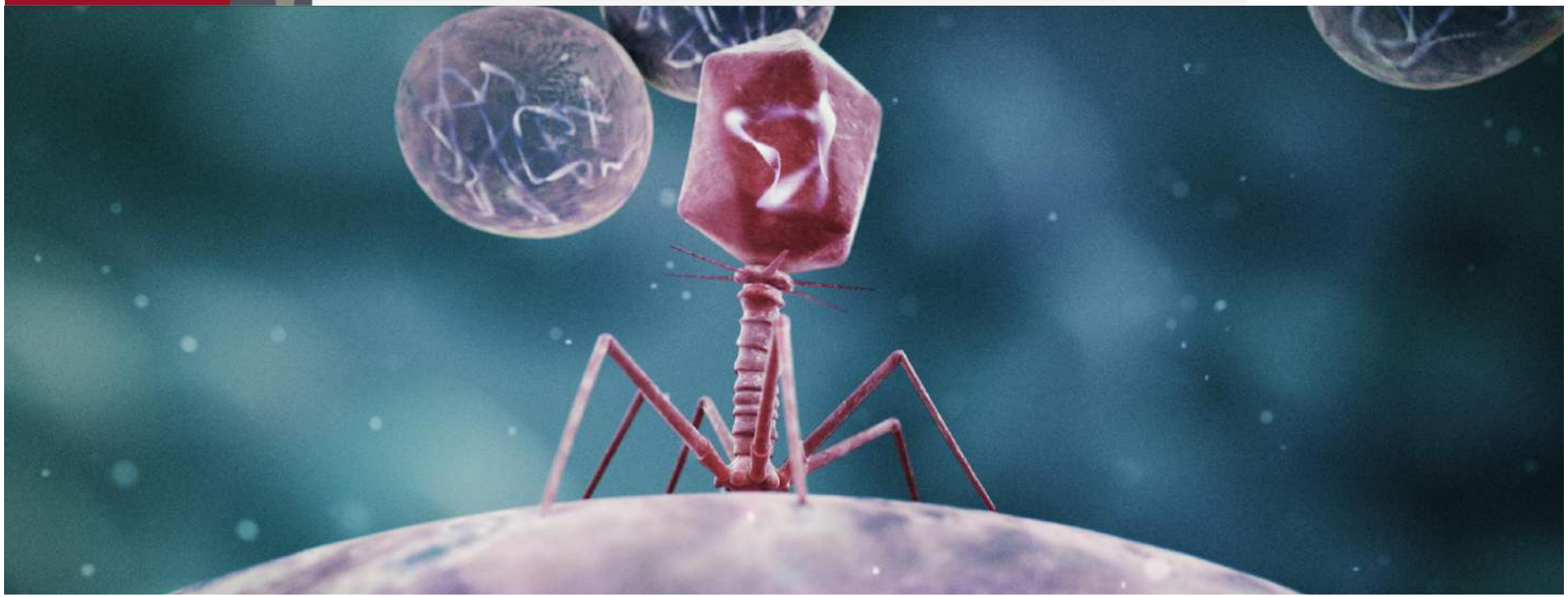
Discovery of CRISPR-Cas, the bacterial immune system

FROM FUNDAMENTAL RESEARCH TO INDUSTRIAL APPLICATIONS

Université de Bordeaux, 6 April 2016

Philippe HORVATH - philippe.horvath@dupont.com

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David Pujadas presenting the news on France 2 (8 March 2016)

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CRISPR-Cas9

L'enzyme qui révolutionne la génétique

L'ADN est devenu modifiable à volonté

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Génétique

CRISPR-Cas9 l'outil qui révolutionne la génétique

Emmanuelle Charpentier et Pierre Kaldy

Un système de défense immunitaire bactérien, CRISPR-Cas9, est devenu un outil précis, simple et universel pour modifier les gènes de n'importe quelle cellule à volonté. Un frein majeur de la génétique est levé.

Depuis 2012, un rêve des généticiens s'accomplit. Un mécanisme a été découvert chez les bactéries, qui permet de modifier à volonté le patrimoine génétique des organismes vivants. Jusqu'à présent, les chercheurs restaient assez démunis pour agir directement sur la séquence d'ADN, cette longue molécule qui code le développement et le fonctionnement des cellules et des organismes. C'était d'autant plus frustrant que, grâce aux progrès fulgurants des techniques de séquençage des génomes, le patrimoine génétique de dizaines d'espèces animales et végétales avait été décrypté. Pour obtenir une souris portant une mutation responsable d'une maladie génétique humaine, par exemple, il fallait des mois, voire des années. Avec le mécanisme bactérien CRISPR-Cas9, ce délai se trouve raccourci à quelques semaines. Pour la première fois, un accès direct, facile et précis à l'ADN contenu dans les cellules vivantes devient possible à un grand nombre de laboratoires. Pour découvrir ce nouvel outil, il suffisait de se pencher sur les bactéries, expertes en manipulation de l'ADN depuis des milliards d'années.

Les bactéries peuvent-elles être vaccinées ? Cette question peut paraître futile, mais elle a une grande importance pour l'industrie alimentaire, celle qui utilise des quantités massives de bactéries pour faire du fromage ou du yaourt. Les virus de bactéries – les bactériophages – sont nombreux, et beaucoup peuvent compromettre la production d'une usine en infectant

L'ESSENTIEL

- Un mécanisme de défense bactérien, des biologistes ont mis au point un outil pour modifier avec précision l'ADN de n'importe quelle cellule.
- Seuls deux ingrédients sont nécessaires pour couper l'ADN à l'endroit voulu : une enzyme, Cas9, et un petit ARN spécifique de la séquence à modifier.
- Recherche fondamentale, thérapie génique, agriculture, cette technique ouvre des horizons dans de nombreux domaines.

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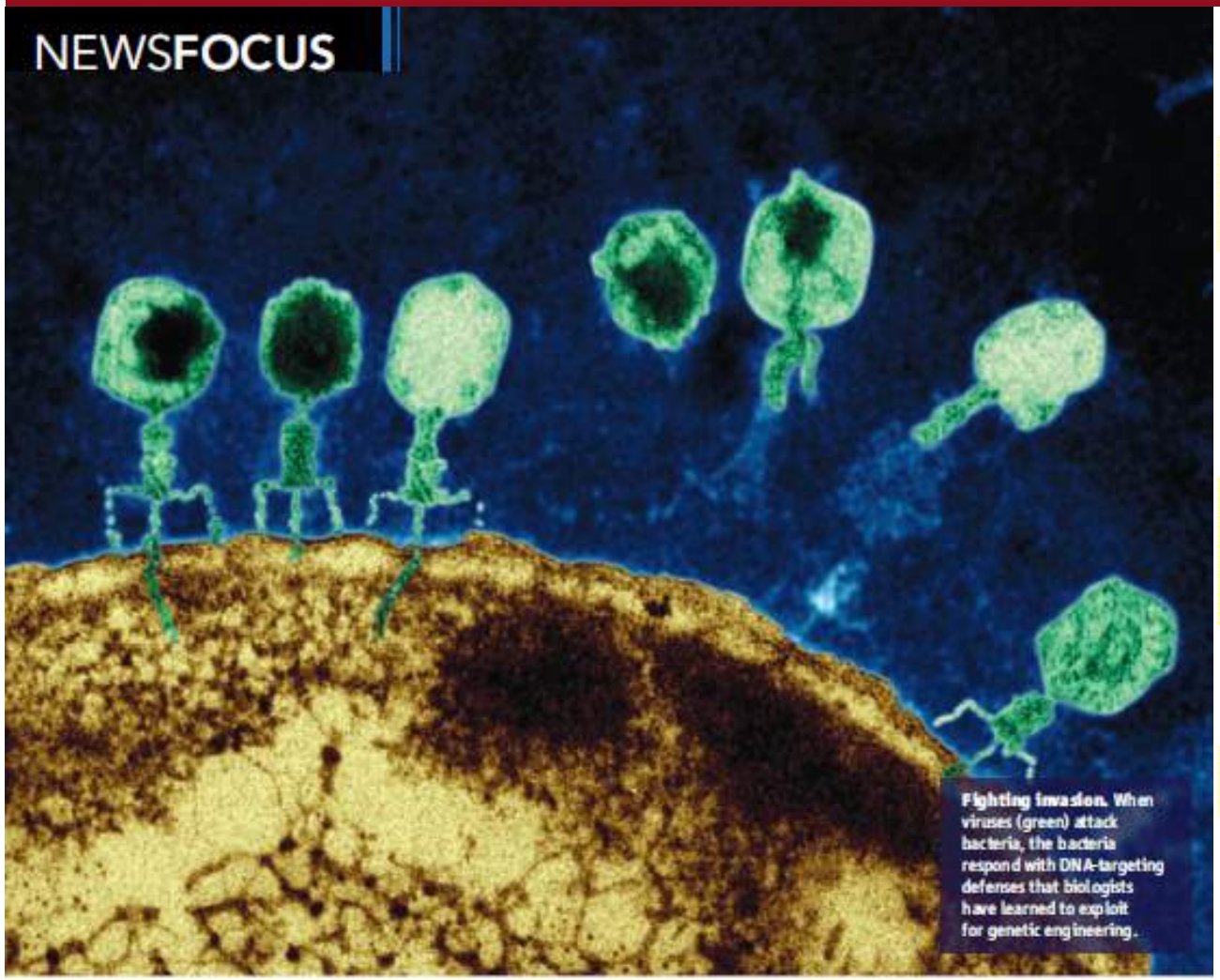

Fighting invasion. When viruses (green) attack bacteria, the bacteria respond with DNA-targeting defenses that biologists have learned to exploit for genetic engineering.

The CRISPR Craze

A bacterial immune system yields a potentially revolutionary genome-editing technique

BACTERIA MAY NOT ELICIT MUCH SYMPA-thy from us eukaryotes, but they, too, can get sick. That's potentially a big problem for the dairy industry, which often depends on bacteria such as *Streptococcus thermophilus* to make yogurts and cheeses. *S. thermophilus* breaks down the milk sugar lactose into tangy lactic acid. But certain viruses—bacteriophages, or simply phages—can debilitate the bacterium, wreaking havoc on the quality or quantity of the food it helps produce.

In 2007, scientists from Danisco, a Copenhagen-based food ingredient company now owned by DuPont, found a way to boost the phage defenses of this workhouse microbe. They exposed the bacterium to a phage and showed that this essentially vaccinated it against that virus (*Science*, 23 March 2007, p. 1650). The trick has enabled DuPont to create heartier bacterial strains for food production. It also revealed something fundamental: Bacteria have a kind of adaptive immune system, which enables them to fight off repeated attacks by specific phages.

That immune system has suddenly become important for more than food scientists and microbiologists, because of a valuable feature: It takes aim at specific DNA sequences.

What is CRISPR?

- Clustered
- Regularly
- Interspaced
- Short
- Palindromic
- Repeats

taagatattctcagacacctgataaggaactattacataaatttttagaaagtaaggatt
gacaaggacagttattgattttataatcactatgtgggtataaaaacgtcaaaatttcat
ttgag**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**tgtttgacagcaaatcaag
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tattggcacaacttaca**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**cgatttg
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cacttggcaggcttattactcaacagcga**GTTTTTGTACTCTCAAGATTTAAGTAACTGT**
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AACTGTACAACttcattcttccgttttgtttgcgaatcct**GTTTTTGTACTCTCAAGAT**
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TACTCTCAAGATTTAAGTAACTGTACAACataatgccgttgaattacacggcaaggtca**G**
TTTTTGTACTCTCAAGATTTAAGTAACTGTACAACgagcgagctcgaaataatcttaatt
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tgattaatacttactcg**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**aacacag
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gacacaagaacgtatgcaagagttcaag**GTTTTTGTACTCTCAAGATTTAAGTAACTGTA**
CAACacaattcttcatccggtaactgctcaagtg**GTTTTTGTACTCTCAAGATTTAAGTA**
ACTGTACAACaattaagggcatagaaagggagacaacatg**GTTTTTGTACTCTCAAGATT**
TAAGTAACTGTACAACcgatatttaaaatcatttcataacttcat**GTTTTTGTACTCTC**
AAGATTTAAGTAACTGTACAACgcagtatcagcaagcaagctgttagttact**GTTTTTGT**
ACTCTCAAGATTTAAGTAACTGTACAACataaactatgaaatttttataatttttaaga**GT**
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aactaacaattatgct**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**aatcgttc
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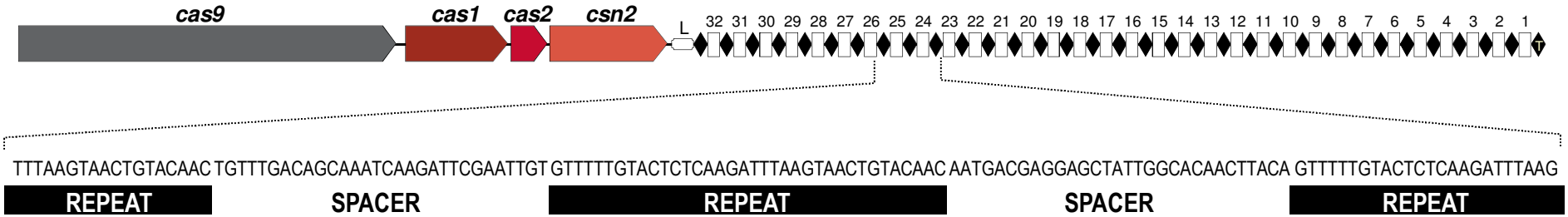
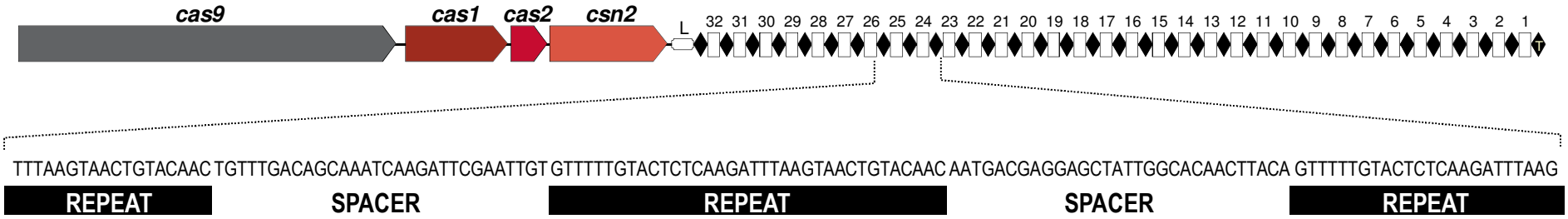


CRISPR basics

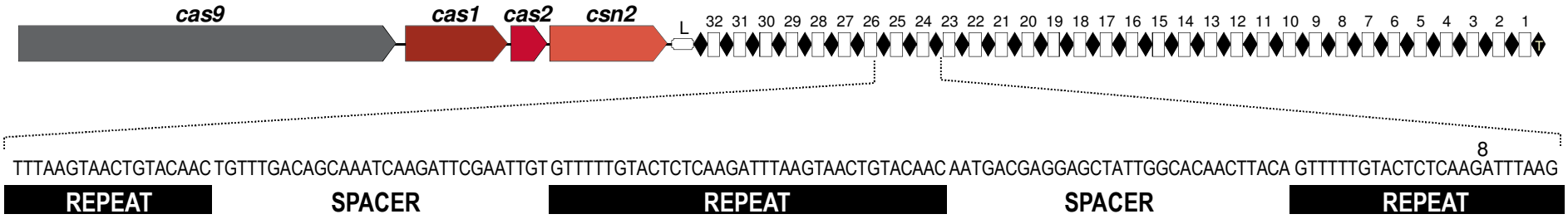
- **CRISPR**: Clustered Regularly Interspaced Short Palindromic Repeats
- Former designations:
  - **DVR** : Direct Variable Repeats | **DR** : Direct Repeats (1993)
  - **TREP** : Tandem REPeats (1995)
  - **LTRR** : Long Tandemly Repeated Repetitive (1996)
  - **SRSR** : Short Regularly Spaced Repeats (2000)
  - **LCTR** : Large Clusters of Tandem Repeats (2001)
  - **SPIDR** : SPacer Interspersed Direct Repeats (2002)
- Family of non-contiguous DNA repeats, separated by "spacers"
- Initially discovered in *E. coli* (1987)
- Found in Bacteria (~48%) and Archaea (~85%)
- Up to: 18 clusters per genome
  588 repeats within a cluster
  815 repeats within a single genome (*Haliangium ochraceum*)



