNA (sgRNA) gene is made, *e.g.* by adding 4 nucleotide synthetic loops (Jinek *et al.*, 2012, Science 337: 816–821); RNA genes residing either on the same plasmid as *cpf1* gene, or on a separate plasmid.

Additionally, a catalytically inactive Cpf1 mutant is made (RuvC active site contains conserved glutamate (E) as well as GID motif).

Additionally, a catalytically inactive Cpf1 mutant is made (RuvC active site contains conserved glutamate (E) as well as SID motif).

Also, N-terminal or C-terminal fusions are made of the Cpf1 mutant with FokI nuclease domain with differently connecting linkers (as described for Cas9; see Guilinger *et al.*, 2014, Nat. Biotechnol. 32: 577-82).

Example 2 - Biochemical characterization of Cpf1 nucleases

These experiments characterize guide surveillance and target cleavage. The CRISPR system is an adaptive immunity system in bacteria and archaea. The CRISPR arrays consist of identical repeats (*e.g.* 30 bp) and variable spacers (e.g. 35 bp). The adaptive nature of the CRISPR system relies on regular acquisition of new spacers, often corresponding to fragments (protospacers) derived from viruses. Acquisition generally depends on the selection of a protospacer based on the presence of a protospacer adjacent motif (PAM). The presence of this motif is crucial for the eventual interference by the CRISPR-associated effector complex (*e.g.* Cas9) with its crRNA guide. The PAM motif allows for self versus non-self discrimination: the potential target sequences (i.e. complementary to the crRNA guide sequence) reside both on the host's genome (the self CRISPR array) as well as on the invader's genome (the non-self protospacer); the presence of the protospacer in the invader DNA triggers the effector complex to bind it in a step-wise manner; when perfect base pairing occurs between the sequence of the protospacer immediately adjacent to the PAM (the so-called seed sequence), then base pairing as a zipper, eventually leading to a state of Cas9 to catalyse cleavage of the target DNA strands (see Jinek *et al.*, 2012, Science 337: 816–821; also Gasiunas *et al.*, 2012, PNAS 109: E2579–E2586).

In silico analysis of the Cpf1-associated PAM by BLAST analysis of the CRISPR spacers of the *cpf1*-loci. BLAST analysis of some spacers shows several homologous sequences (90-100% identity), (Table 1). The most promising hits concern identical sequences of virus genes in general, and genes of prophages in particular. Prophages are derived from lysogenic viruses, the genomes of which have integrated in the genome of bacteria. As is the case with eukaryotic viruses, the host range of prokaryotic viruses is often rather limited; hence, when the matching prophage is found in a bacterium that is closely related to the bacterium that has the corresponding spacer sequence in its CRISPR array, this gives some

confidence that it is a real hit. In other words, it may well be that the prophage resembles a virus that has attempted to infected the CRISPR-containing bacterium, but the invasion has resulted in spacer acquisition and virus immunity of the latter bacterium.

Table 1. BLAST results with FnU112 cpf1-associated CRISPR spacers as query sequences. The nucleotide sequence of both spacer (top) and protospacer are shaded; the 5' and 3' flanks of the protospacers are unshaded; Tool: CRISPR Target (bioanalysis.otago.ac.nz/CRISPRTarget/). Query: Entire CRISPR array from *Francisella novicida* sub species. Target database: Genbank-NT. Gap open -10, Extend -2; Nucleotide match 1, mismatch -1; E-value 1; Word size 7; Cutoff score 20; 3' end flanking protospacer 8 bp; 5' end flanking protospacer 8 bp.

Fn sub species Spacer #	Host of prophage, target gene accession number		Alignment of Fn sub species spacer with protospacer (plus 8 nt flanks on both sides)
Francisella novicida U112 #1	*Francisella novicida 3523,* hypo prot AEE26301.1	spacer protospacer	5′ AGATTAAAAGGTAATTCTATCTTGTTGAG |||||||||||||||||o||||||| 5′ATAATTTAAGATTAAAAGGTAATTCTATTTTGTTGAGATCTGAGC
Francisella novicida U112 #2	*Francisella novicida 3523,* intergenic sequence in prophage	Spacer protospacer	5′ TAGCGATTTATGAAGGTCATTTTTTT ||||||||||||||||||||||| 5′CTAAATTATAGCGATTTATGAAGGTCATTTTTTTAAAAAGTT
Francisella novicida Fx1 #1	*Francisella novicida 3523,* hypo prot	spacer protospacer	5′ ATGGATTATTACTTAACTGCAGTGTTTAC |||||||||||||o|||||||o||| 5′AATGTTCAATGGATTATTACTTAATTGCAGTGTCTACGTCGATGG

	AEE26295.1, "phage major tail tube protein"		
Francisella novicida FTG #1	*Francisella novicida 3523,* hypo prot YP_005824059.1	spacer protospacer	5′ GCCACAAATACTACAAAAAATAACTTAA ||oo|||||||||||||||||||||| 5′ATTTTTTGGCTCCAAATACTACAAAAAATAACTTAAACTTTGAA
Francisella novicida GA99-3549 #1	*Francisella novicida 3523,* hypo prot FN3523_1009, "baseplate_J"	spacer protospacer	5′ ATTGTCAAAACATAAGCAGCTGCTTCAAATAT |o|||o|oo||||||||||o|||||||||| 5′GGTCTTTTACTGTTATTACATAAGCAGCCGCTTCAAATATCTTAGCAA

