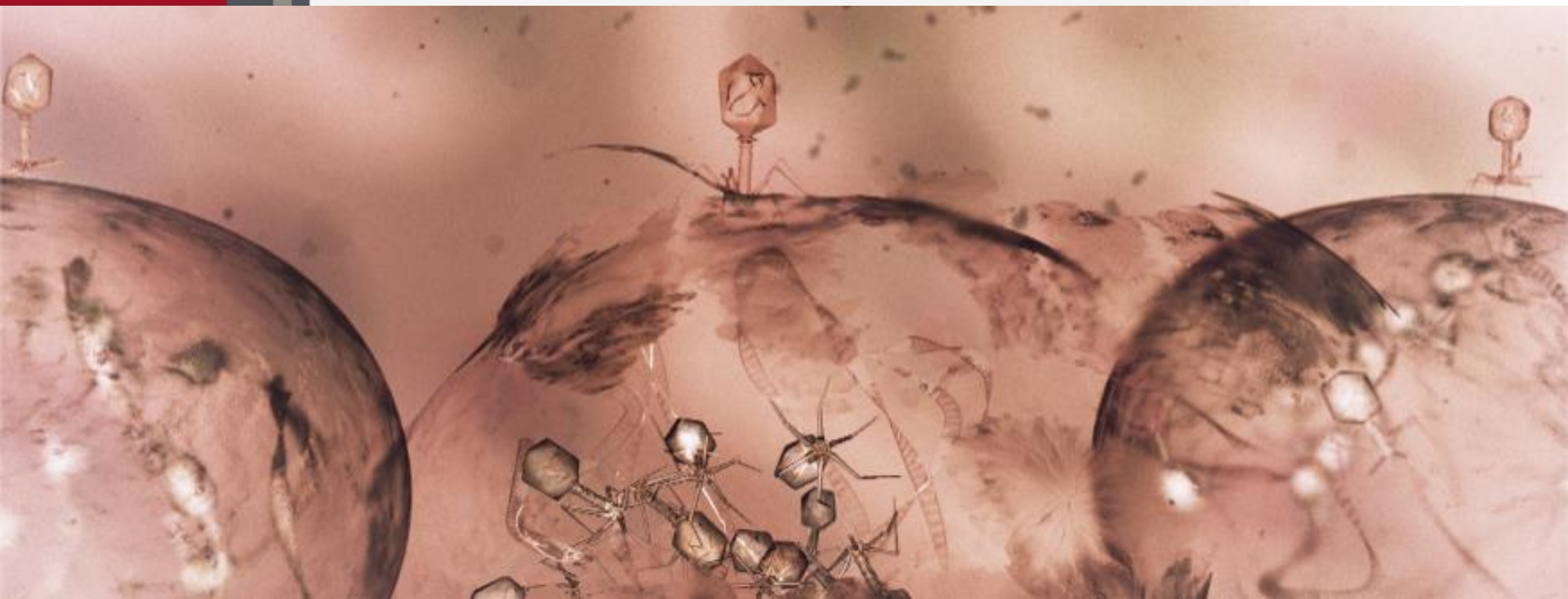
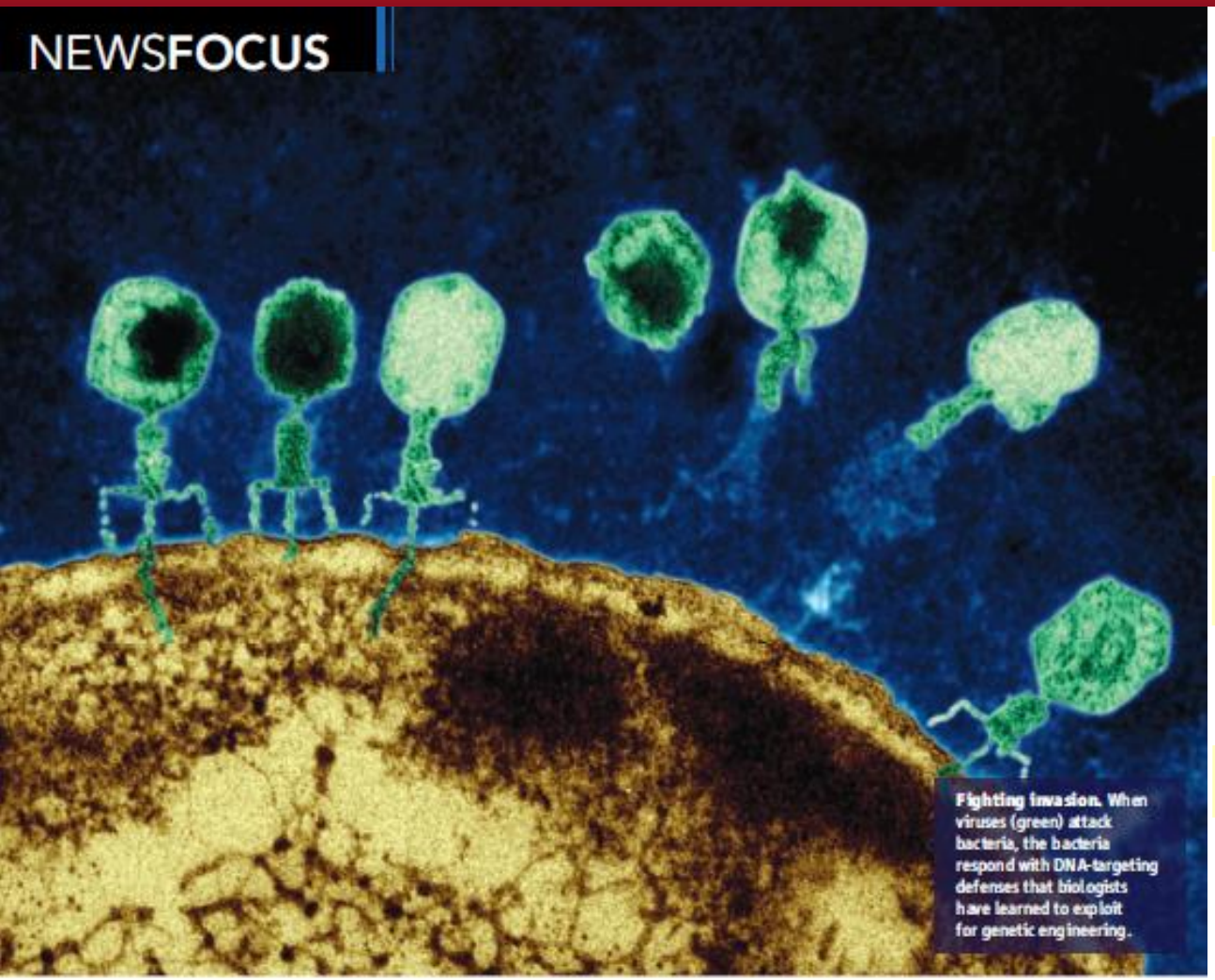
Le système CRISPR-Cas : découverte et applications