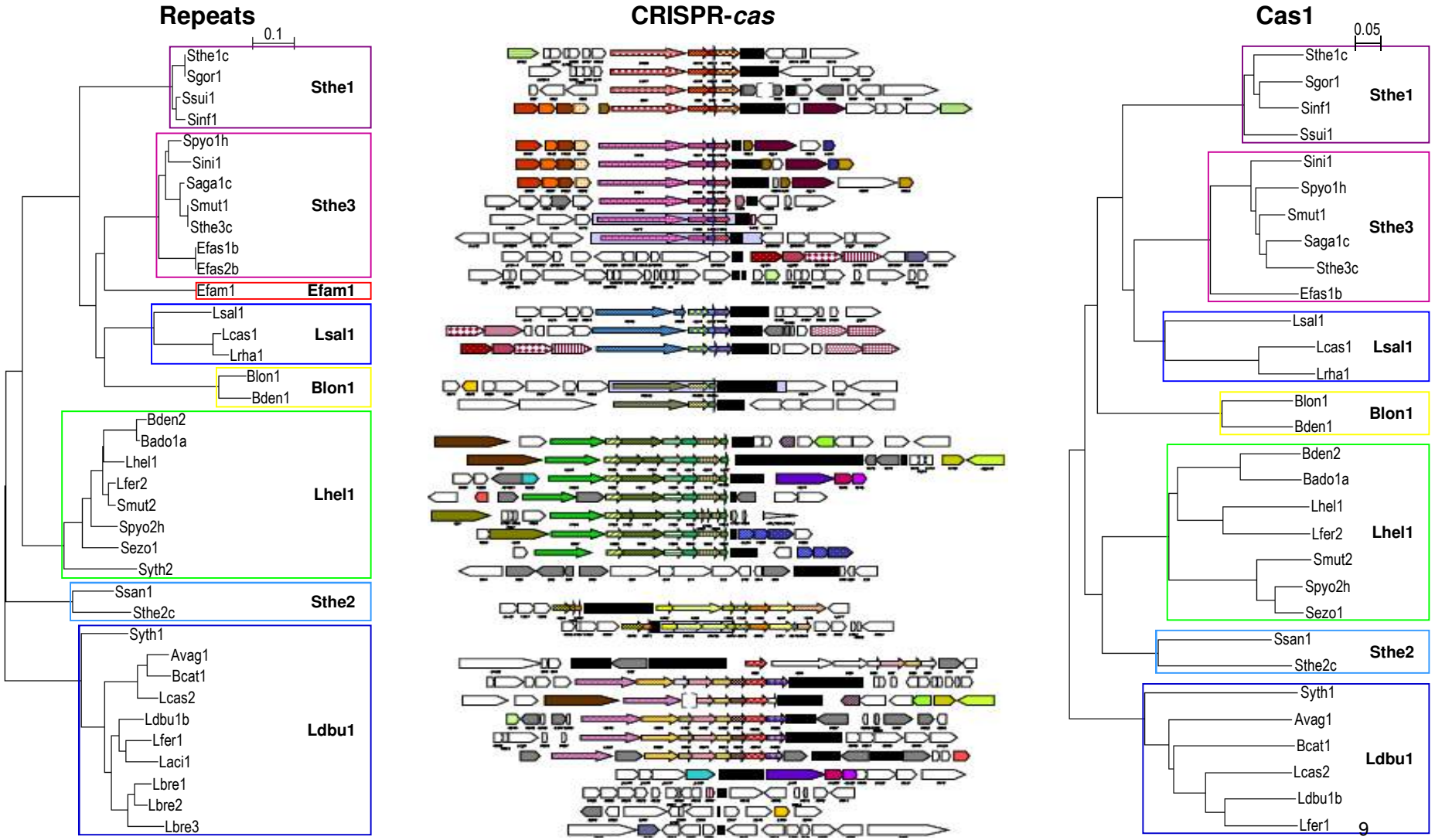
CRISPR basics

- **Cas**: CRISPR-associated sequences
  - Vague definition for a large set of extremely different gene families
  - Many Cas proteins bear domains involved in nucleotide interaction (DNA/RNA-binding): helicases, endo/exo-nucleases, polymerases, transcriptional regulators…
  - Structure and function determined for numerous Cas proteins and complexes
  - Congruence between Cas and CRISPR repeats → "CRISPR-Cas system"

- **Leader**: A/T-rich sequence located upstream of the CRISPR array (12 to >300 bp)
  - Sometimes conserved among distinct arrays of the same system, within a genome
  - Contains a promoter for the transcription of the array into RNA ("pre-crRNA")
  - Transcription level is generally higher at the leader end



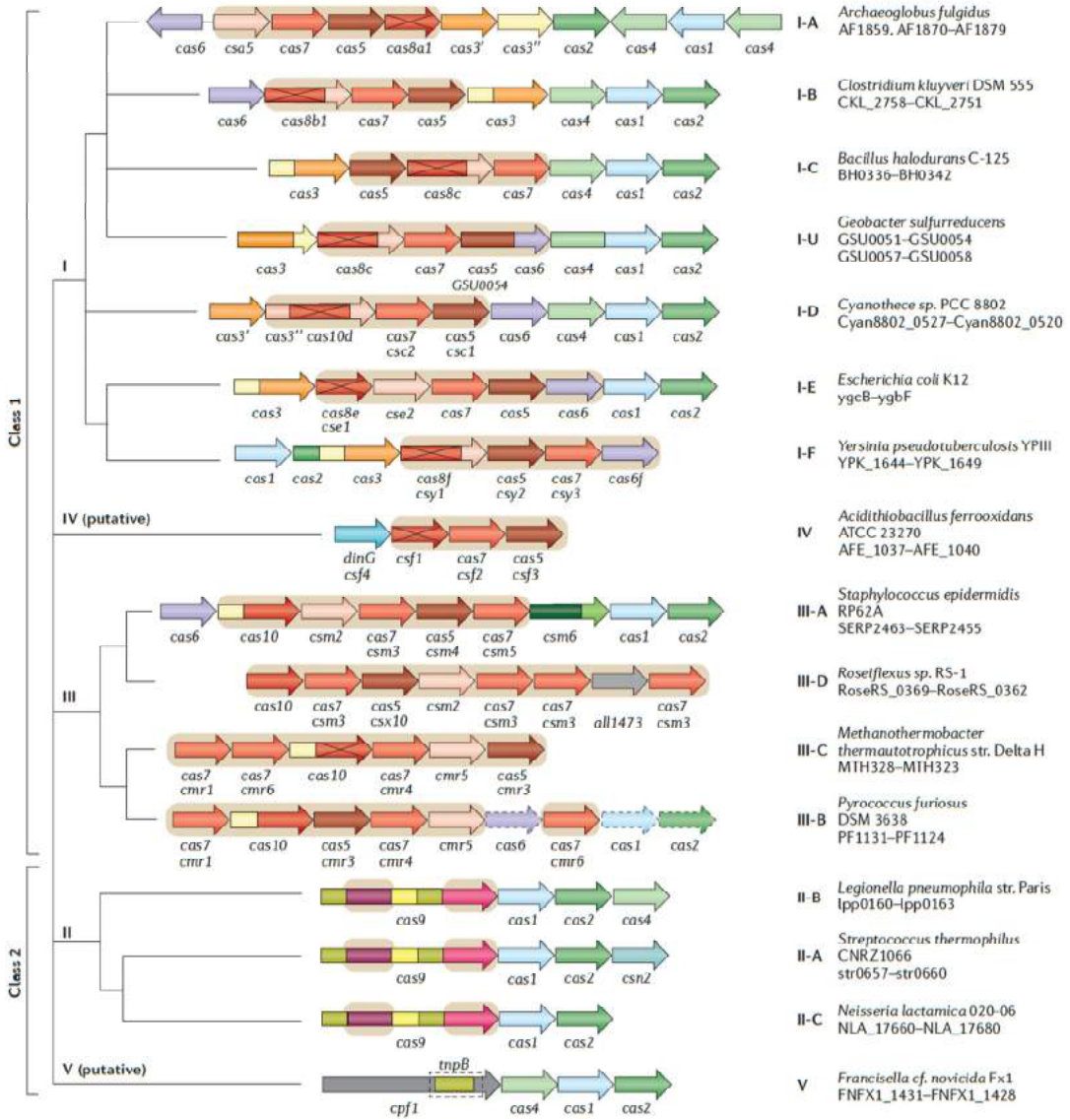
Congruence between Cas and CRISPR repeats



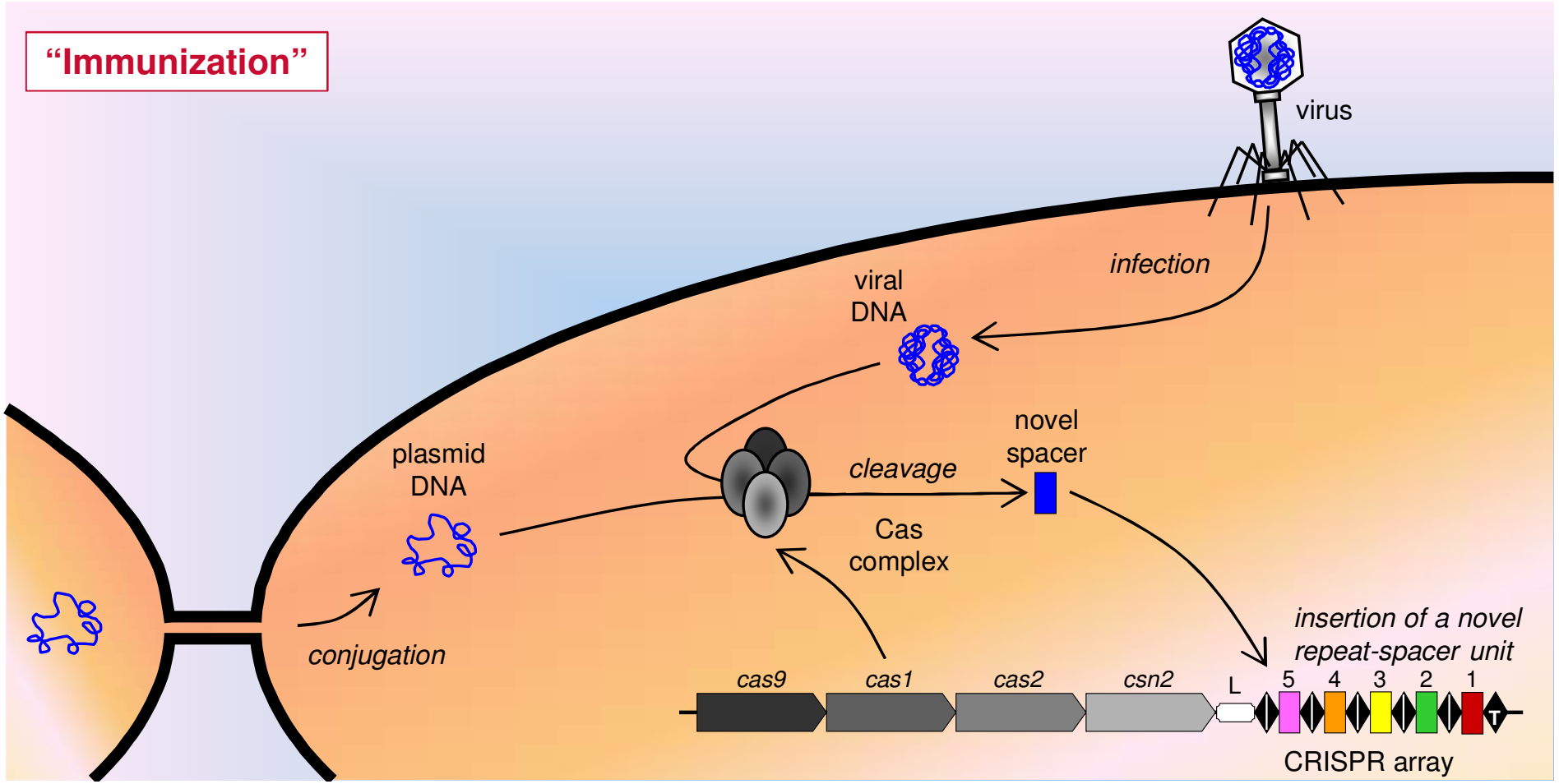
Horvath *et al.*, 2009

CRISPR-Cas systems

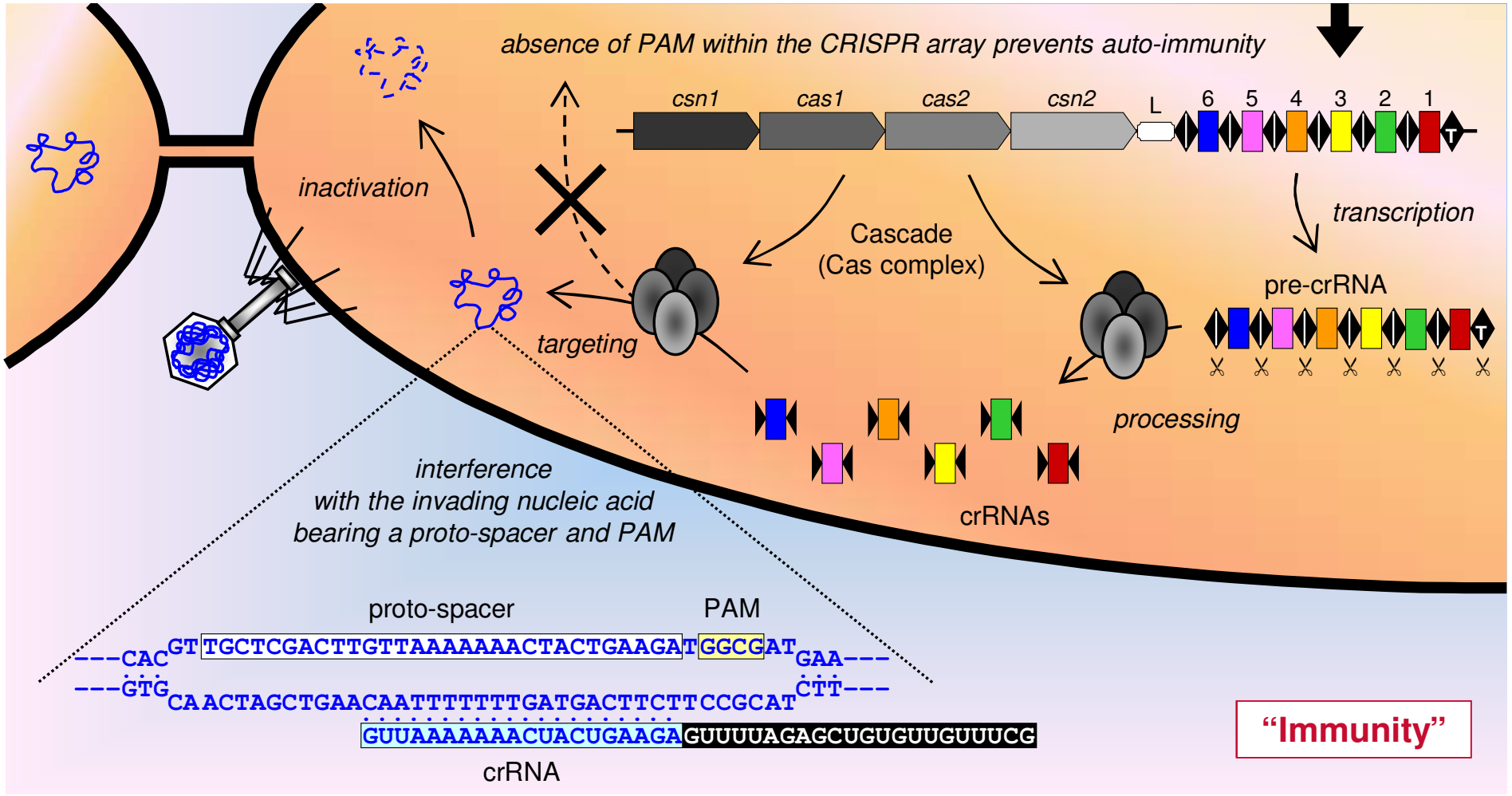
- High variability in *cas* gene content and organization
- Complex classification, revised multiple times
- Simplified into:
  - 2 main **classes**
  - 5 **types**
  - Several **subtypes** identified by signature genes
- *cas1–cas2* almost "universal"
- CRISPR-Cas systems are **strain-specific features**