Analysis of the sequences flanking the protospacers in the prophage genes resulted in a T-rich conserved motif; interestingly, this motif does not reside downstream the protospacer (as in the Cas9 system), but rather upstream. Though not wishing to be bound by particular theory, the inventors find that Cpf1 of the invention requires a PAM-like motif (3-4 nucleotides) for binding a target DNA molecule that is complementary to the guide, has a seed sequence (8-10 nucleotides) in which no mismatches are allowed, and has a single nuclease site that allows for nicking of the base paired target DNA strand.

PAM motifs of Cpf1 and variants of the invention were also characterized using the approach of Jiang *et al.*, 2013, Nat. Biotechnol. 31: 233-239). Two derivatives of *E. coli* BL21(DE3) were used, initially transformed either with a target-plasmid or with a non-target plasmid; two variant target plasmids used have a similar part (GFP marker, KmR marker, origin of replication) and a variable part with target sequence

(protospacer) with an associated degenerate PAM (5-8 variable nucleotides) either upstream or downstream of the protospacer); next, this strain was transformed with a Cpf1-expression plasmid (includes design-CRISPR with single-guide RNA (sgRNA, CmR-marker); screening for transformants was on plates with chloramphenicol (Cm) (not kanamycin (Km)), and screening for non-fluorescent colonies, indicating loss-of-target-plasmid. As the plasmids with the correct PAMs will be lost, DNA Deep Seq was performed of appropriate PCR products of the entire pool of target plasmid, before and after transformation. The differences reveal the PAM (Bikard *et al.*, 2013, Nucleic Acids Res. 41: 7429–7437).

PAM signatures were confirmed by *in vitro* characterization of cleavage activity of BsCas9/sgRNA; assays reveal optimal conditions (temperature, buffer/pH, salt, metals).

Presence of a seed sequence in the PAM was established according to methods described by Jinek *et al.*, 2012, Science 337: 816–821.

Example 3 – Bacterial Engineering

Performing of high-throughput engineering of bacterial genome with nuclease variants. Without wishing to be bound by particular theory, the inventors expect that Cpf1/guide complexes of the invention allow for specific targeting of genomic DNA. Multiplex targeting can be established by using a design CRISPR together with a matching crRNA.

The experiments provide application of Cpf1 and variants of the invention. Cas9 is tested in parallel as a reference.

Gene knock-in/knock-out (insertion/disruption of any sequence) is performed. The host strain *E. coli* K12 (LacZ+, GFP-) was engineered as follows: the gene encoding a variant of the Green Fluorescent Protein (GFPuv) is inserted in the lacZ gene, resulting in a clear phenotype (LacZ-, GFP+). The *cpf1* gene was introduced on a plasmid (or derivatives of those plasmids), together with a fragment that allows for homologous recombination of the target sequence. A target (protospacer) sequence

was selected, with an appropriate adjacently located PAM sequence; a corresponding guide designed, consisting of the crRNA (with spacer complementary to target protospacer) and the crRNA gene (as adapted from the method described for Cas9 by Jiang *et al.* (2013a) *RNA-guided editing of bacterial genomes using CRISPR-Cas systems*. Nat. Biotechnol. 31: 233-239).

Gene expression silencing (using catalytically inactivated Cas9, was as described: dCas9 derivative of Spy-Cas9; (Bikard *et al.*, 2013, Nucleic Acids Res. 41: 7429-7437; Qi *et al.*, 2013, Cell 152: 1173-1183);) by binding at promoter (RNA polymerase binding site) of target gene, or of target genes using a multiplex approach (using a design CRISPR).

Gene expression activation; as above (silencing); binding upstream binding site of RNA polymerase, with Cas9 fused to activation domain (as has been described for Spy-Cas9) (Bikard *et al.*, 2013, Nucleic Acids Res. 41: 7429–7437).

Fusion of inactivated Cpf1 and the FokI nuclease domain (described in Example 1 were compared with an active Cpf1 in different experimental set-ups. This required two simultaneous interactions of guides and targets, that results in a major improvement of cleavage at the desired site.

Example 4 - Human Stem cell engineering

Targeted editing of disease-causing genetic mutations would be an elegant and effective treatment for genetic disease. Recently discovered gene editing systems such as Cas9, allow the specific targeting of disease-causing mutations in the genome, and can be used to functionally repair or permanently disable mutated genes. The efficiency of gene editing systems has been demonstrated in a laboratory setting, and are now routinely used in genome editing of a wide variety of cell types from many different species, including human. However, despite the success of these systems in the research setting, clinical application of gene editing systems is hampered by the lack of a suitable delivery system to introduce gene-editing technologies into patient cells in a safe, transient and efficient manner. Several labs are working on the development of recombinant viral vectors which can

be used to deliver gene editing systems into patient cells, but prolonged expression of for example CRISPR/Cas9 from such vectors will increase the likelyhood of off-target effects and is therefore not ideal. Intracellular delivery of recombinant gene editing protein and synthetic CRISPR RNA would be an effective, non-integrating and transient method for the application of gene editing technology in patient cells.