Académie d'Agriculture de France
Société Nationale d'Horticulture de France, Paris, 22 novembre 2018

Philippe HORVATH – philippe.horvath@dupont.com





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 SYMPAthy 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.

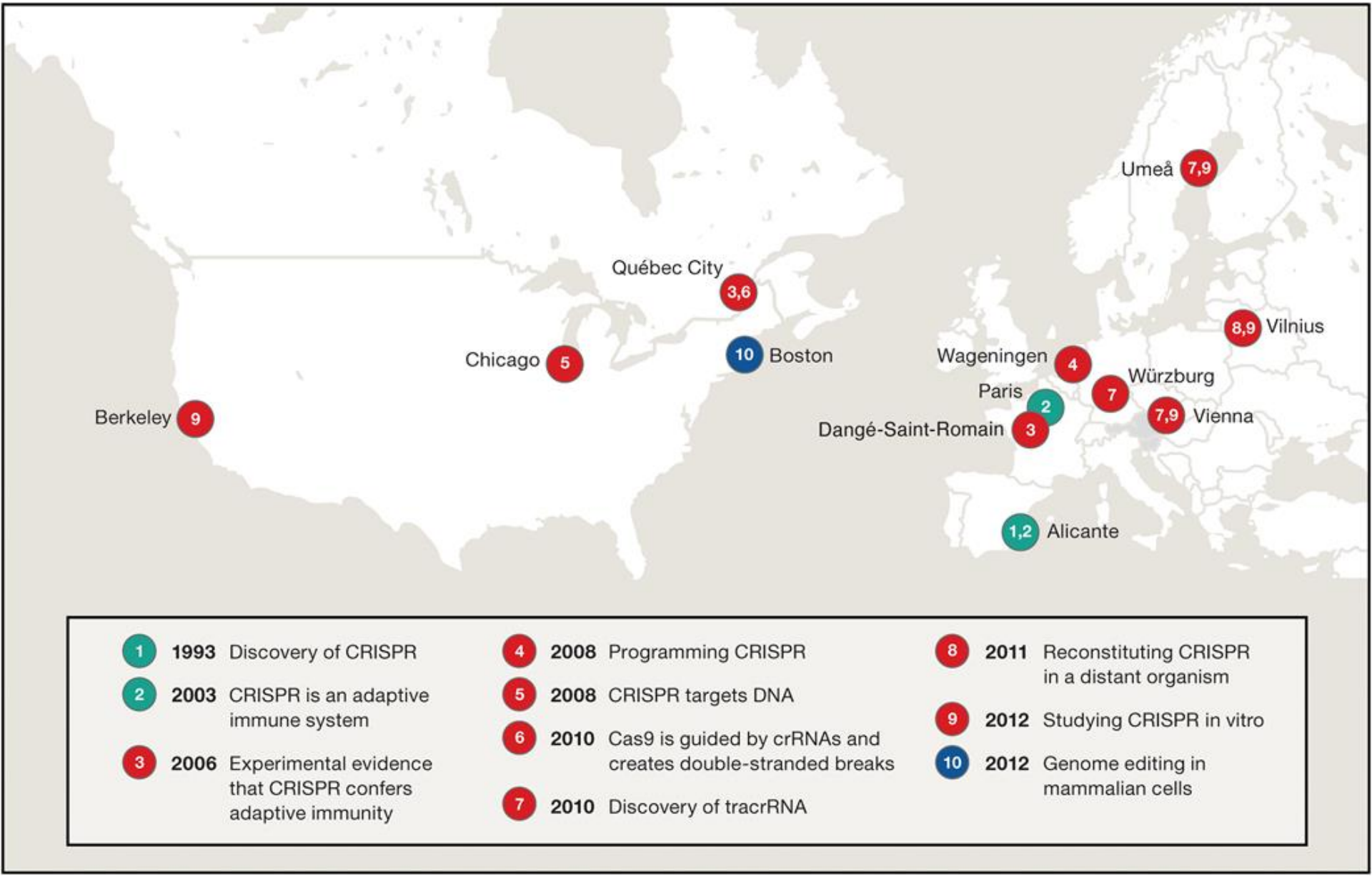


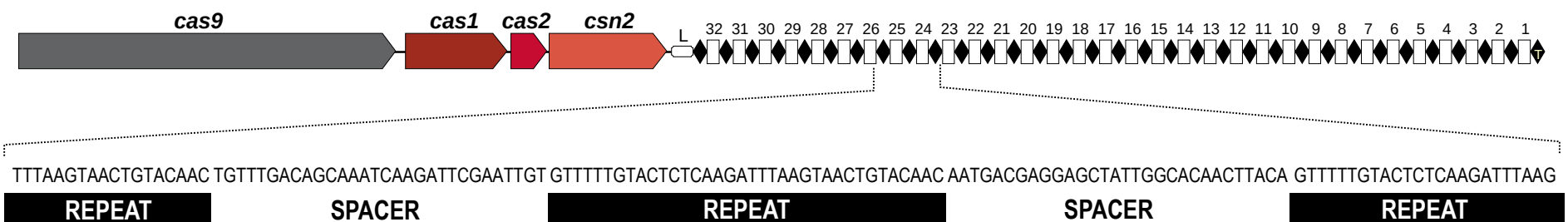
Figure 2 de l'article "**The Heroes of CRISPR**" (2016), Lander ES, *Cell* **164**, pp. 18-28.

CRISPR-Cas

- **C**lustered
- **R**egularly
- **I**nterspaced
- **S**hort
- **P**alindromic
- **R**epeats