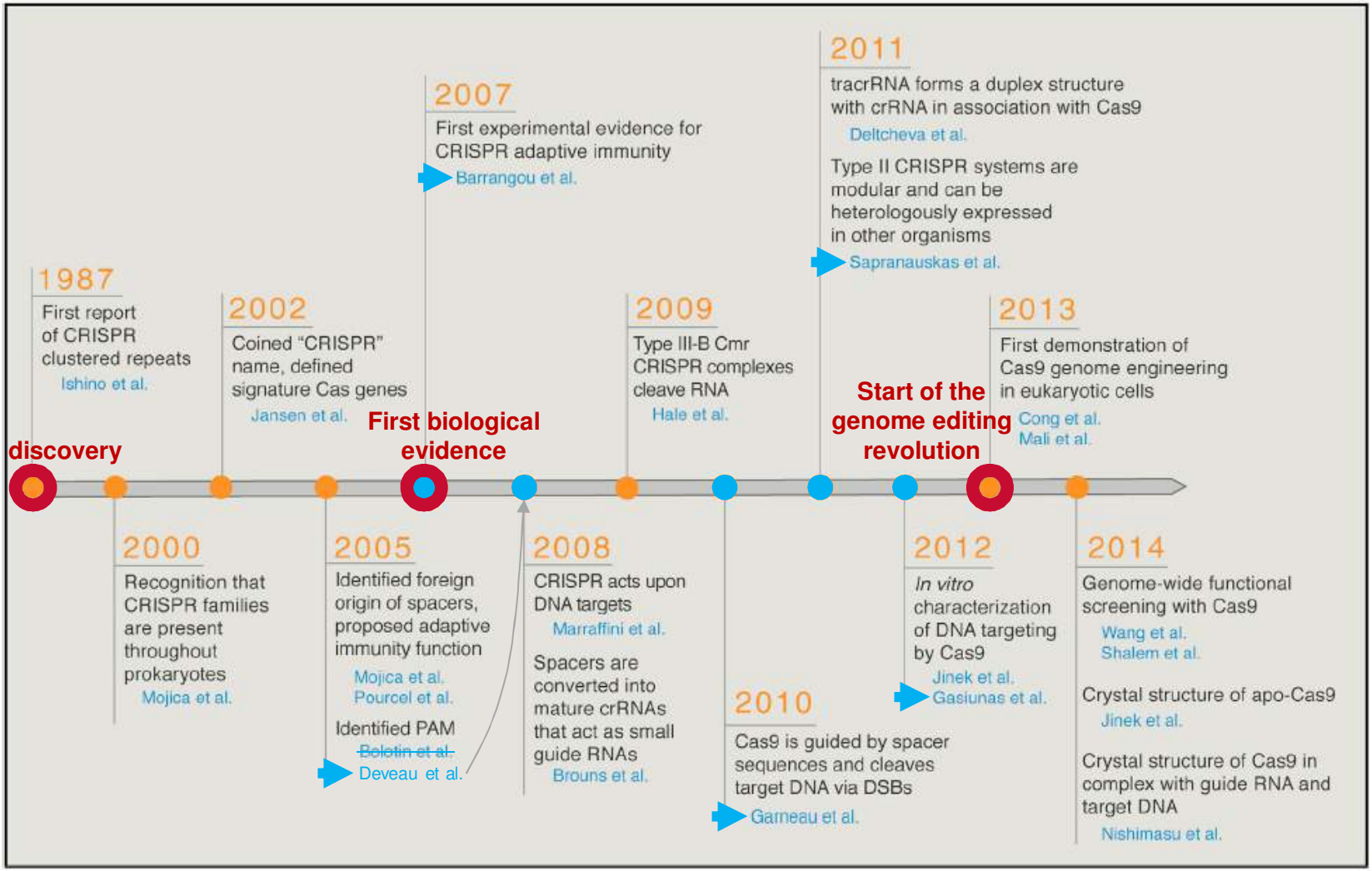
CRISPR mechanism of action – Adaptation



CRISPR mechanism of action – Interference



CRISPR timeline



Adapted from Hsu *et al.*, 2014, *Cell*

CRISPR ("SPIDR") as a new family of DNA repeats (2002)

Identification of a Novel Family of Sequence Repeats among Prokaryotes

RUND JANSEN,[1] JAM D.A. VAN EMBDEN,[2] WIM GAASTRA,[1] and LEO M. SCHOULS[2]

Identification of genes that are associated with DNA repeats in prokaryotes

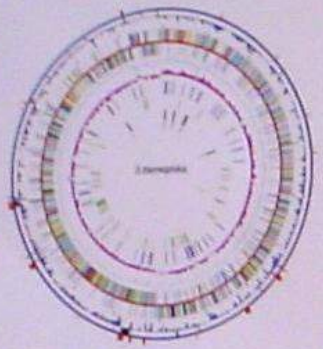
Ruud. Jansen,[1*] Jan. D. A. van Embden,[2] Wim. Gaastra[1] and Leo. M. Schouls[2]

Molecular Microbiology (2002) 43(6), 1565–1575

CRISPR ("SPIDR") in *S. thermophilus*

- **Poster presented by INRA** at the 7th Symposium on Lactic Acid Bacteria (The Netherlands, 1-5 September 2002)

Integrated Genomics



INRA

Multilocus characterization of different strains of *Streptococcus thermophilus* *Streptococcus vestibularis* *Streptococcus salivarius*

Alexander Bolotin[1,3], Benois Quinquis[1], Pierre Renault[1], Alexei Sorokin[1], Dusko S.Ehrlich[1]

Eugene Goltsman[2], Mikhail Mazur[2], Alla Lapidus[2], Michail Fonstein[2]

[1] Genetique Microbienne, INRA, Domaine de Vilvert, Jouy en Josas, France
[2] Integrated Genomics Inc. Chicago, USA
[3] e-mail: bolotine@jouy.inra.fr

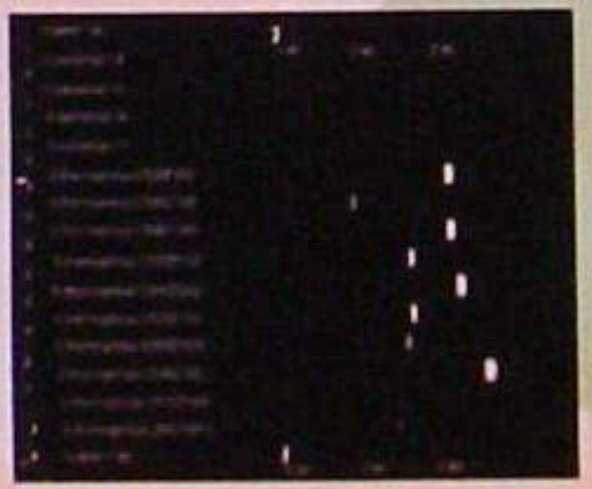
Complete genome of *Streptococcus thermophilus* CNRZ1066 was assembled using 25000 random reads and 1600 sequences of multiplex PCR generated substrates. Analysis of the genome revealed about hundred genes present as truncated ORFs when compared with the orthologs in other completely sequenced *Streptococci*. Among these are genes involved in sugar transport and utilization, central carbon metabolism, protein secretion, hydrolysis of polymers. Comparison of a representative set of such genes from different strains of *Streptococcus salivarius Streptococcus vestibularis* and *Streptococcus thermophilus* is presented. We also show a repeated region which can be used for strain differentiation.

CRISPR ("SPIDR") in *S. thermophilus*

- **Poster presented by INRA** at the 7th Symposium on Lactic Acid Bacteria (The Netherlands, 1-5 September 2002)

4.
Two *S. thermophilus* complete genomes contain ~3 kb size locus consisting on a battery of highly homologous 39-40 bp direct repeats (42 and 34 respectively for CNRZ1066 and LMG18311) separated by nonrepetitive DNA spacers (NS) of similar size. Similar structures were described for other genomes and were designed as SPacers Interspersed Direct Repeats (SPIDR) or DVR (direct variant repeats) [Jansen R et al. (2002) OMICS, 26(1) 23-33]. SPIDR locus was present in all analyzed *S.thermophilus* strains showing high polymorphism of NS sequences and numbers of DR that varied between 15 and 44 (Fig.3). Selected primers are specific for *S.thermophilus* and can be used for strain typing and discriminative analysis.

Fig 3. SPIDR locus specific PCR test
S.thermophilus strains are easily discriminated from evolutionary closed *Streptococcus* and can be identified using PCR analysis with SPIDR locus specific primers



First *S. thermophilus* genome

- **"Lactic Acid Bacteria"** genome project
- 10 LAB species sequenced by the Joint Genome Institute (DOE)

- *S. thermophilus* LMD-9, an industrially-relevant strain provided by Danisco
- Draft genome released in Sept. 2002

- Identification of **CRISPR arrays**
- Design of PCR primers
- **CRISPR-based genotyping** of collection strains

JGI Microbes — *Streptococcus thermophilus* — DRAFT GENOME

BLAST | Download | Annotation | Info | Home | HELP!

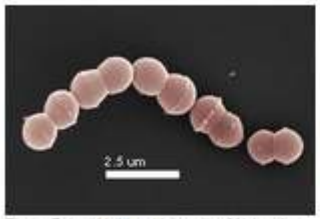


Photo: Robert Hutkins, University of Nebraska

Streptococcus thermophilus was once described as a bacterium "marked more by the things which it cannot do than by it's positive actions" (Sherman, 1937). Although it may be certainly be true that *S. thermophilus* is physiologically and biochemically less versatile than other lactic acid bacteria, the reality is that this organism can actually "do" quite a bit. In fact, research during the past two decades has revealed that *S. thermophilus* has properties that make it one of the most commercially important of all lactic acid bacteria.

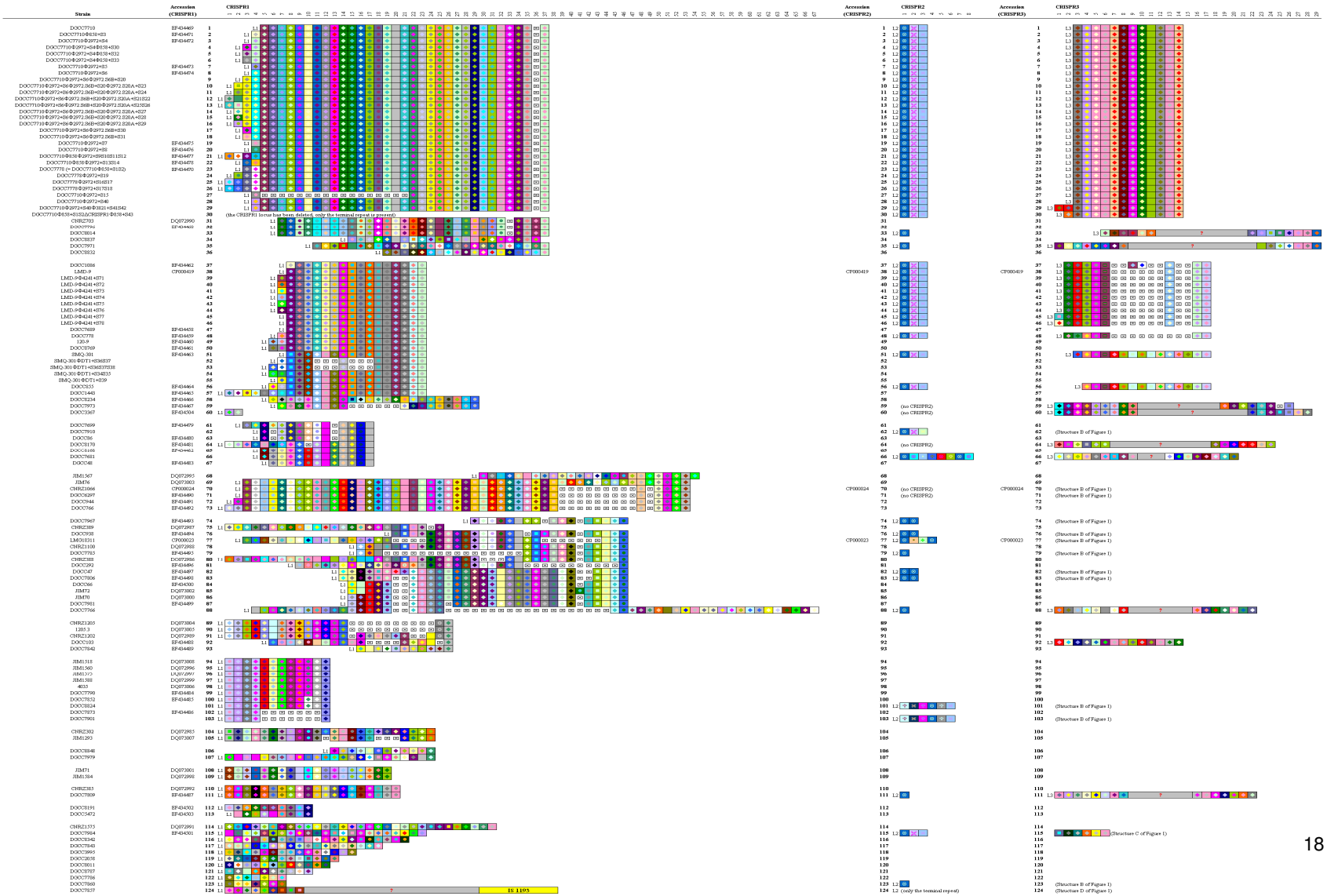
Streptococcus thermophilus is used, along with *Lactobacillus* spp., as a starter culture for the manufacture of several important fermented dairy foods, including yogurt and Mozzarella cheese. Its use has increased significantly during the past two decades, as a result of the tremendous increase in consumption of these products. According to USDA statistics, in 1998, more than 2.24 billion pounds Mozzarella cheese and 1.37 billion pounds of yogurt were produced, respectively, with a combined economic value of nearly $5 billion.

The substantial increase in production of Mozzarella cheese and yogurt have led not only to increased use of *S. thermophilus* cultures, but also to new demands on their performance and production requirements. Industrial strains, for example, should be insensitive to bacteriophage, have stabile fermentation characteristics, and produce products having consistent flavor and texture properties. Although research on the physiology of *S. thermophilus* has revealed important information on some of these properties, including sugar and protein metabolism, polysaccharide production, and flavor generation, only recently has the genetic basis for many of these traits been determined. Clearly, future efforts aimed at improving this important industrial strain will require information that can only be obtained by genome analysis.

Currently, several traits in *S. thermophilus* have been targeted for strain improvement programs (Delcour et al., 2000). Since bacteriophage are responsible for considerable economic losses during cheese manufacture, efforts are underway to engineer restriction and other phage resistance systems into commercial strains. Enhancing stability and expression of exopolysaccharides that act as natural thickening agents has also attracted significant attention. Finally, *S. thermophilus* has an important role as a probiotic, alleviating symptoms of lactose intolerance and other gastrointestinal disorders.

The genome of *S. thermophilus* is 1.8 Mb, making it among the smallest genomes of all lactic acid bacteria. Although a moderate thermophile, it is phylogenetically related to the more mesophilic lactococci and has a comparable low G+C ratio (40%). Genes coding for metabolic pathways involved in sugar catabolism (Poolman et al., 1989; Vaughan et al., 2001), protein and peptide utilization (Fernandez-Espla et al., 2000; Garault et al., 2002), polysaccharide production (Almirón-Roig et al., 2000), the stress response system (Perrin et al., 1999), and phage resistance mechanisms (Burrus, 2001; Solow and Somkuti, 2000) have been sequenced and characterized. More than 100 DNA sequence entries are currently listed in GenBank. Although most strains do not harbor plasmids, other mobile elements have been reported (Guedon et. al., 1995), and techniques for gene transfer and mutagenesis have been developed (Baccigalupi et al., 2000; Coderre and Somkuti, 1999). A genome sequencing project using an industrially-relevant strain will undoubtedly reveal valuable information that could have substantial impact on agriculture, the food industry, and the consuming public.