Recently a novel method has been developed that allows the transduction of native proteins into virtually any cell type (D'Astolfo *et al.*, 2015, Cell, 161: 674-690). This technology, termed iTOP, for Induced Transduction by Osmocytosis and Propanebetaine, is based on a combination of small molecule compounds, which trigger the uptake and intracellular release of native protein. iTOP is highly efficient, routinely achieving transduction efficiencies of >90% of cells, and works on a wide variety of primary cell types. It has been demonstrated that iTOP-mediated transduction of recombinant Cas9 protein and *in vitro* transcribed sgRNA allows for highly efficient gene editing in difficult-to-transfect cell types including human stem cells. Upon iTOP-CRISPR/Cas9 transduction, >70% bi-allelic gene targeting has been reported in human ES cells without the need for drug-selection of transduced cells.

Key advantages of iTOP over existing technologies are: (i) the ability to transduce primary (stem) cells with native protein at very high efficiency, (ii) the non-integrating, transient nature of protein mediated gene editing, ensuring safety and minimizing off-target effects, and (iii) the tight control of dosage and timing of the delivered protein. We have demonstrated that iTOP-CRISPR/Cas9 is an effective tool to modify a large variety of primary (patient) cell types. However, due to size and protein solubility issues, production of recombinant Cas9 is hampering broad-scale (clinical) adoption of this system. Cpf1 could solve these problems and pave the way for the development of novel therapies to treat genetic disease.

The iTOP technology will be used to allow efficient intracellular delivery of Cpf1 into human stem cells. The advantage of iTOP is its highly flexible approach. First, NaCl-mediated hypertonicity induces intracellular uptake op protein via a process called macropinocytosis (D'Astolfo *op. cit.*)). Second, a propanebetaine transduction compound (NDSB-201 or gamma-aminobutyric acid (GABA) or others triggers the intracellular release of protein from the macropinosome vesicles. In addition to these

compounds, osmoprotectants such as glycerol and glycine are added to help cells to cope with the NaCl-induced hypertonic stress. By varying the concentration of NaCl, the concentration and type of transduction compound and/or the concentration and type of osmoprotectants, the iTOP system can be adapted and optimised to meet the specific requirements of the cargo protein and/or the target cells. iTOP parameters were optimized to allow efficient gene editing of human embryonic stem cells (hESCs), targeting the endogenous WDR85 gene by Cpf1 (equipped with an N- or C-terminal nuclear localization signal (NLS)), as recently shown for Cas9.

In the following sequence listing, the amino acid residues Glu Xaa Asp (single underlined) are the GID motif of an RuvC domain. Therefore in the SEQ ID NO: 1, the Xaa residue may be I.

The amino acid residues Ile Asp Arg Gly Glu Arg (double underlined) include the IDR residues of an RuvC domain.

The amino acid residues Phe Glu Asp (triple underlined) include the E residue making up part of the active site residues of an RuvC domain.

Example 5 Multiple alignment of Cpf1 proteins

Figure 3 shows the results of an Multiple alignment of Cpf1 proteins. The alignment was built using MUSCLE program and modified manually on the basis of local PSI-BLAST pairwise alignments and HHpred output. Each sequence is labelled with GenBank Identifier (GI) number and systematic name of an organism. Five sequences analysis in this work are marked by the respective numbers. Secondary structure (SS) was predicted by Jpred and is shown is shown by shading. CONSENSUS was calculated for each alignment column by scaling the sum-of-pairs score within the column between those of a homogeneous column (the same residue in all aligned sequences) and a random column with homogeneity cutoff 0.8. Active site residues of RuvC-like domain(s) are shown as bold and double underlined. Potential bridge helix is shown by shading and with single underline. The amino acid sequence FQIYN is also indicated in bold, by shading and dotted underline.

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CLAIMS

1. An expression vector comprising a nucleotide sequence encoding a Cpf1 polypeptide, wherein the Cpf1 polypeptide comprises the amino acid sequence YLFQIYNKDF corresponding to amino acid residues 784-793 of SEQ ID NO:1, wherein the Cpf1 polypeptide comprises a RUV-C domain and does not comprise an HNH domain, and wherein the Cpf1 polypeptide has nuclease activity.

2. The expression vector of claim 1, wherein the Cpf1 polypeptide has at least 60% sequence identity with SEQ ID NO:1.

3. The expression vector of claim 1, wherein the Cpf1 polypeptide comprises the amino acid sequence GKLYLFQIYNKDFS corresponding to amino acid residues 781-794 of SEQ ID NO:1.

4. The expression vector of any one of claims 1-3, wherein the Cpf1 polypeptide is fused at its N or C terminus to an additional protein domain.

5. The expression vector of claim 4, wherein the additional protein domain is a helicase, a nuclease, a nuclease-helicase, a DNA methyltransferase, a DNA demethylase, a histone methyltransferase, a histone demethylase, an acetylase, a deacetylase, a phosphatase, a kinase, a transcription (co-)activator, an RNA polymerase subunit, a transcription repressor, a DNA binding protein, a DNA structuring protein, a marker protein, a reporter protein, a fluorescent protein, a ligand binding protein, a signal peptide, a subcellular localization sequence, or an antibody epitope.