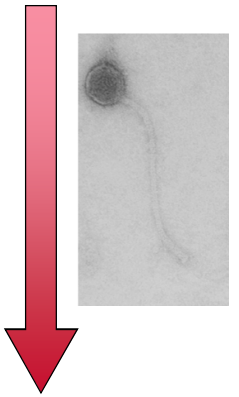
An outstanding spacer polymorphism

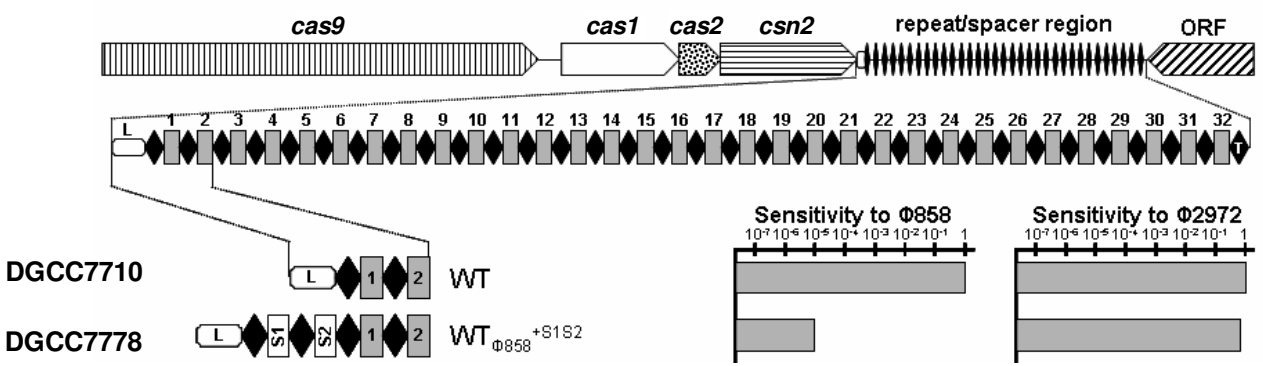


Horvath *et al.*, 2008, *J. Bacteriol.*

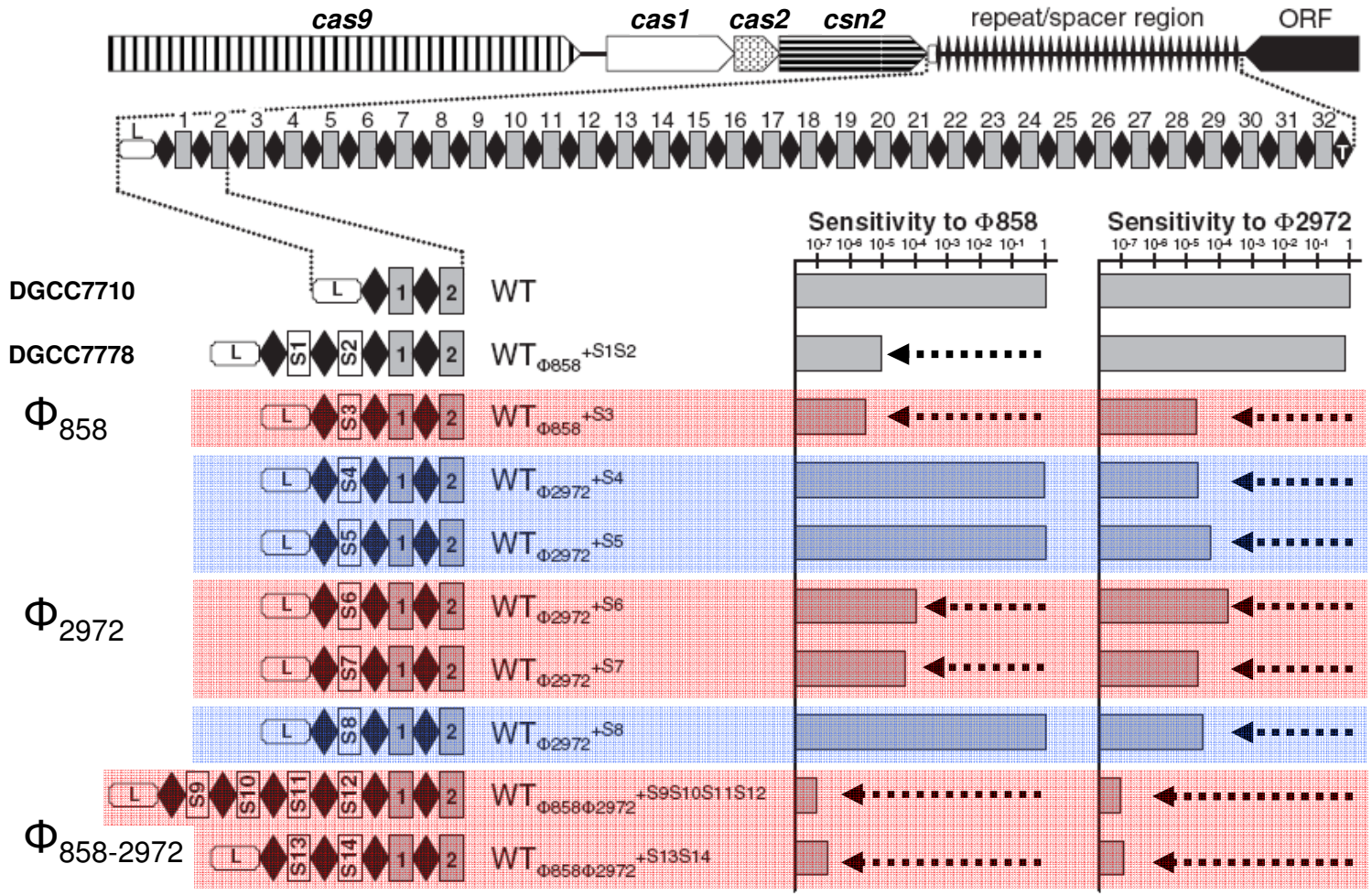
First evidence that novel CRISPR spacers provide phage resistance

- Strain **DGCC7710** isolated in 1985, industrialized in 1989
- In April 1990, isolation of a **virulent phage** against DGCC7710, **named 858** (sequenced in 2006)
- In 1990, after challenge of DGCC7710 with phage 858, selection of a BIM (Bacteriophage Insensitive Mutant) named **DGCC7778**
- In 1999, isolation of a **virulent phage** against DGCC7778 and DGCC7710, **named 2972** (sequenced in 2004)





Reproduction of challenges with phages 858 and/or 2972



Emergence of the anti-viral hypothesis (2005)

Intervening Sequences of Regularly Spaced Prokaryotic Repeats Derive from Foreign Genetic Elements

Francisco J.M. Mojica, César Díez-Villaseñor, Jesús García-Martínez, Elena Soria

CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies

C. Pourcel,[1] G. Salvignol[1] and G. Vergnaud[1,2]

Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin

Alexander Bolotin, Benoit Quinquis, Alexei Sorokin and S. Dusko Ehrlich

First biological evidence published in *Science* (2007)

CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes

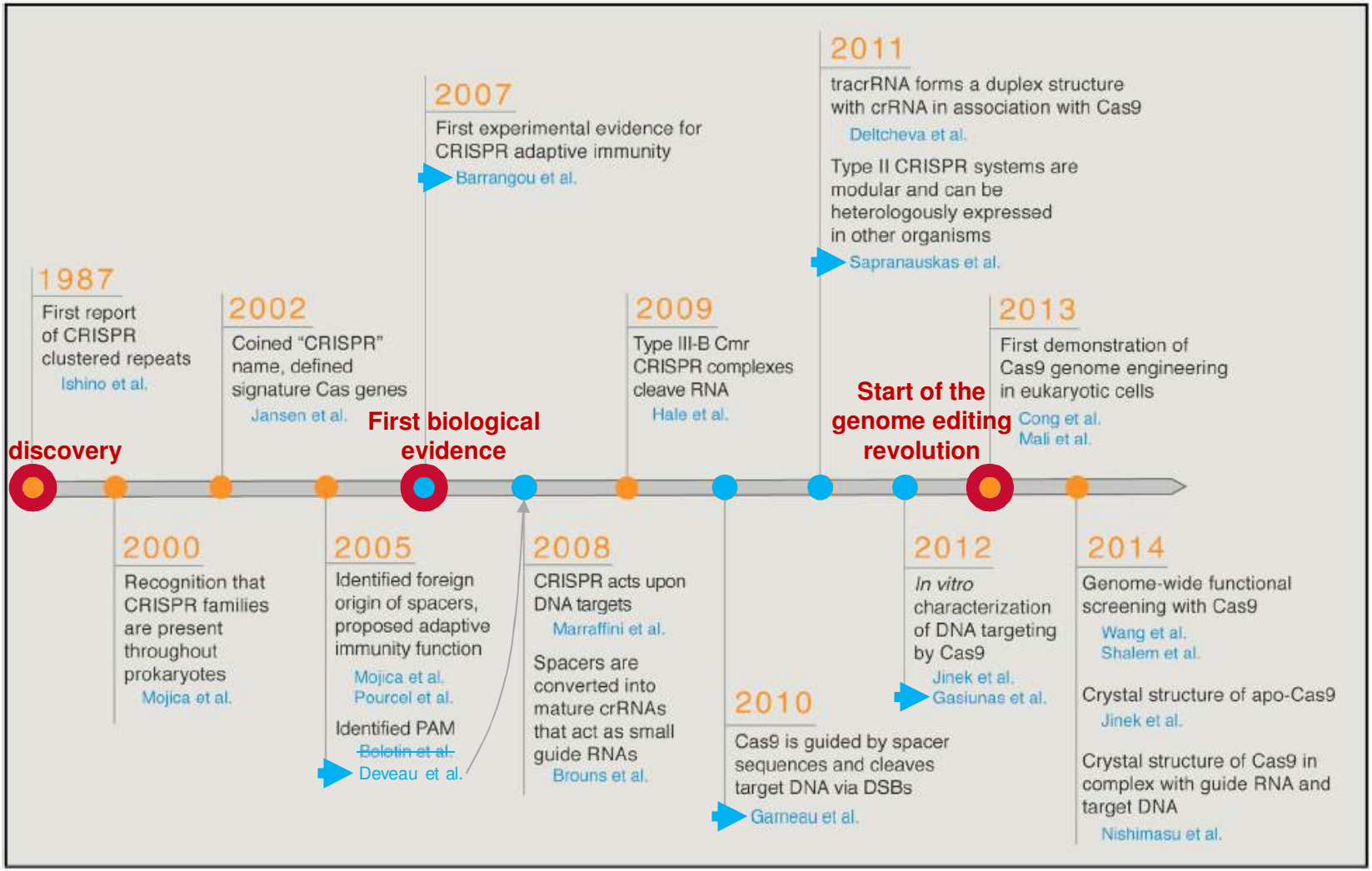
Rodolphe Barrangou,[1] Christophe Fremaux,[2] Hélène Deveau,[3] Melissa Richards,[1] Patrick Boyaval,[2] Sylvain Moineau,[3] Dennis A. Romero,[1] Philippe Horvath[2]*

Clustered regularly interspaced short palindromic repeats (CRISPR) are a distinctive feature of the genomes of most Bacteria and Archaea and are thought to be involved in resistance to bacteriophages. We found that, after viral challenge, bacteria integrated new spacers derived from phage genomic sequences. Removal or addition of particular spacers modified the phage-resistance phenotype of the cell. Thus, CRISPR, together with associated *cas* genes, provided resistance against phages, and resistance specificity is determined by spacer-phage sequence similarity.

SCIENCE VOL 315 23 MARCH 2007

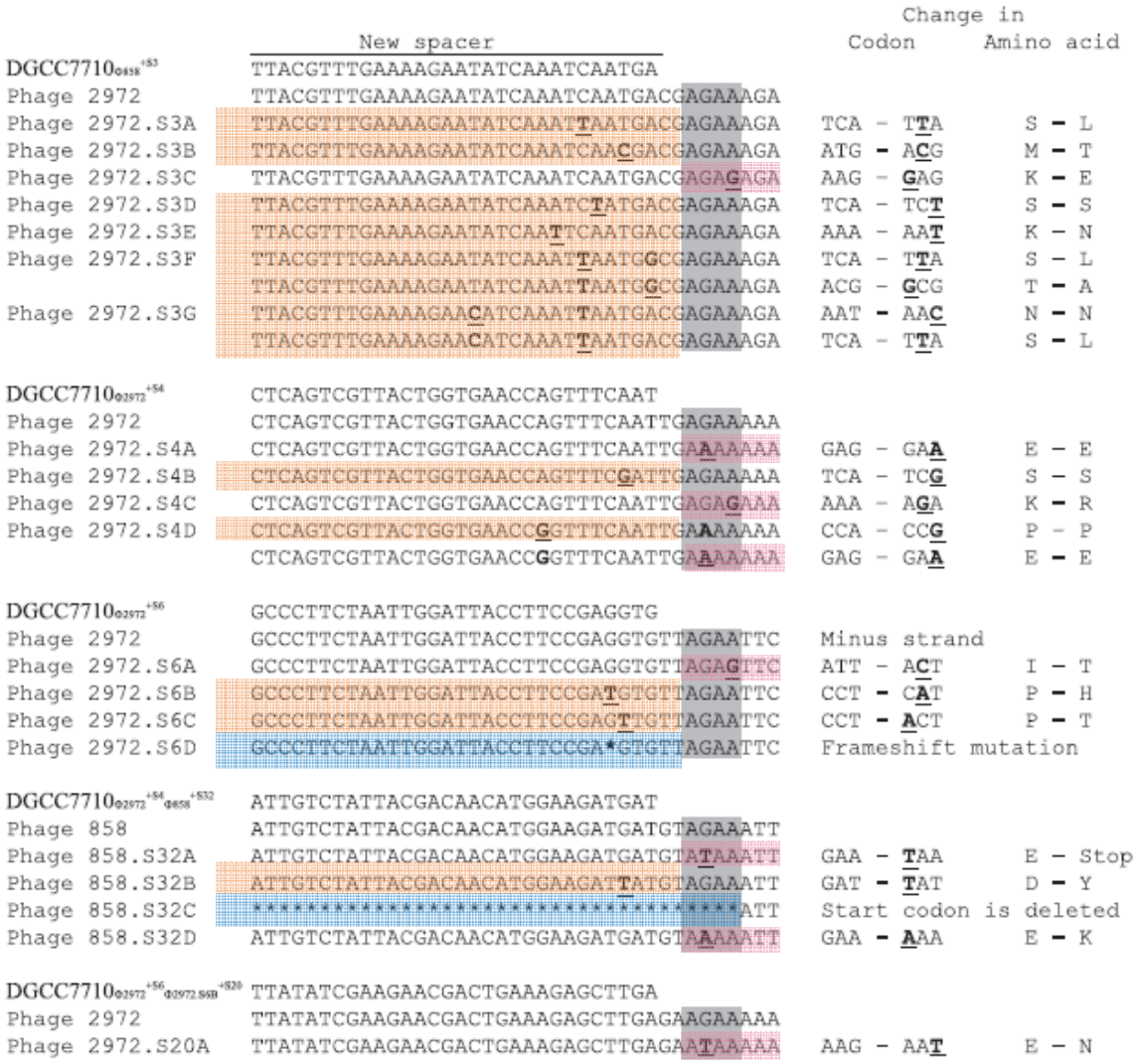
- Gain of phage resistance when spacers are acquired, loss when removed
- Exchanges of spacers switch phage resistance profiles
- **Role of *cas* genes**, notably *cas9* (aka *cas5*, or *csn1*), in phage resistance
- **Most highly cited article** in the CRISPR field until March 2015

CRISPR timeline

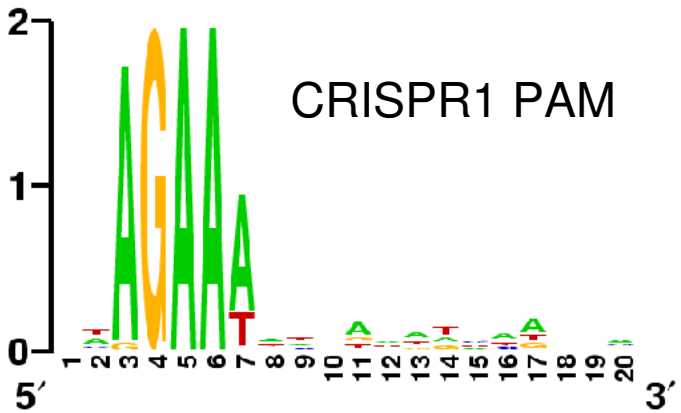


Adapted from Hsu *et al.*, 2014, *Cell*

The Proto-spacer Adjacent Motif (PAM)



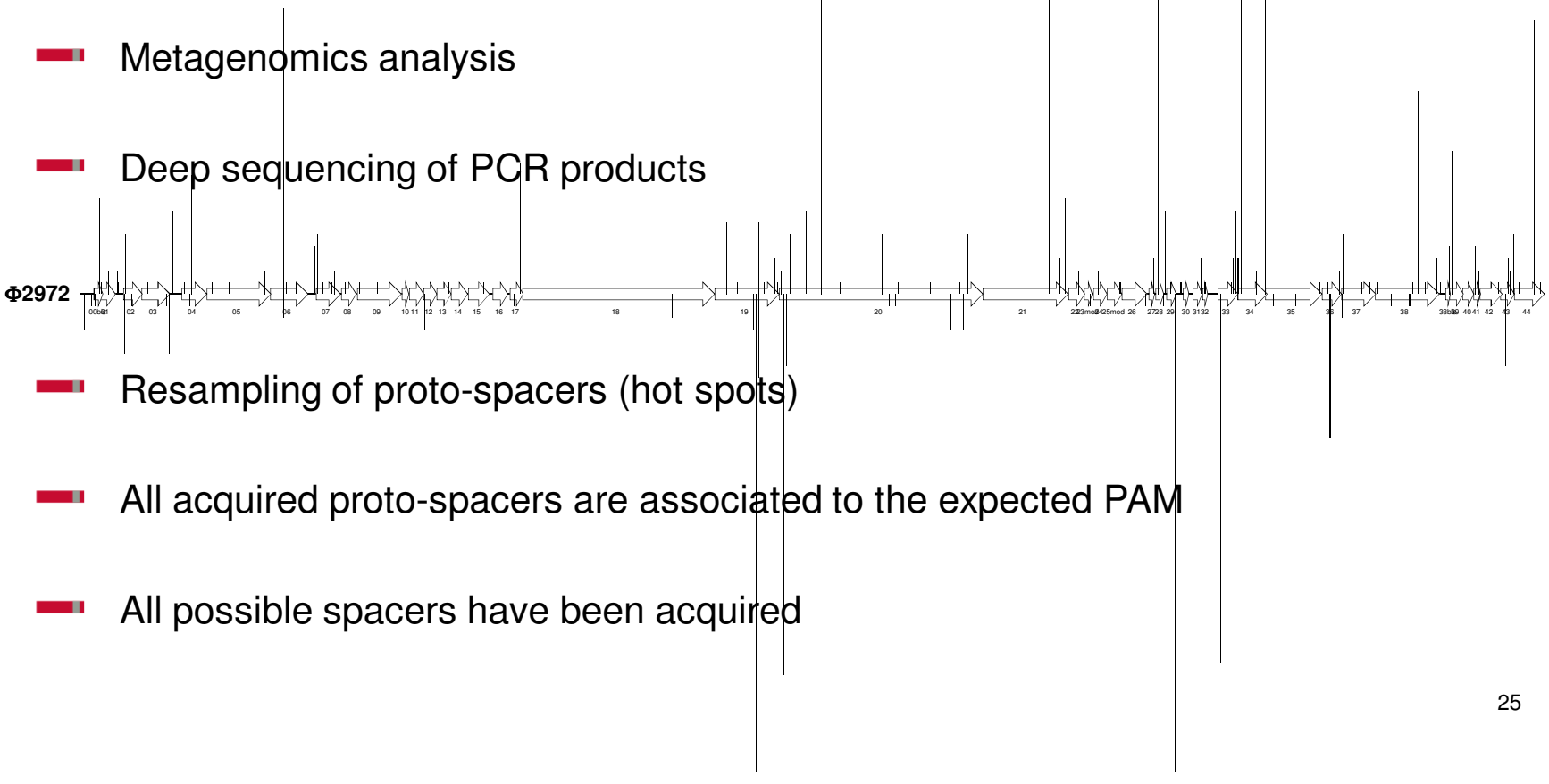
- SNP in proto-spacer
- SNP in PAM
- deletion



Deveau *et al.*, 2008; Horvath *et al.* 2008

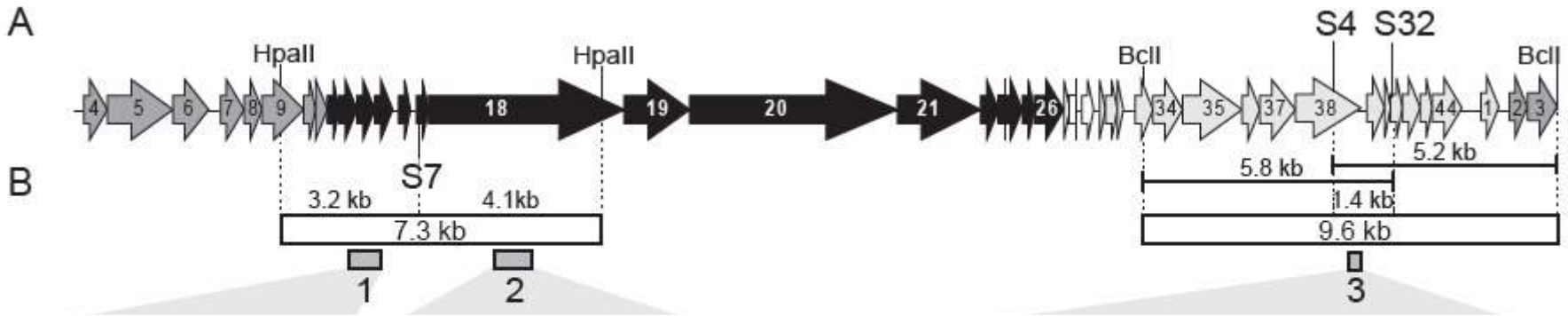
Spacer acquisition is not random, but PAM-dependent

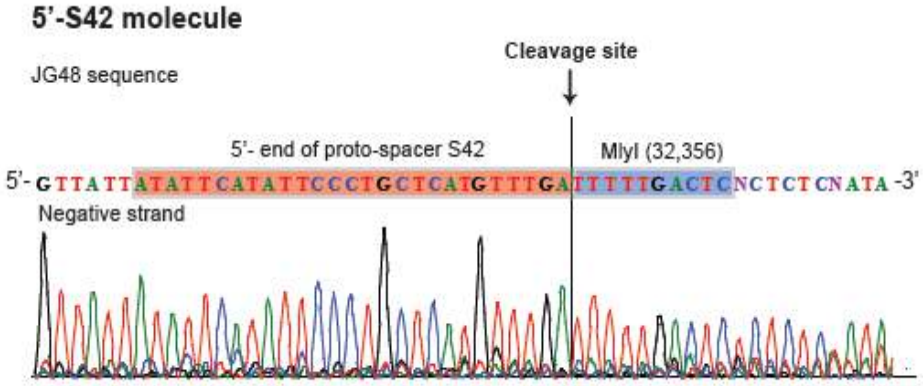
- Host and phage co-culture (closed system)
- "Long-term" experiment
- Metagenomics analysis
- Deep sequencing of PCR products

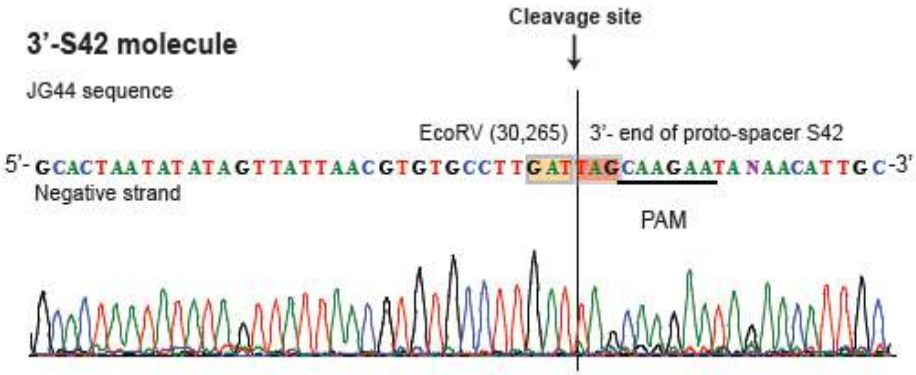


- Resampling of proto-spacers (hot spots)
- All acquired proto-spacers are associated to the expected PAM
- All possible spacers have been acquired

Cleavage of the dsDNA target







Garneau *et al.*, 2010

Heterologous transfer of a CRISPR-Cas system

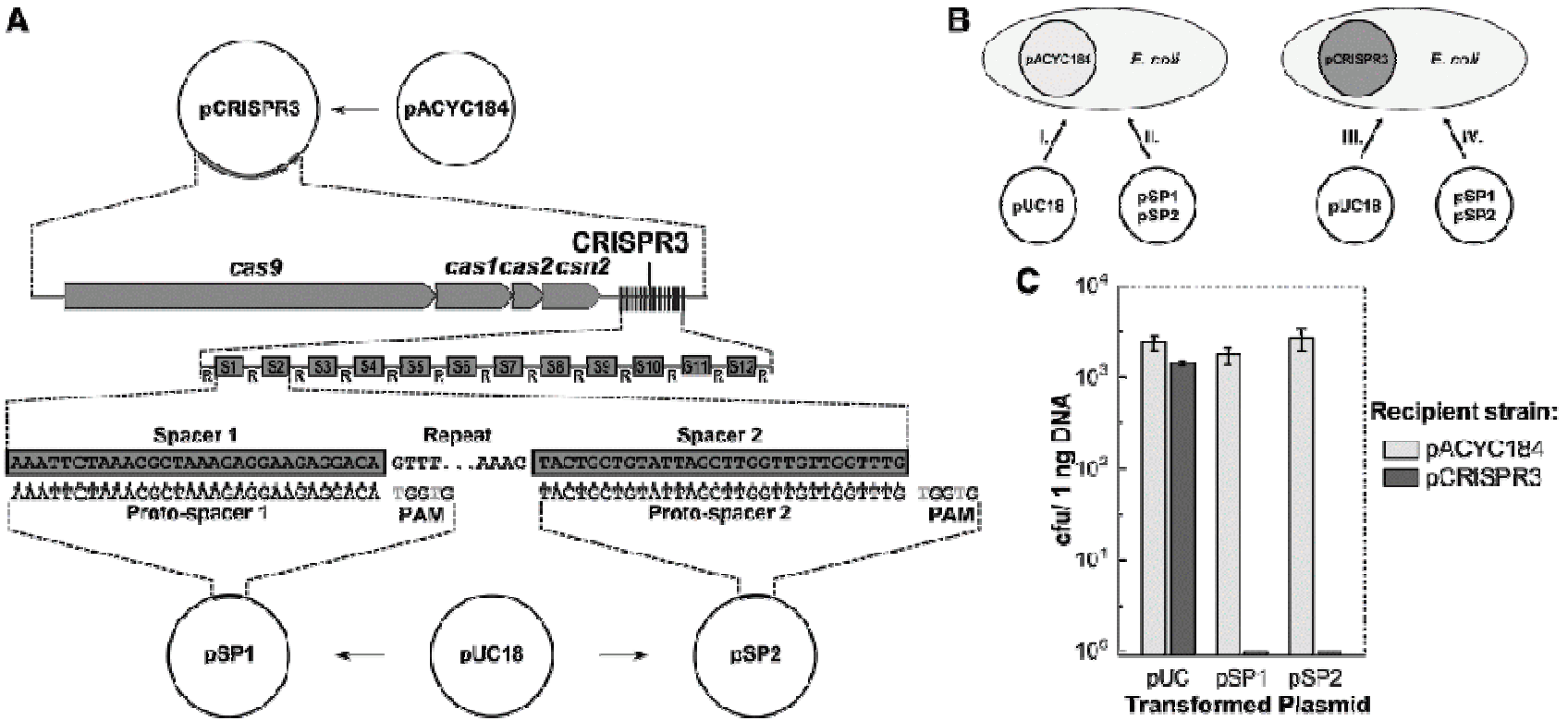


Figure 2. CRISPR3/Cas system of *S. thermophilus* provides immunity against plasmid transformation in *E. coli* cells. **(A)** Schematic representation of CRISPR3/Cas system cloning and construction of the plasmids for interference assay. *Streptococcus thermophilus* CRISPR3/Cas system was cloned into *E. coli* plasmid pACYC184. Plasmids for interference assays were obtained by inserting a proto-spacer and PAM into pUC18 plasmid. **(B)** Schematic representation of the plasmid transformation interference assay. *Escherichia coli* RR1 recipient strains carrying plasmids pCRISPR3 and pACYC184 with and without the *S. thermophilus* CRISPR3/Cas system, respectively, were transformed with plasmids pSP1 and pSP2 carrying proto-spacers and PAMs or pUC18. **(C)** Interference of plasmid transformation by *S. thermophilus* CRISPR3/Cas system in *E. coli* cells. Transformation efficiency is expressed as cfu per nanogram of plasmid DNA (mean ± SD).

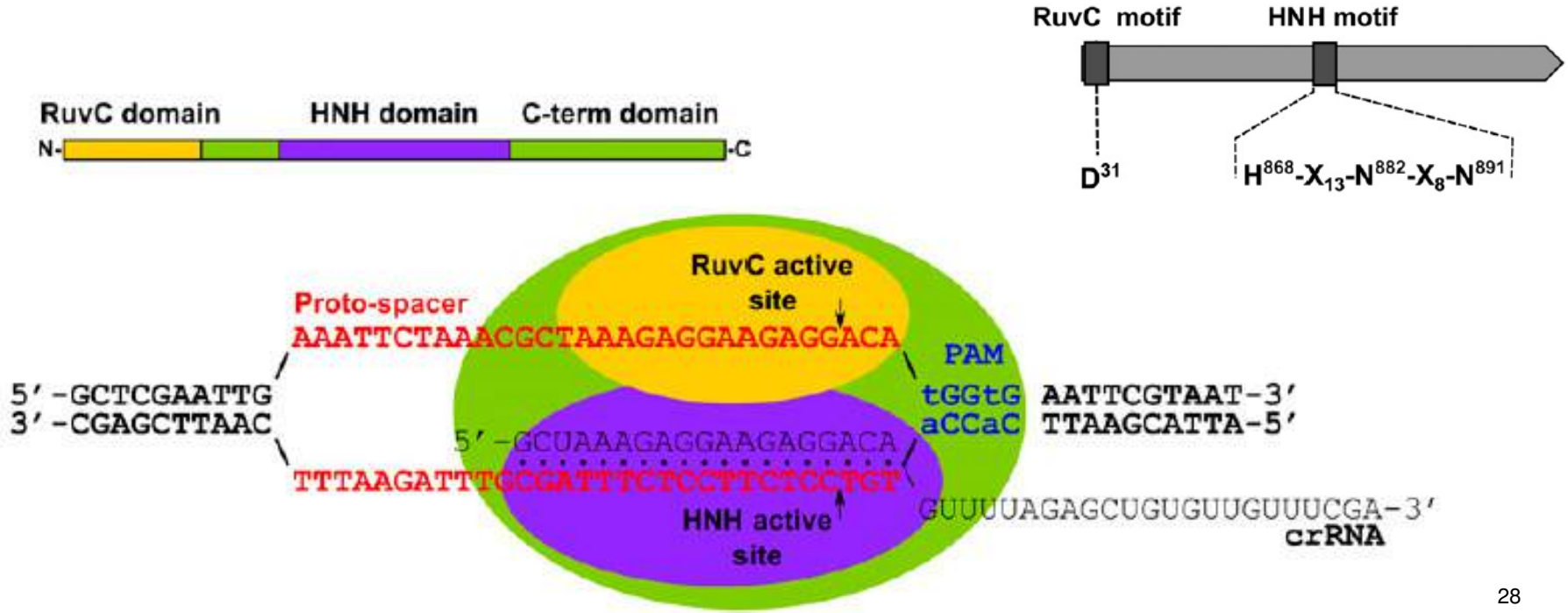
Sapranauskas *et al.*, 2011

Re-programming of the Cas9 nuclease

Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria

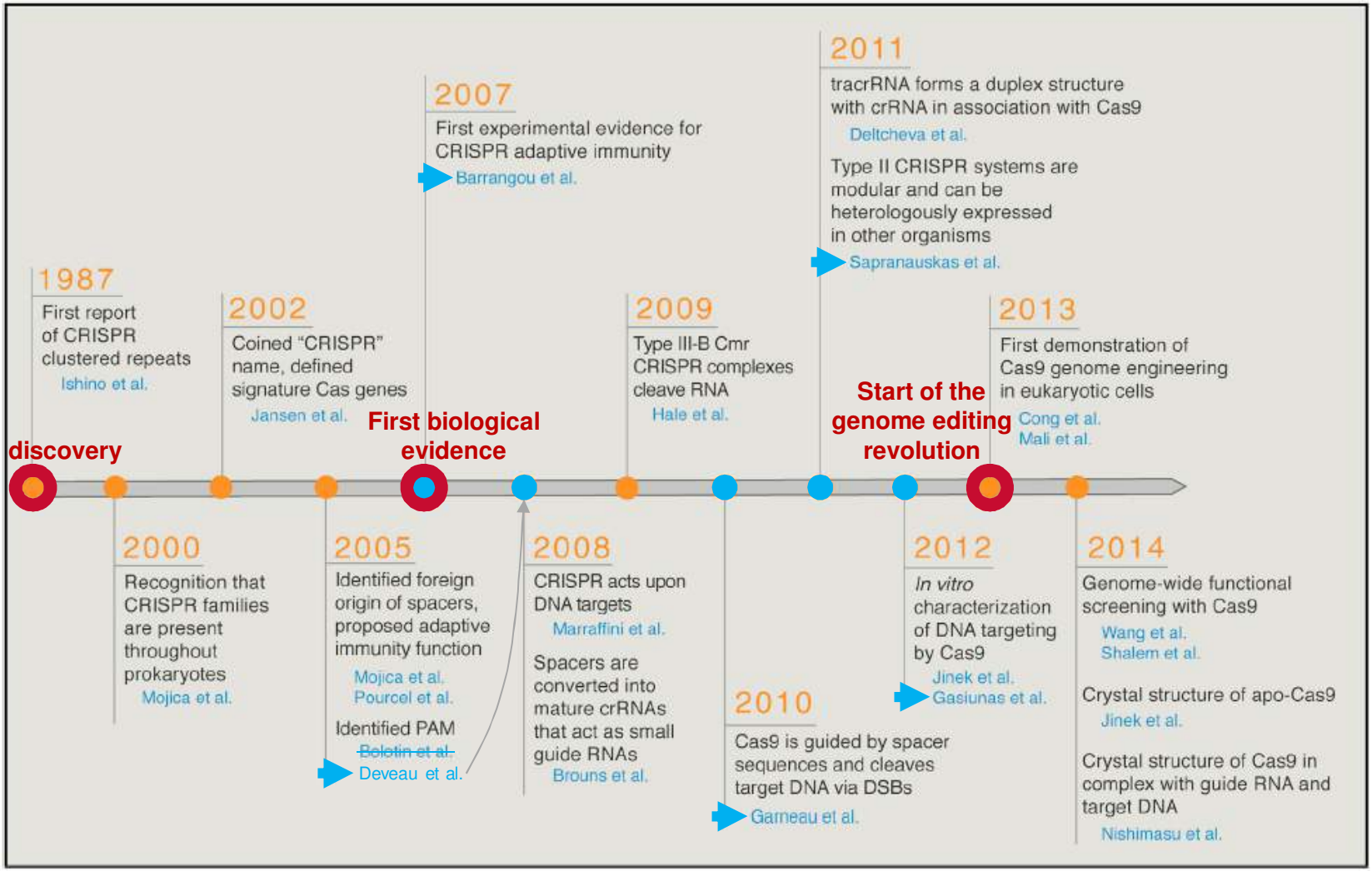
Giedrius Gasiunas[a], Rodolphe Barrangou[b], Philippe Horvath[c], and Virginijus Siksnys[a,1]