6. The expression vector of claim 5, wherein the additional protein domain is a nuclear localization sequence.

7. The expression vector of claim 5, wherein the additional protein domain is a FokI domain.

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8. A vector system comprising one or more vectors comprising

a) a nucleotide sequence encoding a Cpf1 polypeptide, wherein the Cpf1 polypeptide comprises the amino acid sequence YLFQIYNKDF corresponding to amino acid residues 784-793 of SEQ ID NO:1, wherein Cpf1 polypeptide comprises a RUV-C domain and does not comprise an HNH domain, and wherein the Cpf1 polypeptide has nuclease activity; and

b) a nucleotide sequence encoding a guide RNA wherein said Cpf1 polypeptide has affinity for said guide RNA .

9. The vector system of claim 8, wherein said one or more vectors are viral vectors.

10. The vector system of claim 9, wherein said viral vectors are AAV vectors.

11. The expression vector of claim 1, wherein said Cpf1 polypeptide has at least 85% identity with SEQ ID NO:1.

12. The expression vector of claim 1, wherein said Cpf1 polypeptide has at least 95% identity with SEQ ID NO:1.

Figure 1

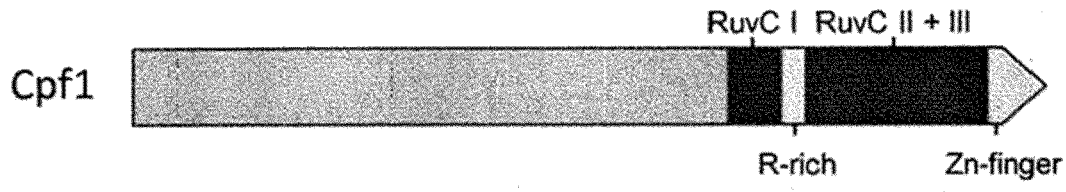

Figure 2

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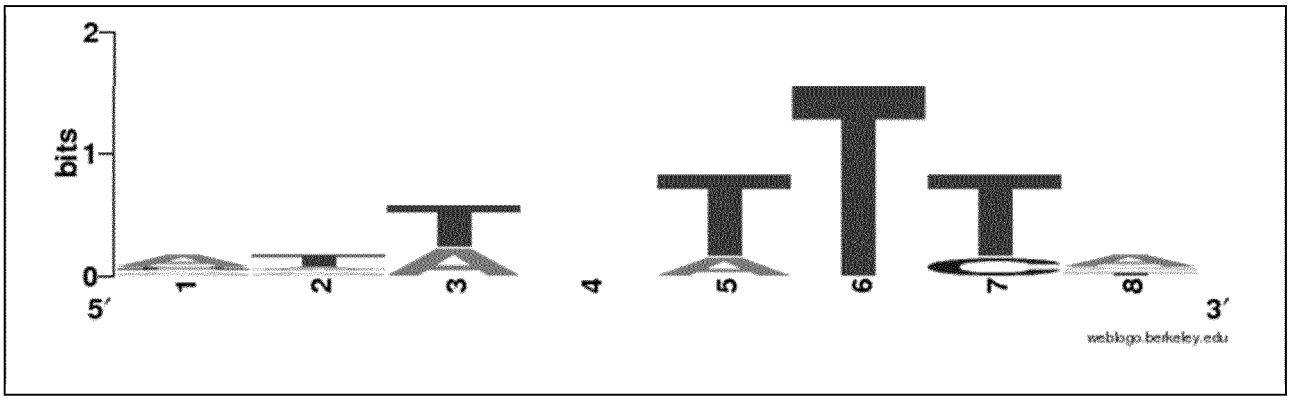
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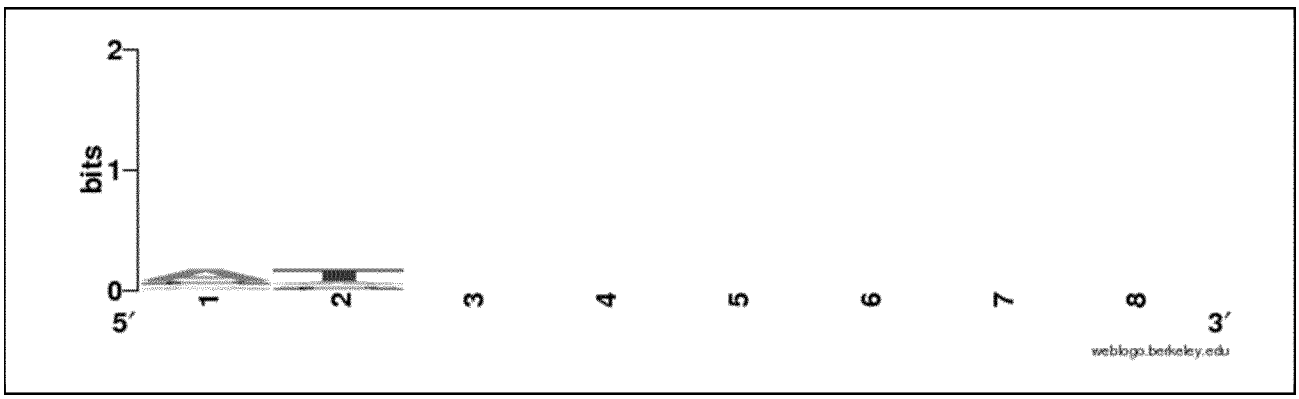
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Figure 3A