[a]Institute of Biotechnology, Vilnius University, LT-02241 Vilnius, Lithuania; [b]DuPont Nutrition and Health, Madison, WI 53716; and [c]DuPont Nutrition and Health, F-86220 Dangé-Saint-Romain, France



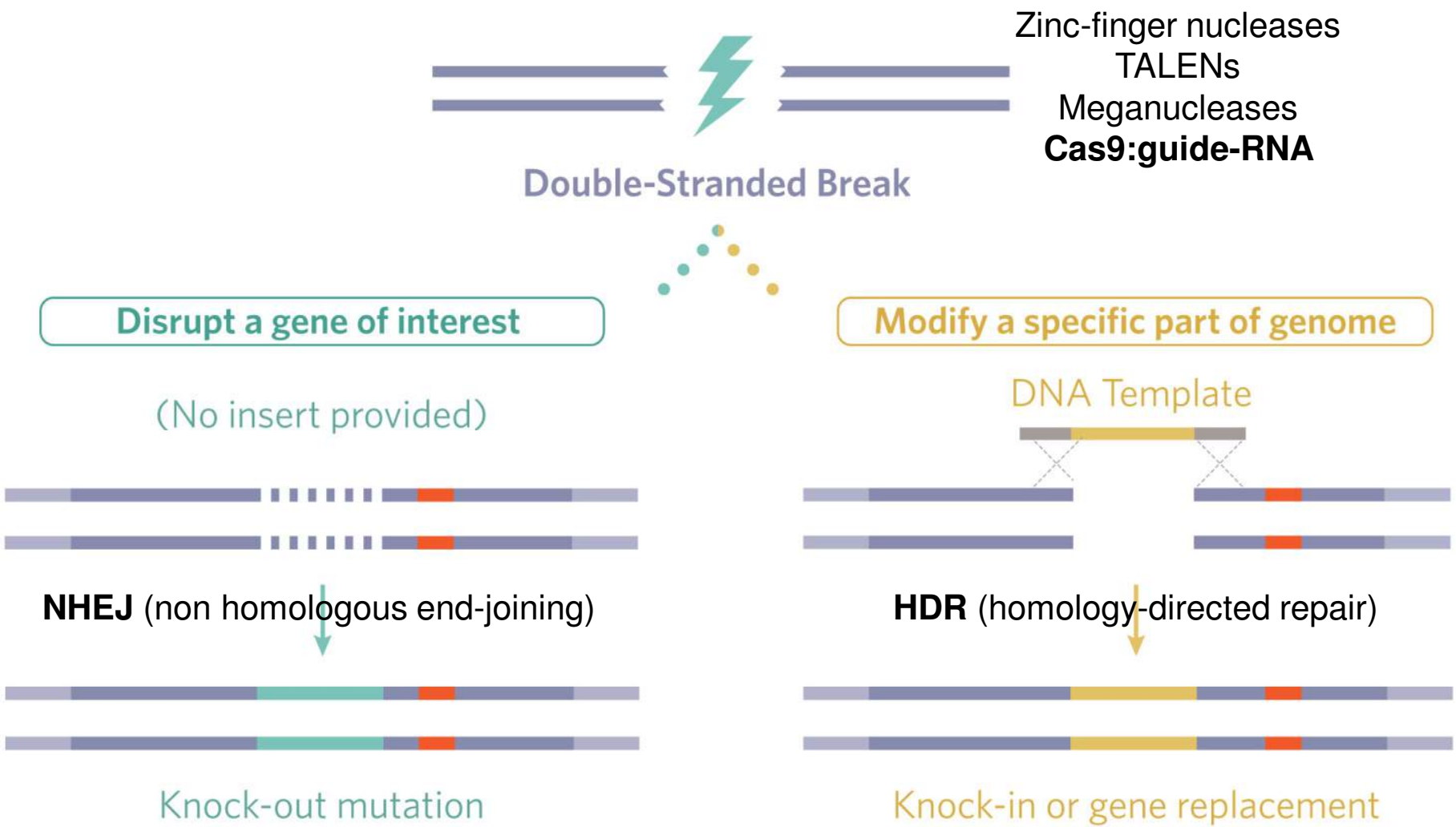
Gasiunas *et al.*, 2012

CRISPR timeline



Adapted from Hsu *et al.*, 2014, *Cell*

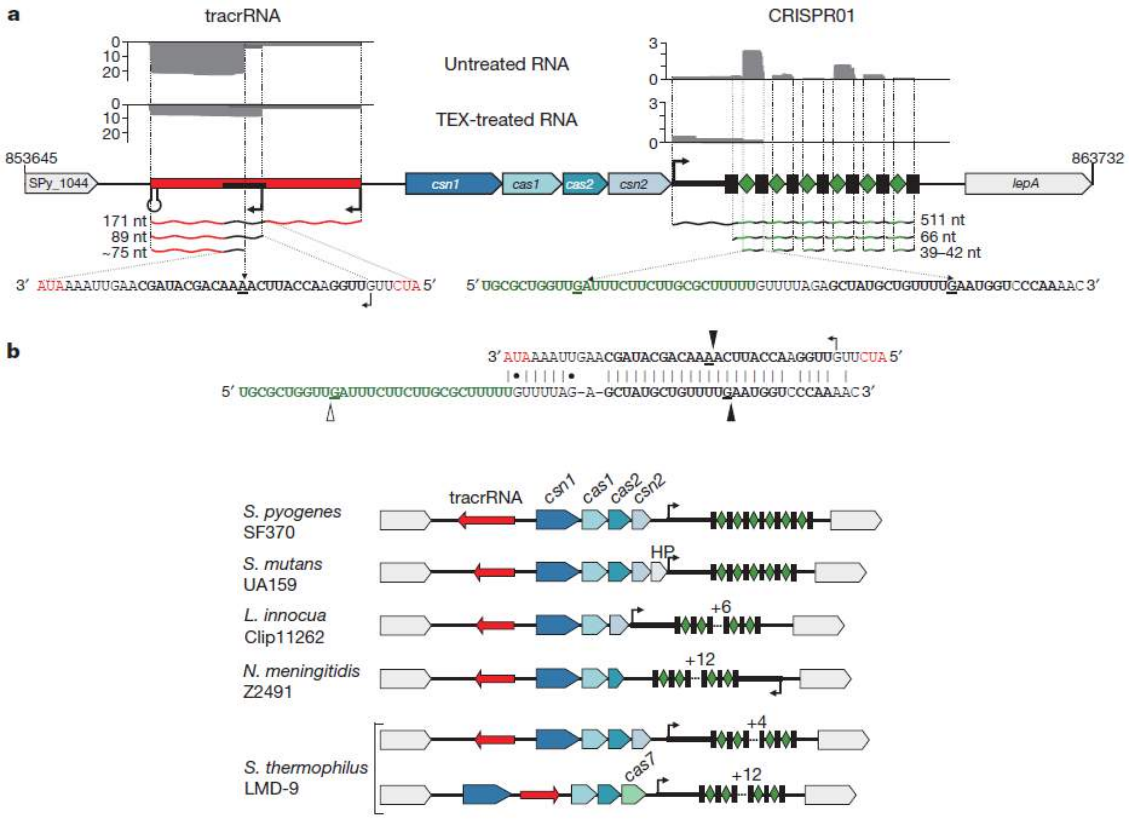
Genome editing



Discovery of tracrRNA, the "missing link" for Cas9 activity

CRISPR RNA maturation by *trans*-encoded small RNA and host factor RNase III

Elitza Deltcheva[1,2], Krzysztof Chylinski[1,2]*, Cynthia M. Sharma[3]*, Karine Gonzales[2], Yanjie Chao[3,4], Zaid A. Pirzada[2], Maria R. Eckert[2], Jörg Vogel[3,4] & Emmanuelle Charpentier[1,2]



Deltcheva *et al.*, 2011

The single guide-RNA breakthrough

A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity

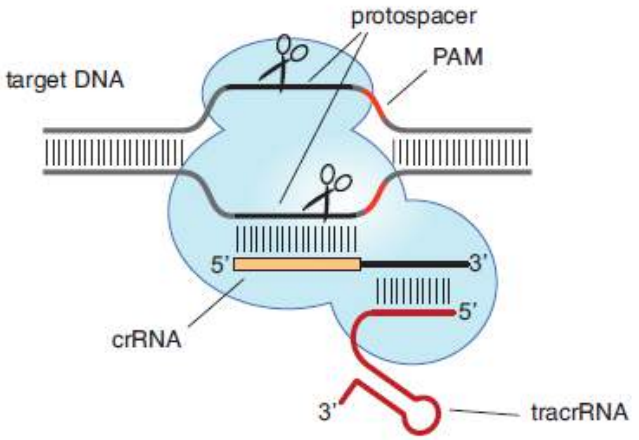
Martin Jinek,[1,2*] Krzysztof Chylinski,[3,4*] Ines Fonfara,[4] Michael Hauer,[2†] Jennifer A. Doudna,[1,2,5,6‡] Emmanuelle Charpentier[4‡]

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems provide bacteria and archaea with adaptive immunity against viruses and plasmids by using CRISPR RNAs (crRNAs) to guide the silencing of invading nucleic acids. We show here that in a subset of these systems, the mature crRNA that is base-paired to trans-activating crRNA (tracrRNA) forms a two-RNA structure that directs the CRISPR-associated protein Cas9 to introduce double-stranded (ds) breaks in target DNA. At sites complementary to the crRNA-guide sequence, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the noncomplementary strand. The dual-tracrRNA:crRNA, when engineered as a single RNA chimera, also directs sequence-specific Cas9 dsDNA cleavage. Our study reveals a family of endonucleases that use dual-RNAs for site-specific DNA cleavage and highlights the potential to exploit the system for RNA-programmable genome editing.

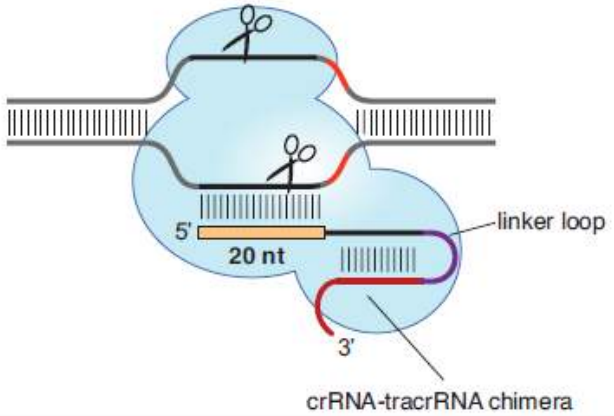
[1]Howard Hughes Medical Institute (HHMI), University of California, Berkeley, CA 94720, USA.
[2]Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA.
[3]Max F. Perutz Laboratories (MFPL), University of Vienna, A-1030 Vienna, Austria.
[4]The Laboratory for Molecular Infection Medicine Sweden, Umeå Centre for Microbial Research, Department of Molecular Biology, Umeå University, S-90187 Umeå, Sweden.
[5]Department of Chemistry, University of California, Berkeley, CA 94720, USA.
[6]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA.
*These authors contributed equally to this work.
†Present address: Friedrich Miescher Institute for Biomedical Research, 4058 Basel, Switzerland.
‡Corresponding author.
E-mail: doudna@berkeley.edu (J.A.D.); emmanuelle.charpentier@mims.umu.se (E.C.)

Read full article: scim.ag/1piiXv7

Cas9 programmed by crRNA:tracrRNA duplex



Cas9 programmed by single chimeric RNA



First reports on genome editing in mammalian cells

Multiplex genome engineering using CRISPR/Cas systems

Le Cong,[1,2]* F. Ann Ran,[1,4,5]* David Cox,[1,3] Shuailiang Lin,[1,3] Robert Barretto,[6] Naomi Habib,[1] Patrick D. Hsu,[1,4] Xuebing Wu,[7] Wenyan Jiang,[8] Luciano A. Marraffini,[8] Feng Zhang[1]†

Functional elucidation of causal genetic variants and elements requires precise genome editing technologies. The type II prokaryotic CRISPR (clustered regularly interspaced short palindromic repeats)/Cas adaptive immune system has been shown to facilitate RNA-guided site-specific DNA cleavage. We engineered two different type II CRISPR/Cas systems and demonstrate that Cas9 nucleases can be directed by short RNAs to induce precise cleavage at endogenous genomic loci in human and mouse cells. Cas9 can also be converted into a nicking enzyme to facilitate homology-directed repair with minimal mutagenic activity. Lastly, multiple guide sequences can be encoded into a single CRISPR array to enable simultaneous editing of several sites within the mammalian genome, demonstrating easy programmability and wide applicability of the RNA-guided nuclease technology.

Precise and efficient genome-targeting technologies are needed to enable systematic reverse engineering of causal genetic variations by allowing selective perturbation of individual genetic elements. Although genome-editing technologies such as designer zinc fingers (ZFs) (*1–4*), transcription activator–like effectors (TALEs)

ularly interspaced short palindromic repeats (CRISPR) adaptive immune system (*15–18*).

The *Streptococcus pyogenes* SF370 type II CRISPR locus consists of four genes, including the Cas9 nuclease, as well as two noncoding CRISPR RNAs (crRNAs): trans-activating crRNA (tracrRNA) and a precursor crRNA (pre-crRNA) array containing nuclease guide sequences (spacers) interspaced by identical direct repeats (DRs) (fig. S1) (*19*). We sought

[1]Broad Institute of MIT and Harvard, 7 Cambridge Center, Cambridge, MA 02142, USA; and McGovern Institute for Brain Research, Department of Brain and Cognitive Sciences, Department of Biological Engineering, Massachusetts Institute of Technology (MIT), Cambridge, MA 02139, USA.
[2]Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, MA 02115, USA.
[3]Harvard-MIT Health Sciences and Technology, Harvard Medical School, Boston, MA 02115, USA.
[4]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA.

RNA-guided human genome engineering via Cas9

Prashant Mali,[1]* Luhan Yang,[1,3]* Kevin M. Esvelt,[2] John Aach,[1] Marc Guell,[1] James E. DiCarlo,[4] Julie E. Norville,[1] George M. Church,[1,2]†

Bacteria and archaea have evolved adaptive immune defenses, termed clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems, that use short RNA to direct degradation of foreign nucleic acids. Here, we engineer the type II bacterial CRISPR system to function with custom guide RNA (gRNA) in human cells. For the endogenous AAVS1 locus, we obtained targeting rates of 10 to 25% in 293T cells, 13 to 8% in K562 cells, and 2 to 4% in induced pluripotent stem cells. We show that this process relies on CRISPR components; is sequence-specific; and, upon simultaneous introduction of multiple gRNAs, can effect multiplex editing of target loci. We also compute a genome-wide resource of ~190 K unique gRNAs targeting ~40.5% of human exons. Our results establish an RNA-guided editing tool for facile, robust, and multiplexable human genome engineering.

we also tested a Cas9D10A mutant that is known to function as a nickase in vitro, which yielded similar HR but lower nonhomologous end joining (NHEJ) rates (fig. S3) (*4*, *5*). Consistent with (*4*), in which a related Cas9 protein is shown to cut both strands 3 bp upstream of the PAM, our NHEJ data confirmed that most deletions or insertions occurred at the 3′ end of the target sequence (fig. S3B). We also confirmed that mutating the target genomic site prevents the gRNA from effecting HR at that locus, which demonstrates that CRISPR-mediated genome editing is sequence-specific (fig. S4). Finally, we showed that two gRNAs targeting sites in the GFP gene, and also three additional gRNAs targeting fragments from homologous regions of the DNA methyl transferase 3a (DNMT3a) and DNMT3b genes could sequence-specifically induce significant HR in the engineered reporter cell lines (figs. S5

First targeted modification of the human germline

Protein Cell 2015, 6(5):363–372
DOI 10.1007/s13238-015-0153-5

Protein & Cell

RESEARCH ARTICLE

CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes

Puping Liang, Yanwen Xu, Xiya Zhang, Chenhui Ding, Rui Huang, Zhen Zhang, Jie Lv, Xiaowei Xie, Yuxi Chen, Yujing Li, Ying Sun, Yaofu Bai, Zhou Songyang, Wenbin Ma, Canquan Zhou✉, Junjiu Huang✉

Guangdong Province Key Laboratory of Reproductive Medicine, the First Affiliated Hospital, and Key Laboratory of Gene Engineering of the Ministry of Education, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, China
✉ Correspondence: hjunjiu@mail.sysu.edu.cn (J. Huang), zhoucanquan@hotmail.com (C. Zhou)

Received March 30, 2015 Accepted April 1, 2015

Call for a moratorium

COMMENT

Don't edit the human germ line

Heritable human genetic modifications pose serious risks, and the therapeutic benefits are tenuous, warn **Edward Lanphier, Fyodor Urnov** and colleagues.

It is thought that studies involving the use of genome-editing tools to modify the DNA of human embryos will be published shortly[1].

There are grave concerns regarding the ethical and safety implications of this research. There is also fear of the negative impact it could have on important work involving the use of genome-editing techniques in somatic (non-reproductive) cells.

We are all involved in this latter area of work. One of us (F.U.) helped to develop the first genome-editing technology, zinc-finger nucleases[2] (ZFNs), and is now senior scientist at the company developing them, Sangamo BioSciences of Richmond, California. The Alliance for Regenerative Medicine (ARM; in which E.L., M.W. and S.E.H. are involved), is an international organization that represents more than 200 life-sciences companies, research institutions, non-profit organizations, patient-advocacy groups and investors focused on developing and commercializing therapeutics, including those involving genome editing.

Genome-editing technologies may offer a powerful approach to treat many human diseases, including HIV/AIDS, haemophilia, sickle-cell anaemia and several forms of cancer[3]. All techniques currently in various stages of clinical development focus on modifying the genetic material of somatic cells, such as T cells (a type of white blood cell). These are not designed to affect sperm or eggs.

In our view, genome editing in human embryos using current technologies could have unpredictable effects on future generations. This makes it dangerous and ethically unacceptable. Such research could be exploited for non-therapeutic modifications. We are concerned that a public outcry about such an ethical breach could hinder a promising area of therapeutic development, namely making genetic changes that cannot be inherited.

At this early stage, scientists should agree not to modify the DNA of human reproductive cells. Should a truly compelling case ever arise for the therapeutic benefit of germline modification, we encourage an open discussion around the appropriate course of action.

EDITING TOOLS

Genome editing of human somatic cells aims to repair or eliminate a mutation that could cause disease. The premise is that corrective changes to a sufficient number of cells carrying the mutation — in which the genetic fixes would last the lifetimes of the modified cells and their progeny — could provide a 'one and done' curative treatment for patients.

For instance, ZFNs are DNA-binding proteins that can be engineered to induce a double-strand break in a section of DNA. Such molecular scissors enable researchers to 'knock out' specific genes, repair a mutation or incorporate a new stretch of DNA into a selected location.

Sangamo BioSciences is conducting clinical trials to evaluate an application of genome editing as a potential 'functional cure' for HIV/AIDS[4]. The hope is that

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CRISPR-Cas9 genome editing applications

Could CRISPR technology be used to cure AIDS and other devastating viral diseases?

Why are viral diseases like AIDS still incurable? Although antiretroviral drugs can effectively control viral load in many patients, the permanent integration of viral DNA into a host genome means that patients remain vulnerable to re-activation of a latent virus. Exciting new research now shows that CRISPR technology can remove HIV DNA that has integrated into the host genome in human cells, re-igniting our hopes for developing a true cure for AIDS.

CRISPR-mediated genome editing is revolutionizing biomedical research due to its precise targeting, high efficiency, and ease of use in any cell type or experimental system. CRISPR has been used to create new transgenic animal models for basic and translational research, and it holds promise fo

GenScript's new GenCRISPR gR

- Take advantage of compli functionally-validated knoc
- Our gene synthesis servic researchers who've pione

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T cell editing using CRISPR/Cas9 could revolutionize HIV therapeutics

September 15, 2015

Reinforcing the immune system by engineering lymphocytes to target and destroy viruses has the potential to be an effective therapy for many diseases. One potential approach to this strategy is to alter the genome of lymphocytes so that proteins that are typically hijacked by viruses are no longer present. While conceptually feasible, editing T cells has been challenging in practice; however, with the advent of mammalian cell editing using CRISPR/Cas9, T-cell editing is closer to becoming a reality.

How can CRISPR/Cas9 bring us closer to finding a cure for HIV?

In a study recently published in PNAS, scientists have optimized a protocol to introduce nucleotide replacements that would inhibit CXCR4 expression. The authors streamlined the CRISPR/Cas9 editing process by electroporating Cas9 ribonucleoproteins (RNPs) into CD4+ T cells. The RNPs, consisting of both a recombinant Cas9 enzyme and guide RNA, vastly improved editing efficiency, ultimately promoting knock-out of the CXCR4 cell-surface receptor. Taken together, these result suggest the potential of a new cell therapy approach for the fight against HIV.

Schumann *et al.* Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. **PNAS.** 2015; 112(33): 10437-10442. DOI: 10.1073/pnas.1512503112

CRISPR-Cas9 genome editing applications

CRISPR gene editing improves the safety of porcine organs for human transplantation

November 16, 2015

Organ shortage for transplantation has been a critical problem for those that experience organ failure. Pig organs are ideal for organ replacement since they are the most comparable in size to humans; however, safety can be an issue for many patients. One reason for this incompatibility is the potential transmission of porcine endogenous retroviruses (PERV), which have the ability to infect human cells. So to address this problem and make pig organs safer for transplant, a group led by George Church at Harvard Medical School turned to the most effective gene editing technique available – CRISPR genome editing.

How were pig transplants made safer using CRISPR?

As a proof of principle, the authors examined the potential of knocking out all copies of the PERV polymerase gene in the porcine cell line PK15 using CRISPR/Cas9 technology. They found that the efficiency of the system was even better than expected: All 62 copies of the PERV *pol* gene were disrupted in the pig cells. In addition, when these edited cells were co-cultured with human cell lines, they found that there was a 1,000-fold reduction in PERV transmission. Together this report demonstrates the potential of CRISPR gene editing for eliminating harmful zoonoses from pig organs.

Yang *et al.* Genome-wide inactivation of porcine endogenous retroviruses. **Science.** 2015; DOI: 10.1126/science.aad1191.

CRISPR-Cas9 to resurrect extinct species

RESURRECTION BIOLOGY

SCIENCE FACT OR SCIENCE FICTION?

With the theatrical release of *Jurassic World*, we began to wonder whether the idea of bringing an extinct species back to life is within the realm of science fact or science fiction.

A little background: mankind has had a large—and largely negative—impact on earth's animal and plant species. Biologists estimate that we are losing thousands of species year after year. And while no one is certain exactly how many species are currently in danger, it's clear that we risk losing many thousands of species to extinction in the coming decades.

How are scientists approaching this problem? One ambitious idea is de-extinction—also called resurrection biology, species revivalism, or zombie zoology—which is the process of re-creating an extinct species from ancient DNA.

Why should we bring these creatures back to life? For the same vital reasons we attempt to protect our currently endangered species: to preserve biodiversity, to restore damaged ecosystems, to strengthen reproductive health through gene pool enrichment, and to mitigate at least some of the harm that we humans have created.

Using cutting-edge developments in genetic technology, molecular biologists and conservation biologists are working on projects that may bring extinct animals back to life, or endangered animals back from the brink. Possible candidates for de-extinction, genetic rescue, or genetic assistance are easy to isolate because the very material required for revival—the animal's DNA—is relatively easy to obtain.

According to The Long Now Foundation (longnow.org), "The "ancient DNA" of many extinct species can be recovered from museum specimens and fossils via Ancient Genome Assembly. New techniques still being further developed (synthetic DNA and CRISPR genome editing) may be able to bring the reassembled genomes back to life via a close living relative. But species that died out so long ago that no DNA remains, such as dinosaurs, are unrecoverably extinct."

The science is not without significant controversy. Opponents of de-extinction posit that efforts and resources used to resurrect extinct species would be better spent in protecting currently endangered species. *Scientific American*, in an editorial condemning de-extinction, opined that research "should be conducted under the mantle of preserving modern biodiversity rather than conjuring extinct species from the grave."

So, while a pet archaeopteryx or dodo is probably not in your future, this research may give us another chance to see some of these species in living, breathing form again.

Sources:
The Long Now Foundation and *Scientific American*.

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Carolina parakeet, Dodo, Great auk, Woolly mammoth, Xerces blue butterfly, Thylacine (Tasmanian tiger), Archaeopteryx

ThermoFisher SCIENTIFIC

CRISPR-Cas9 genome editing applications in plants

Making photosynthesis more efficient

Photosynthesis is such a fundamentally important process that you might think plants are highly efficient at it. However, scientists working to improve crop yields have noticed that one enzyme, Rubisco, represents a weak link in the photosynthetic pathway due to its poor oxygenase activity and slow turnover. A recent paper in

enzyme to support autotrophic ph
concentrating mechanism, inclu
functional CCM in plants. If this s
major breakthrough for sustaina

Plant biologists are increasingly
reasons. CRISPR is highly efficie
methods for creating transgenic
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new plant strains created using
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applications.

GenScript is the global leader in
enzyme expression, biosynthetic

- Gene synthesis starting a
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- *New!* Express Cloning fo
- GenCRISPR gRNA const

Lin MT, Occhialini A, An
Nature. 2014 Sep 25;5

Will CRISPR bring us more nutritious fruit crops without GM worries?

Global nutrition and food security are major concerns as human population rises and the land area devoted to agriculture shrinks. Biotechnology has accelerated the development of improved food crops that can address these issues in order to boost the economic productivity of farms and improve human health. However, genetically modified food crops have met with consumer resistance and increasing regulation due in part to concerns over the long-term safety and environmental effects of transgenes being introduced into plants. One way in which the scientific community is responding is to identify new technologies that can achieve the nutrition and food security goal of prior genetic engineering efforts while avoiding the use of transgenes and the ※GM§ label that is controversial among consumers.

※Superbananas§ have made news headlines as a biofortified crop containing enhanced levels of vitamin A to counteract widespread vitamin A deficiencies in certain parts of the world. A new ※Science & Society§ paper in *Trends in Biotechnology* presents the argument that luxury items such as fruit crops may find more consumer acceptance for bioengineering than do staple crops such as grain and rice, and discusses the possibility that new technologies such as CRISPR-mediated genome editing may elude the regulatory designation as ※GMOs§ since they contain no foreign DNA.[1] Successful CRISPR-mediated genome editing has been demonstrated in citrus fruits[2] and a variety of other food crops and diverse plant species[3].

GenScript offers gRNA constructs for CRISPR-mediated genome editing, including expert gRNA design for any species.

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To be or not to be… GMO

NATURE | NEWS

CRISPR tweak may help gene-edited crops bypass biosafety regulation

Technique deletes plant genes without adding foreign DNA.

David Cyranoski

19 October 2015

Je Wook Woo

These lettuce-plantlets have had their genomes edited with CRISPR/Cas9, but do not contain foreign DNA.

"Green light in the tunnel": Opinion of the Swedish Board of Agriculture – a CRISPR-Cas9-mutant but not a GMO

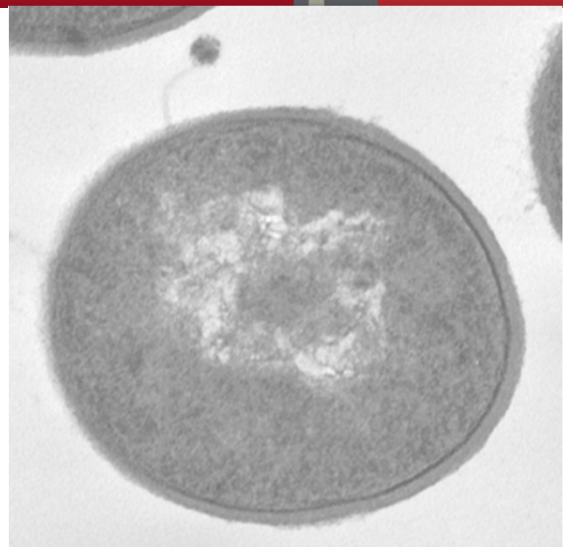
[2015-11-17] The Swedish Board of Agriculture has, after questions from researchers in Umeå and Uppsala, confirmed the interpretation that some plants in which the genome has been edited using the CRISPR-Cas9 technology do not fall under the European GMO definition. This is important for the wide use of such plants to contribute to solving some of the escalating challenges of mankind.

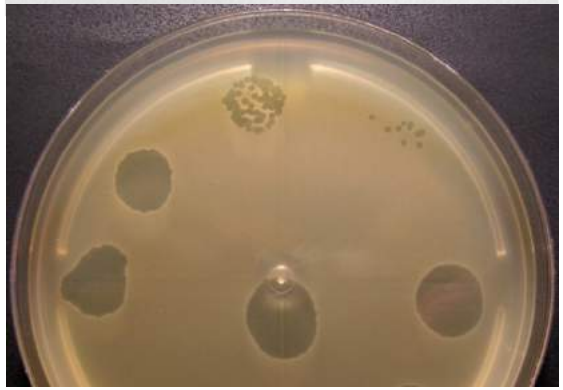
CRISPR-Cas9 is a technique, invented in collaboration with researchers at Umeå University, allowing scientists to make small edits in the genetic material of an organism, edits that can also occur naturally. Instead of hoping that such edits occur by natural recombination, they can now be deliberately introduced in a targeted and precise manner. CRISPR-Cas9 can thus be used in many ways in plant science and breeding.

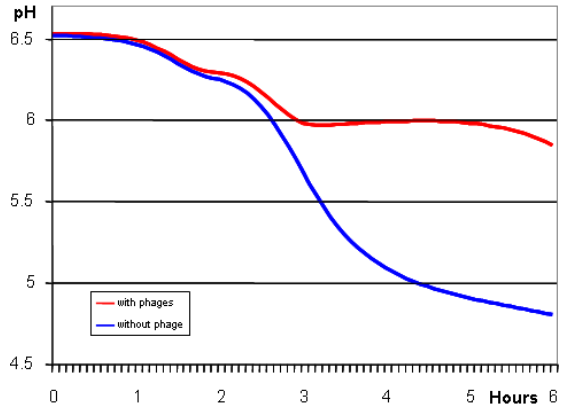
Plants that fall within the scope of EU GMO legislation are subject to a very strict regulatory regime (in reality making it impossible to grow them in the field in most EU countries). Plants that fall outside the scope can be grown without restriction. Since "inside or outside of the GMO definition" will decide whether or not plant scientists

The “phage problem”