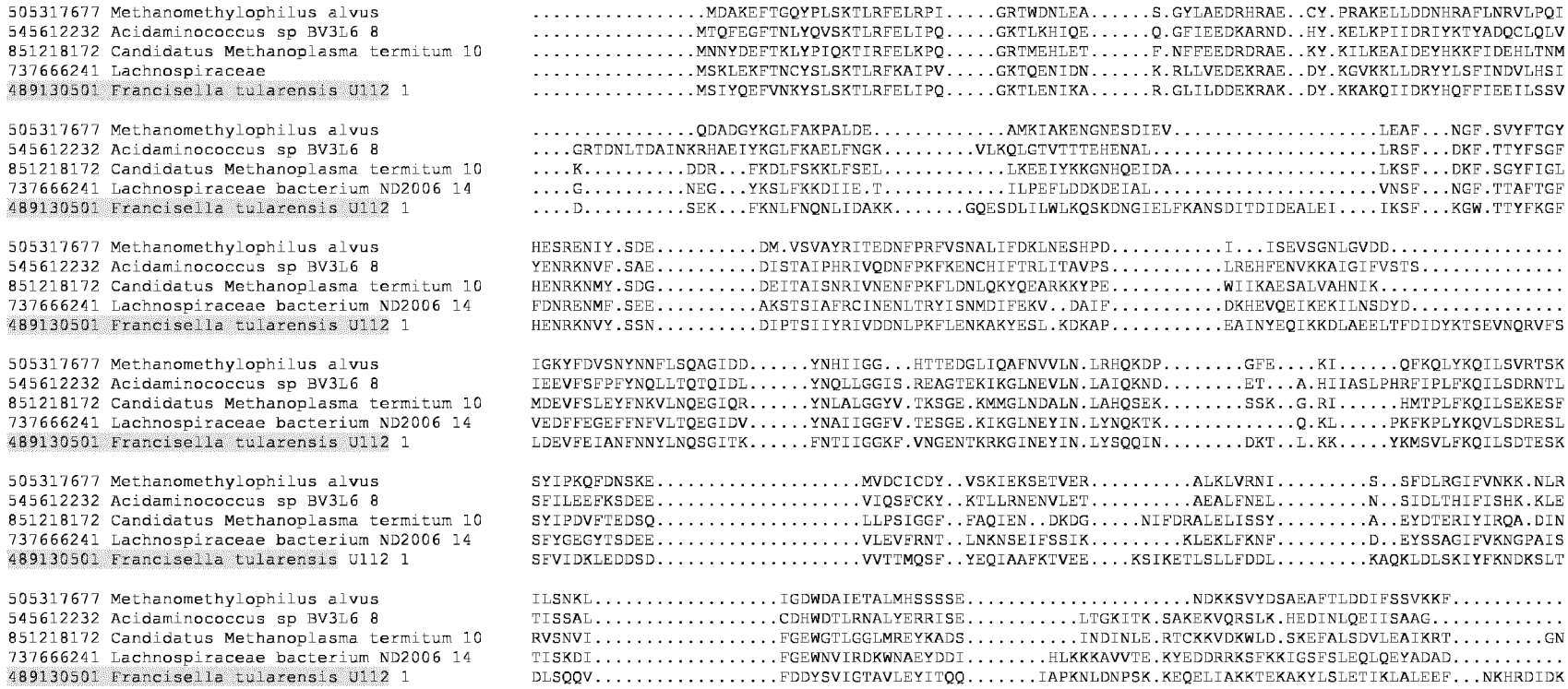
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Figure 3B

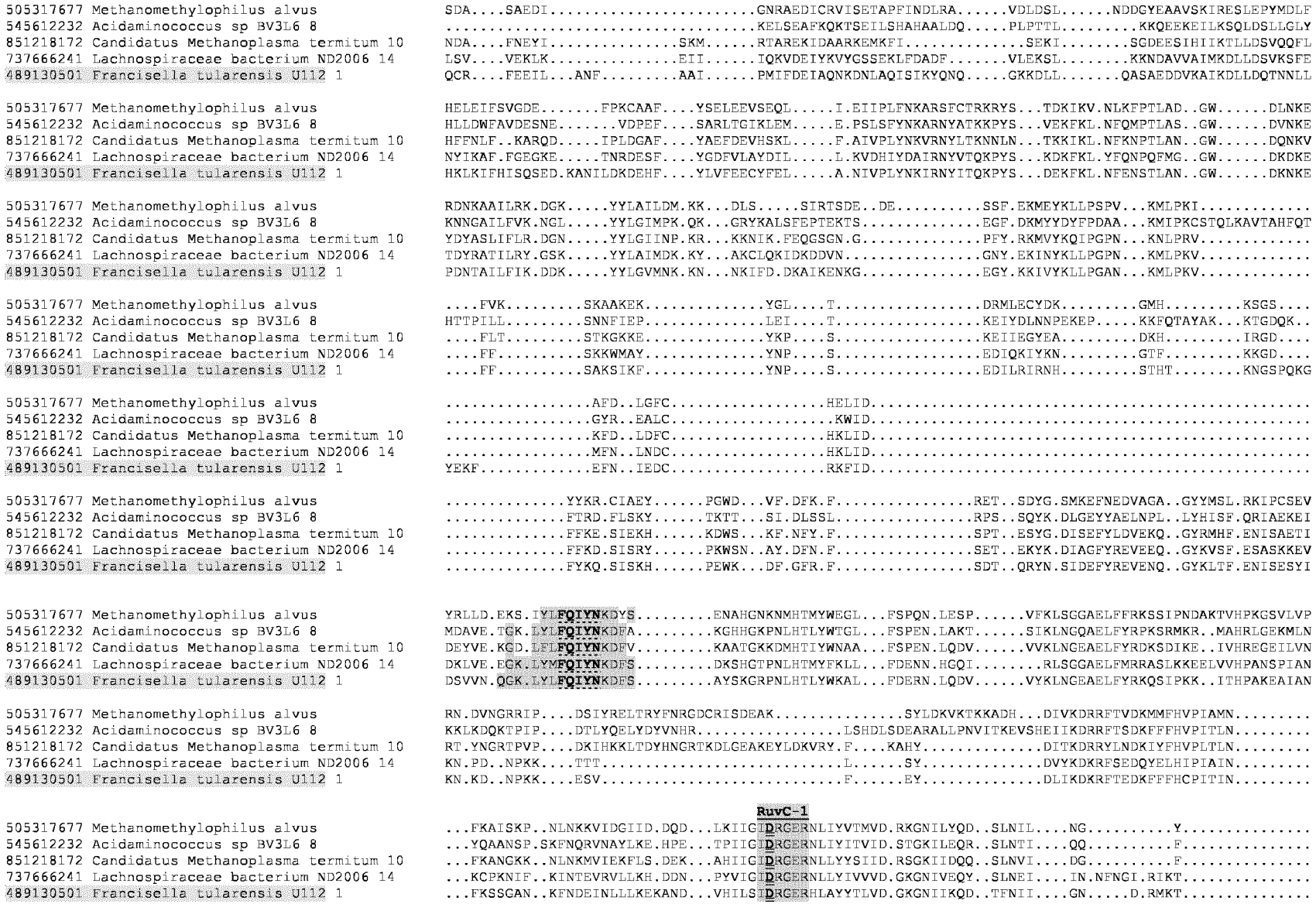
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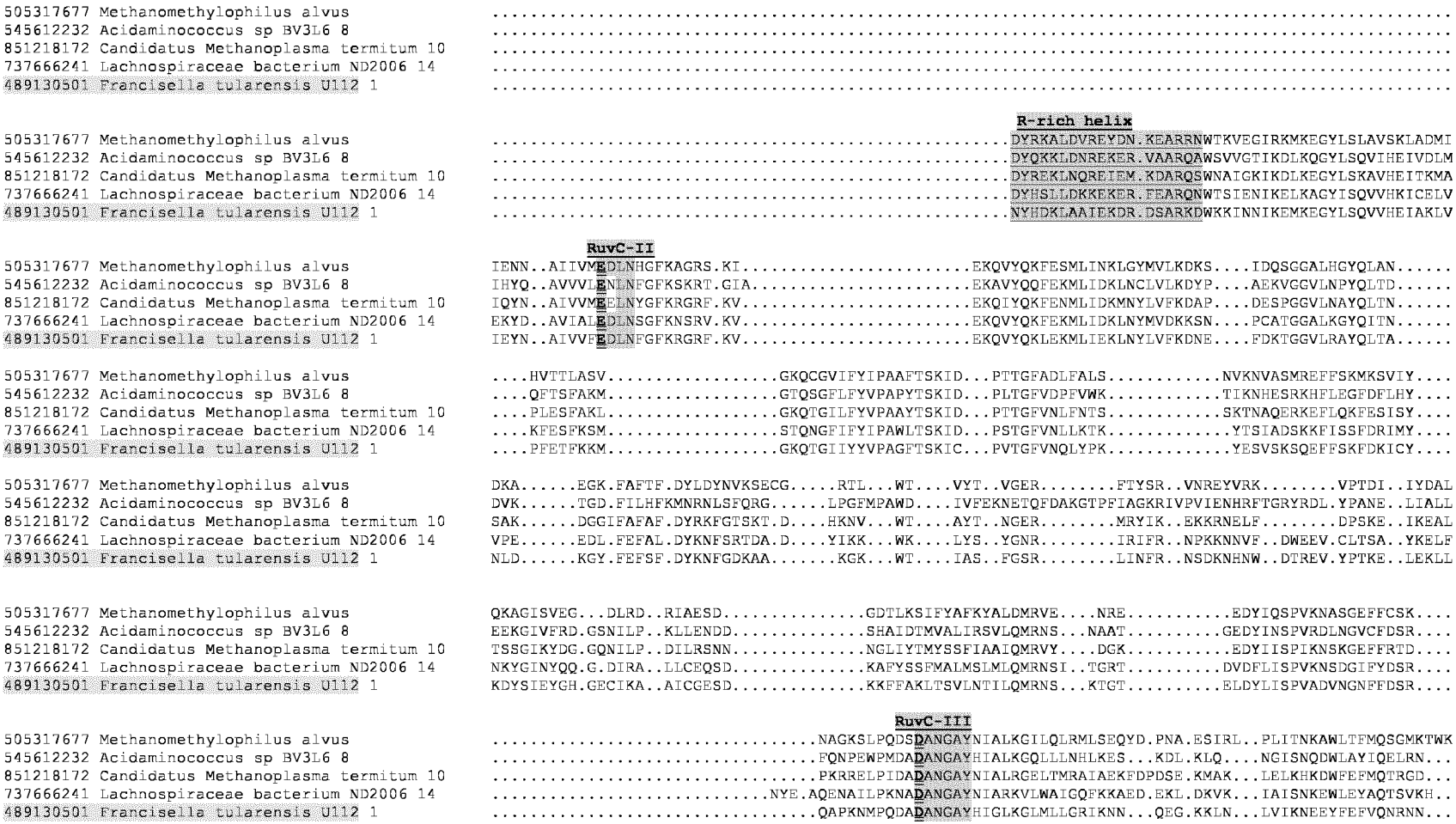
Figure 3C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Ser Ile Lys Glu Thr Leu Ser Leu Leu Phe Asp Asp Leu Lys Ala Gln
    370                 375                 380