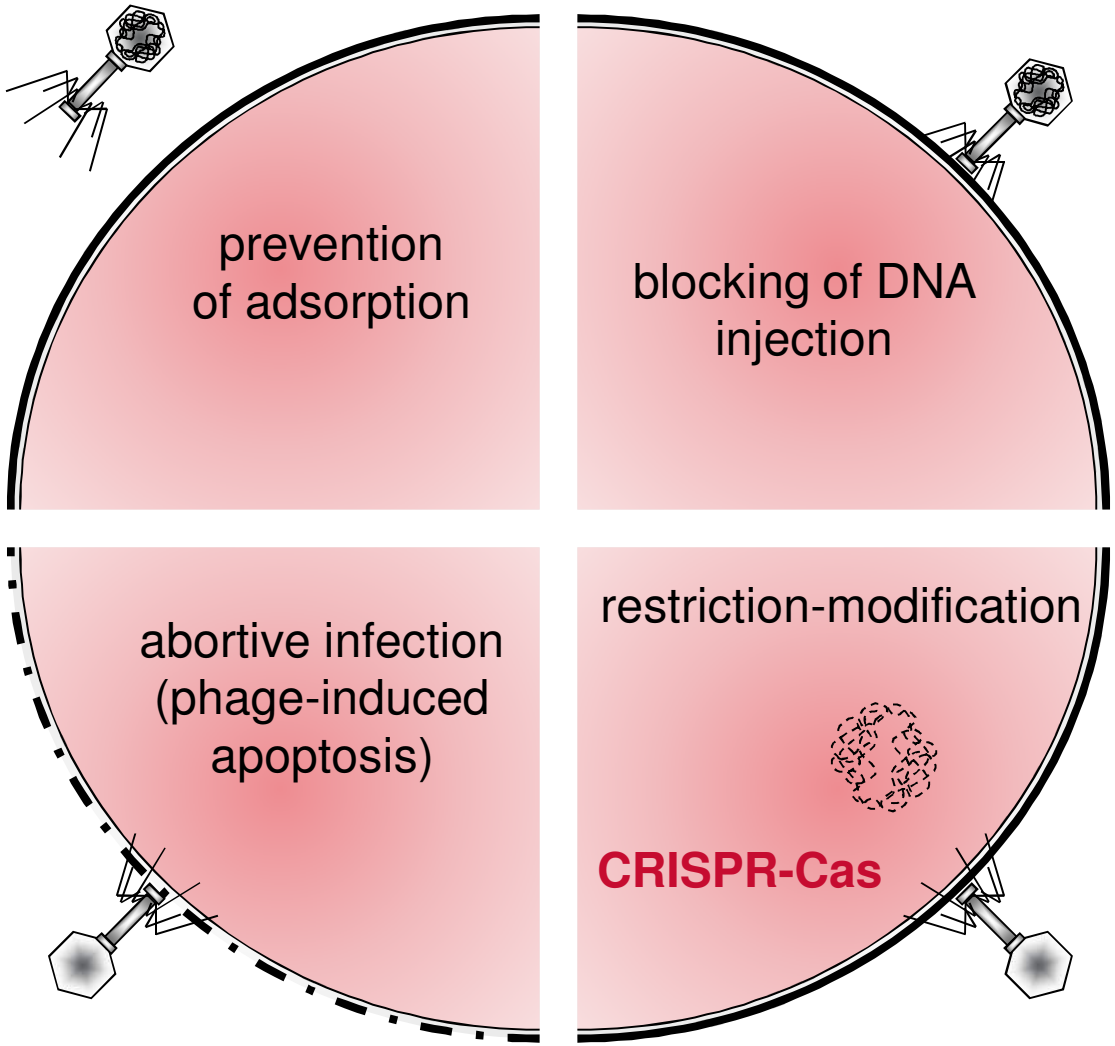
- Bacteriophages (phages) are **bacterial viruses** that are commonly found in the environment, notably in milk and other raw materials
- The non-sterile nature of dairy fermentations allows for the **proliferation of phages** against LAB cultures
- Without proper measures to control their proliferation, phage infections result in loss of productivity and quality in the dairy industry
- Phages are the **major cause of fermentation failures**, with significant economic losses
- **Selection of phage-resistant bacterial strains** is a constant, never-ending activity







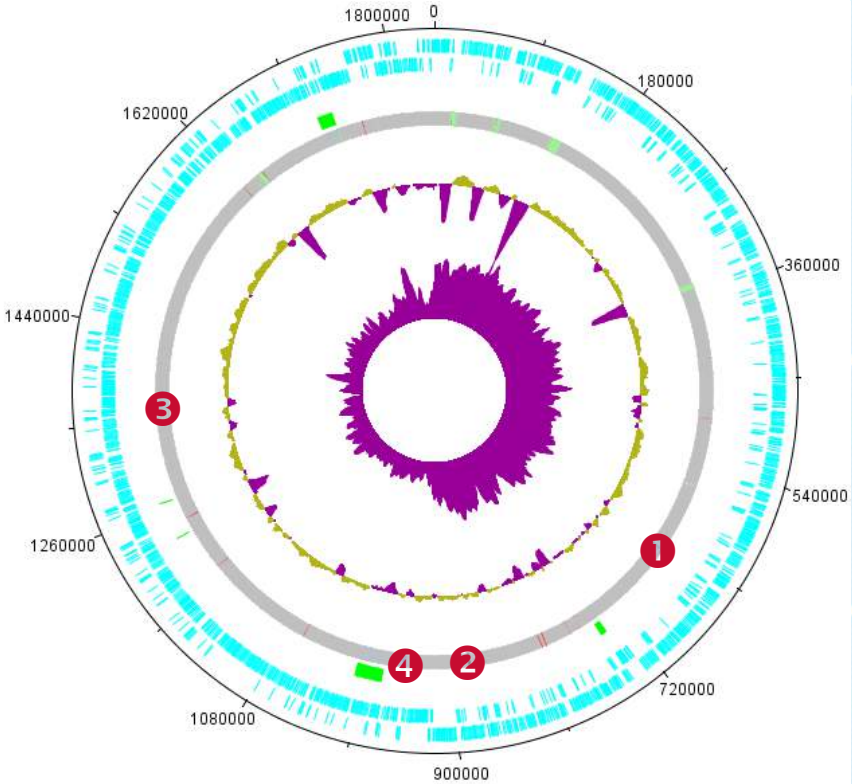
CRISPR-Cas, an additional hurdle against phage infection



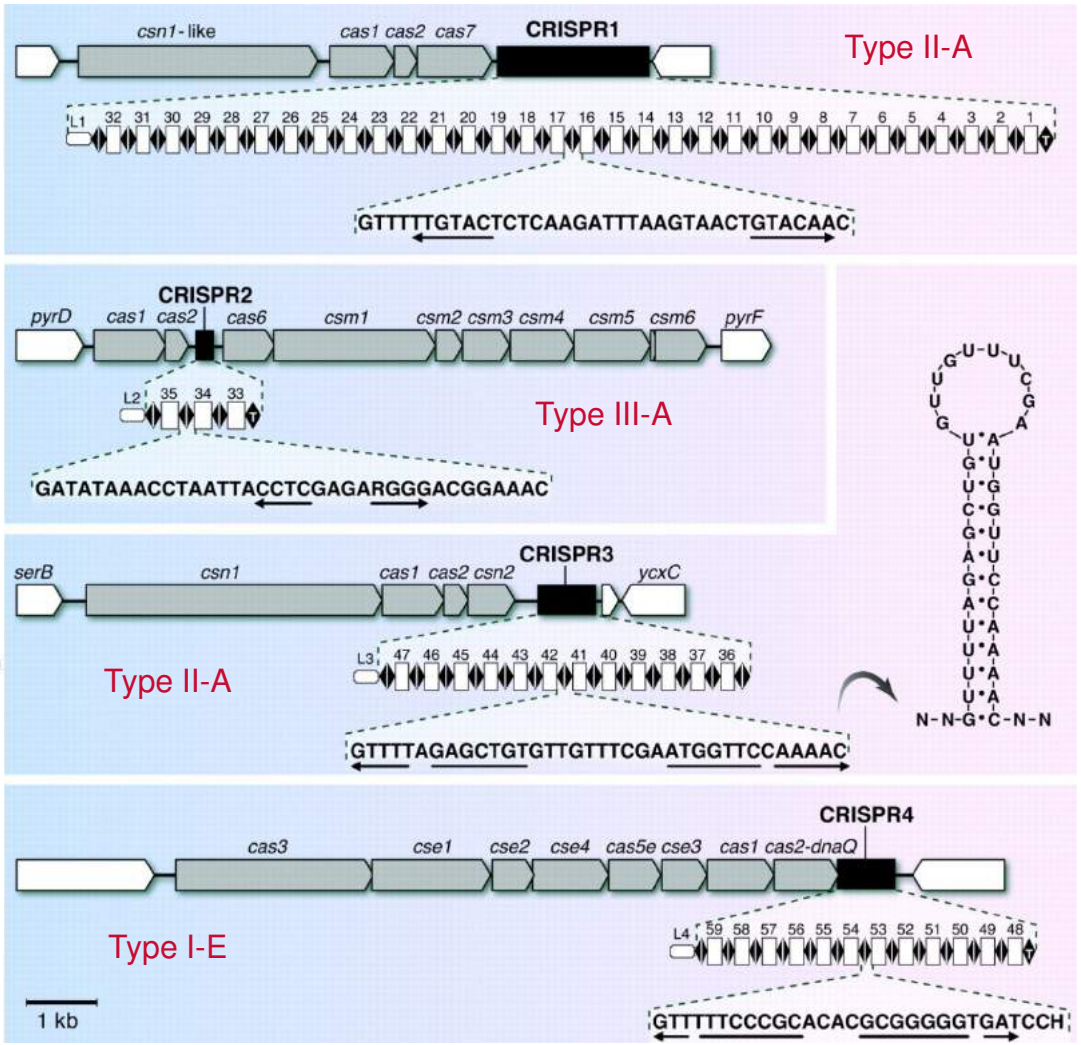
Barrangou & Horvath, 2010, *Stress Responses of Lactic Acid Bacteria*

S. thermophilus, a relevant model system

Up to 4 CRISPR-Cas systems

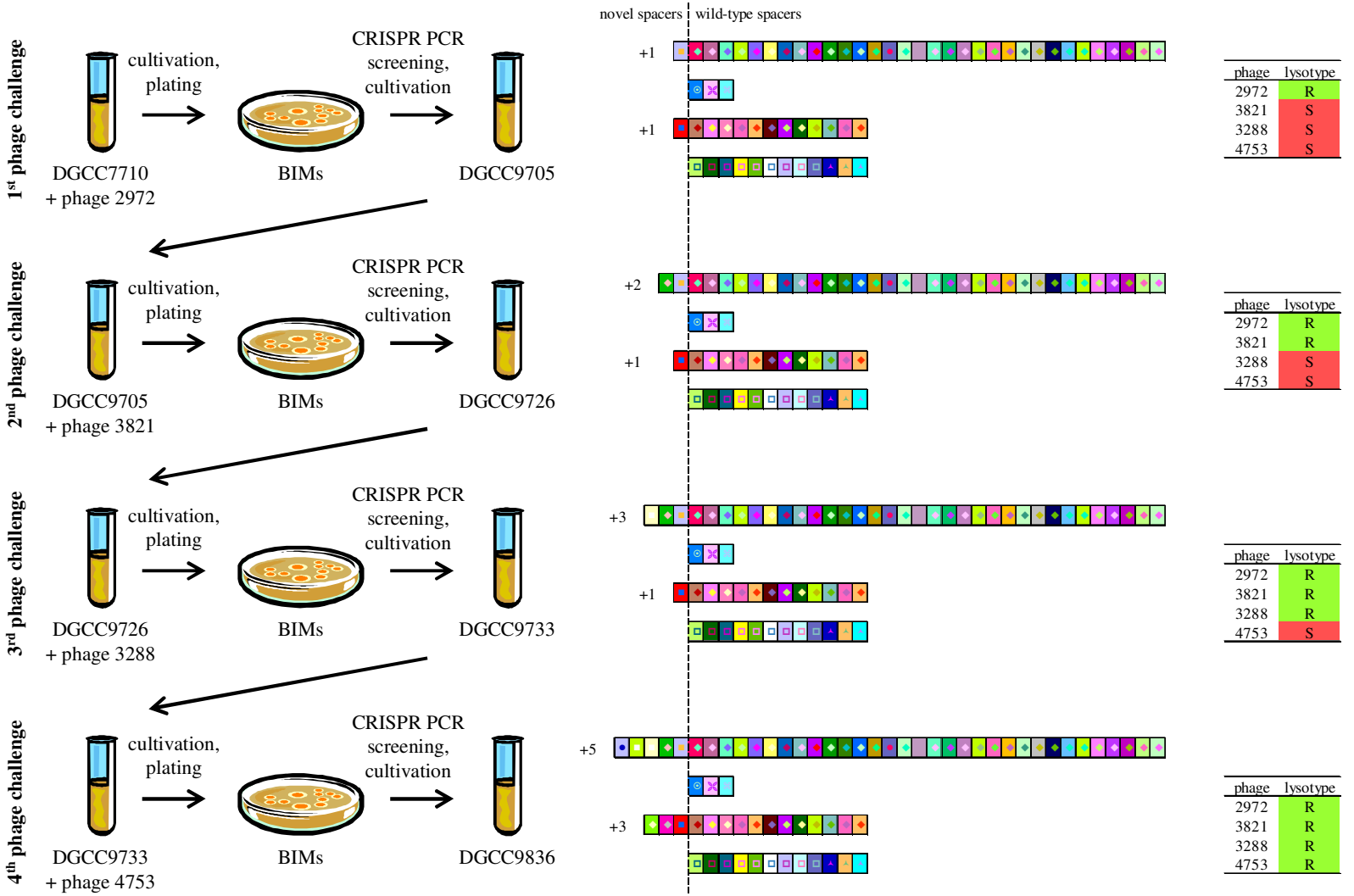


S. thermophilus DGCC7710



Horvath & Barrangou, 2010, *Science*

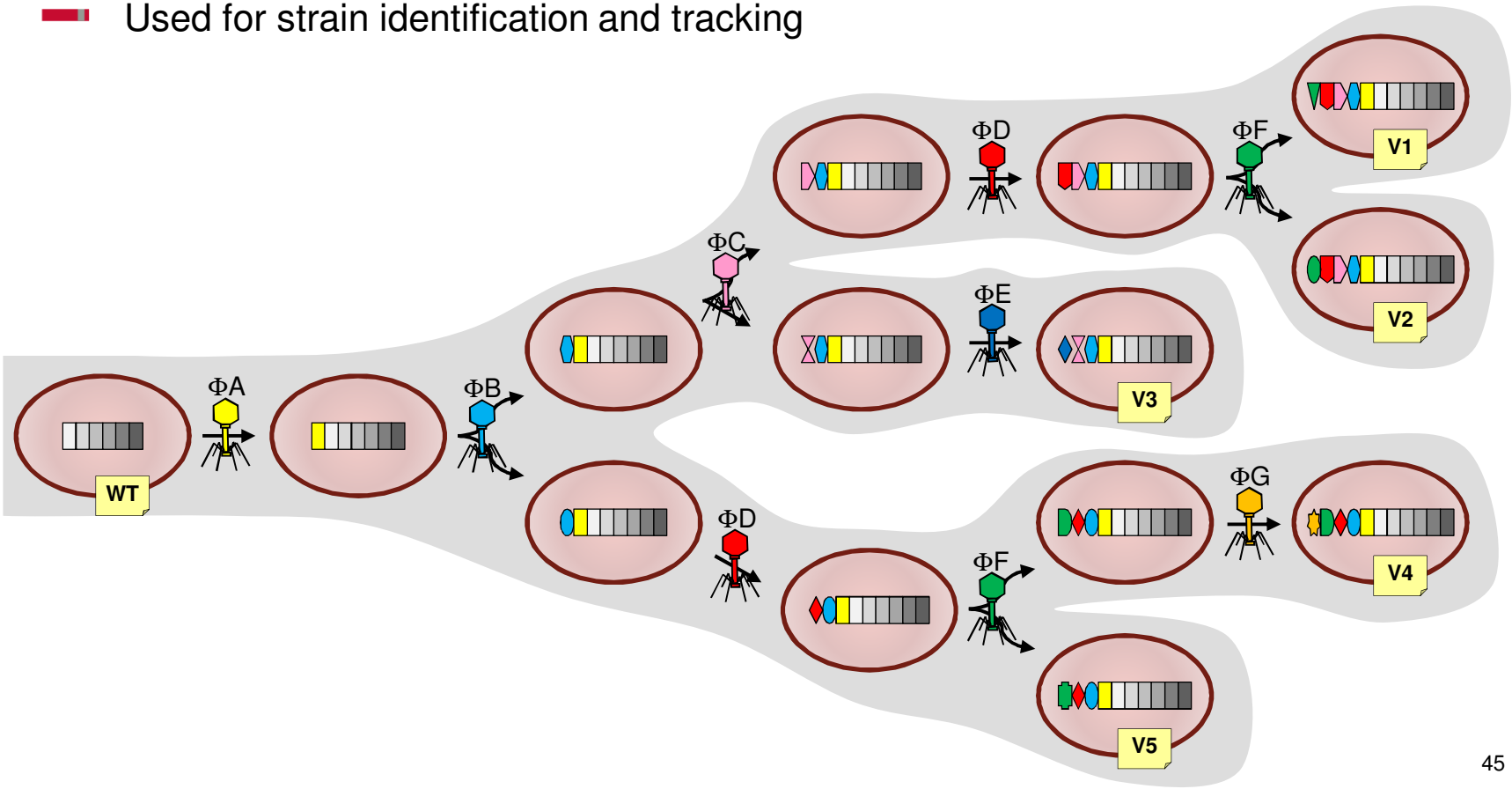
“CRISPerization”: natural vaccination against phages



Barrangou *et al.*, 2013, *Biochem. Soc. Trans.*

Natural genetic tagging

- Newly acquired spacers constitute a **genetic tag**
- Independently acquired **spacer combinations are unique**
- Used for strain identification and tracking



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First disclosure of the potential use of Cas nucleases as customizable restriction enzymes?

The CRISPR System Protects Microbes against Phages, Plasmids

Palindromic DNA repeat sequences immunize microorganisms against phages and plasmids, while also directing their evolution

Rodolphe Barrangou and Philippe Horvath

Moreover, assuming that the target DNA is recognized through sequence identity with crRNAs and then cleaved by a Cas endonuclease, the CRISPR/Cas system could be considered a variable geometry restriction system with broad and adaptive specificity.