Lys Leu Asp Leu Ser Lys Ile Tyr Phe Lys Asn Asp Lys Ser Leu Thr
385                 390                 395                 400

Asp Leu Ser Gln Gln Val Phe Asp Asp Tyr Ser Val Ile Gly Thr Ala
                405                 410                 415

Val Leu Glu Tyr Ile Thr Gln Gln Val Ala Pro Lys Asn Leu Asp Asn
            420                 425                 430

Pro Ser Lys Lys Glu Gln Xaa Leu Ile Ala Lys Lys Thr Glu Lys Ala
        435                 440                 445

Lys Tyr Leu Ser Leu Glu Thr Ile Lys Leu Ala Leu Glu Glu Phe Asn
    450                 455                 460

Lys His Arg Asp Ile Asp Lys Gln Cys Arg Phe Glu Glu Ile Leu Ala
465                 470                 475                 480

Asn Phe Ala Ala Ile Pro Met Ile Phe Asp Glu Ile Ala Gln Asn Lys
                485                 490                 495

Asp Asn Leu Ala Gln Ile Ser Xaa Lys Tyr Gln Asn Gln Gly Lys Lys
            500                 505                 510

Asp Leu Leu Gln Ala Ser Ala Glu Xaa Asp Val Lys Ala Ile Lys Asp
        515                 520                 525

Leu Leu Asp Gln Thr Asn Asn Leu Leu His Xaa Leu Lys Ile Phe His
    530                 535                 540

Ile Ser Gln Ser Glu Asp Lys Ala Asn Ile Leu Asp Lys Asp Glu His
545                 550                 555                 560

Phe Tyr Leu Val Phe Glu Glu Cys Tyr Phe Glu Leu Ala Asn Ile Val
                565                 570                 575

Pro Leu Tyr Asn Lys Ile Arg Asn Tyr Ile Thr Gln Lys Pro Tyr Ser
            580                 585                 590

Asp Glu Lys Phe Lys Leu Asn Phe Glu Asn Ser Thr Leu Ala Asn Gly
        595                 600                 605

Trp Asp Lys Asn Lys Glu Pro Asp Asn Thr Ala Ile Leu Phe Ile Lys
    610                 615                 620

Asp Asp Lys Tyr Tyr Leu Gly Val Met Asn Lys Lys Asn Asn Lys Ile
625                 630                 635                 640
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Phe Asp Asp Lys Ala Ile Lys Glu Asn Lys Gly Glu Gly Tyr Lys Lys
                645                 650                 655

Ile Val Tyr Lys Leu Leu Pro Gly Ala Asn Lys Met Leu Pro Lys Val
            660                 665                 670

Phe Phe Ser Ala Lys Ser Ile Lys Phe Tyr Asn Pro Ser Glu Asp Ile
        675                 680                 685

Leu Arg Ile Arg Asn His Ser Thr His Thr Lys Asn Gly Asn Pro Gln
    690                 695                 700

Lys Gly Tyr Glu Lys Phe Glu Phe Asn Ile Glu Asp Cys Arg Lys Phe
705                 710                 715                 720

Ile Asp Phe Tyr Lys Glu Ser Ile Ser Lys His Pro Glu Trp Lys Asp
                725                 730                 735

Phe Gly Phe Arg Phe Ser Asp Thr Gln Arg Tyr Asn Ser Ile Asp Glu
            740                 745                 750

Phe Tyr Arg Glu Val Glu Asn Gln Gly Tyr Lys Leu Thr Phe Glu Asn
        755                 760                 765

Ile Ser Glu Ser Tyr Ile Asp Ser Val Val Asn Gln Gly Lys Leu Tyr
    770                 775                 780

Leu Phe Gln Ile Tyr Asn Lys Asp Phe Ser Ala Tyr Ser Lys Gly Xaa
785                 790                 795                 800

Pro Asn Leu His Thr Leu Tyr Trp Lys Ala Leu Phe Asp Glu Arg Asn
                805                 810                 815

Leu Gln Asp Val Val Tyr Lys Leu Asn Gly Glu Ala Glu Leu Phe Tyr
            820                 825                 830

Arg Lys Gln Ser Ile Pro Lys Lys Ile Thr His Pro Ala Lys Glu Ala
        835                 840                 845

Ile Ala Asn Lys Asn Lys Asp Asn Pro Lys Lys Glu Ser Val Phe Glu
    850                 855                 860

Tyr Asp Leu Ile Lys Asp Lys Arg Phe Thr Glu Asp Lys Phe Phe Phe
865                 870                 875                 880

His Cys Pro Ile Thr Ile Asn Phe Lys Ser Ser Gly Ala Asn Lys Phe
                885                 890                 895

Asn Asp Glu Ile Asn Leu Leu Leu Lys Glu Lys Ala Asn Asp Val His
            900                 905                 910
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Ile Leu Ser Ile Asp Arg Gly Glu Arg His Leu Ala Tyr Tyr Thr Leu
        915                 920                 925

Val Asp Gly Lys Gly Asn Ile Ile Lys Gln Asp Thr Phe Asn Ile Ile
    930                 935                 940

Gly Asn Asp Arg Met Lys Thr Asn Tyr His Asp Lys Leu Ala Ala Ile
945                 950                 955                 960

Glu Lys Asp Arg Asp Ser Ala Arg Lys Asp Trp Lys Lys Ile Asn Asn
                965                 970                 975

Ile Lys Glu Met Lys Glu Gly Tyr Leu Ser Gln Val Val His Glu Ile
            980                 985                 990

Ala Lys Leu Val Ile Glu Tyr Asn  Ala Ile Val Val Phe  Glu Asp Leu
        995                 1000                1005

Asn Phe  Gly Phe Lys Arg Gly  Arg Phe Lys Val Glu  Lys Gln Val
    1010                1015                1020

Tyr Gln  Lys Leu Glu Lys Met  Leu Ile Glu Lys Leu  Asn Tyr Leu
    1025                1030                1035

Val Phe  Lys Asp Asn Glu Phe  Asp Lys Thr Gly Gly  Val Leu Arg
    1040                1045                1050

Ala Tyr  Gln Leu Thr Ala Pro  Phe Glu Thr Phe Lys  Lys Met Gly
    1055                1060                1065

Lys Gln  Thr Gly Ile Ile Tyr  Tyr Val Pro Ala Gly  Phe Thr Ser
    1070                1075                1080

Lys Ile  Cys Pro Val Thr Gly  Phe Val Asn Gln Leu  Tyr Pro Lys
    1085                1090                1095

Tyr Glu  Ser Val Ser Lys Ser  Gln Glu Phe Phe Ser  Lys Phe Asp
    1100                1105                1110

Lys Ile  Cys Tyr Asn Leu Asp  Lys Gly Tyr Phe Glu  Phe Ser Phe
    1115                1120                1125

Asp Tyr  Lys Asn Phe Gly Asp  Lys Ala Ala Lys Gly  Lys Trp Thr
    1130                1135                1140

Ile Ala  Ser Phe Gly Ser Arg  Leu Ile Asn Phe Arg  Asn Ser Asp
    1145                1150                1155

Lys Asn  His Asn Trp Asp Thr  Arg Glu Val Tyr Pro  Thr Lys Glu
    1160                1165                1170

Leu Glu  Lys Leu Leu Lys Asp  Tyr Ser Ile Glu Tyr  Gly His Gly
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    1175                1180                1185

Glu Cys  Ile Lys Ala Ala Ile  Cys Gly Glu Ser Asp  Lys Lys Phe
    1190                1195                1200

Phe Ala  Lys Leu Thr Ser Val  Leu Asn Thr Ile Leu  Gln Met Arg
    1205                1210                1215

Asn Ser  Lys Thr Gly Thr Glu  Leu Asp Tyr Leu Ile  Ser Pro Val
    1220                1225                1230

Ala Asp  Val Asn Gly Asn Phe  Phe Asp Ser Arg Gln  Ala Pro Lys
    1235                1240                1245

Asn Met  Pro Gln Asp Ala Asp  Ala Asn Gly Ala Tyr  His Ile Gly
    1250                1255                1260

Leu Lys  Gly Leu Met Leu Leu  Asp Arg Ile Lys Asn  Asn Gln Glu
    1265                1270                1275

Gly Lys  Lys Leu Asn Leu Val  Ile Lys Asn Glu Glu  Tyr Phe Glu
    1280                1285                1290

Phe Val  Gln Asn Arg Asn Asn  Ser Ser Lys Ile
    1295                1300
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