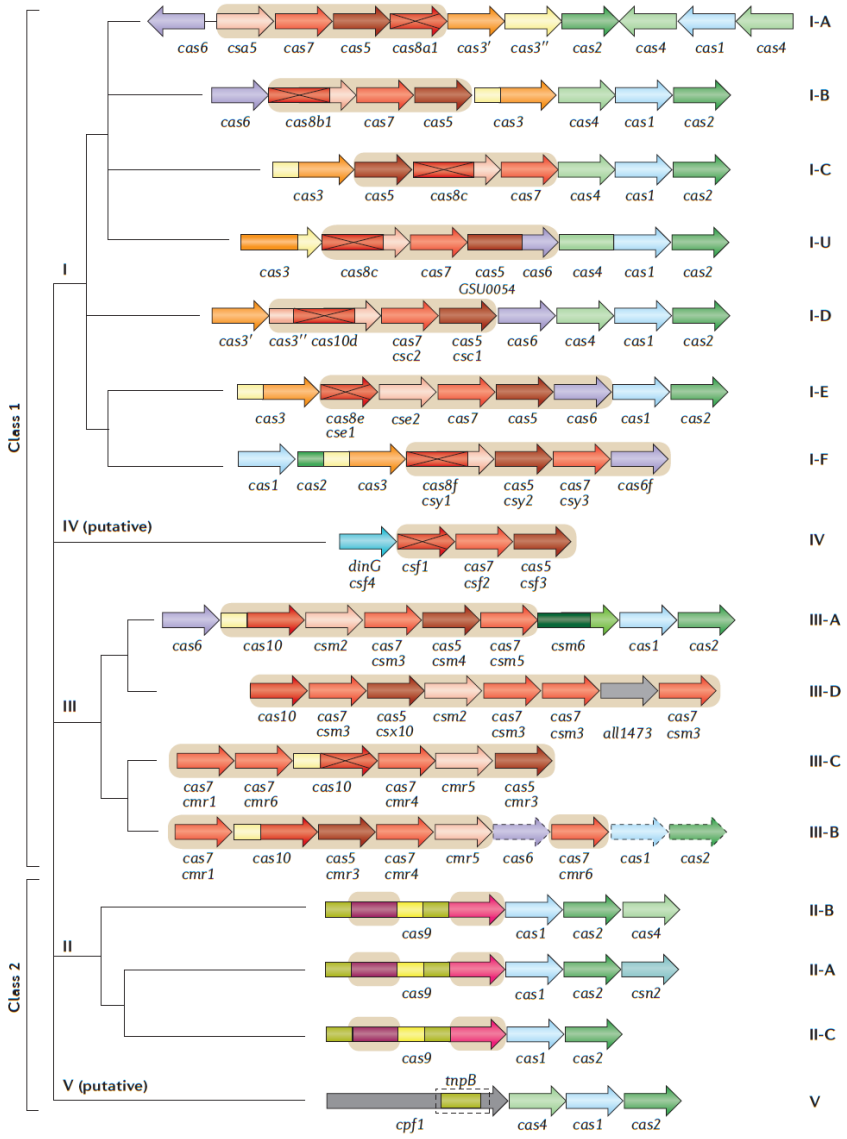
- **C**RISPR-
- **as**sociated

taagatattctcagacacctgataaggaactattacataaatttttagaaagtaaggatt
gacaaggacagttattgattttataatcactatgtgggtataaaaacgtcaaaatttcat
ttgag**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**tgtttgacagcaaatcaag
attcgaattgt**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**aatgacgaggagc
tattggcacaacttaca**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**cgatttg
acaatctgctgaccactgttatc**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**a
cacttggcaggcttattactcaacagcga**GTTTTTGTACTCTCAAGATTTAAGTAACTGT**
ACAACctgttccttgttcttttgttgtatcttttc**GTTTTTGTACTCTCAAGATTTAAGT**
AACTGTACAACttcattcttccgttttgtttgcgaatcct**GTTTTTGTACTCTCAAGAT**
TTAAGTAACTGTACAACgctggcgaggaaacgaacaaggcctcaaca**GTTTTTGTACTCT**
CAAGATTTAAGTAACTGTACAACcatagagtggaaaactagaaacagattcaa**GTTTTTG**
TACTCTCAAGATTTAAGTAACTGTACAACataatgccgttgaattacacggcaaggtca**G**
TTTTTGTACTCTCAAGATTTAAGTAACTGTACAACgagcgagctcgaaataatcttaatt
acaag**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**gttcgctagcgtcatgtgg
taacgtattta**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**ggcgtcccaatcc
tgattaatacttactcg**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**aacacag
caagacaagaggatgatgctatg**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**c
gacacaagaacgtatgcaagagttcaag**GTTTTTGTACTCTCAAGATTTAAGTAACTGTA**
CAACacaattcttcatccggtaactgctcaagtg**GTTTTTGTACTCTCAAGATTTAAGTA**
ACTGTACAACaattaagggcatagaaagggagacaacatg**GTTTTTGTACTCTCAAGATT**
TAAGTAACTGTACAACcgatatttaaaatcattttcataacttcat**GTTTTTGTACTCTC**
AAGATTTAAGTAACTGTACAACgcagtatcagcaagcaagctgttagttact**GTTTTTGT**
ACTCTCAAGATTTAAGTAACTGTACAACataaactatgaaattttataattttaaga**GT**
TTTTGTACTCTCAAGATTTAAGTAACTGTACAACaataatttatggtatagcttaatatc
attg**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**tgcatcgagcacgttcgagt
ttaccgtttc**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**tctatatcgaggtc
aactaacaattatgct**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**aatcgttc
aaattctgttttaggtacattt**GTTTTTGTACTCTCAAGATTTAAGTAACTGTACAAC**aa
tcaatacgacaagagttaaaatggtctt**GTTTTTGTACTCTCAAGATTTAAGTAACTGTA**
CAACgcttagctgtccaatccacgaacgtggatg**GTTTTTGTACTCTCAAGATTTAAGTA**
ACTGTACAACcaaccaacggtaacagctacttttacagt**GTTTTTGTACTCTCAAGATT**



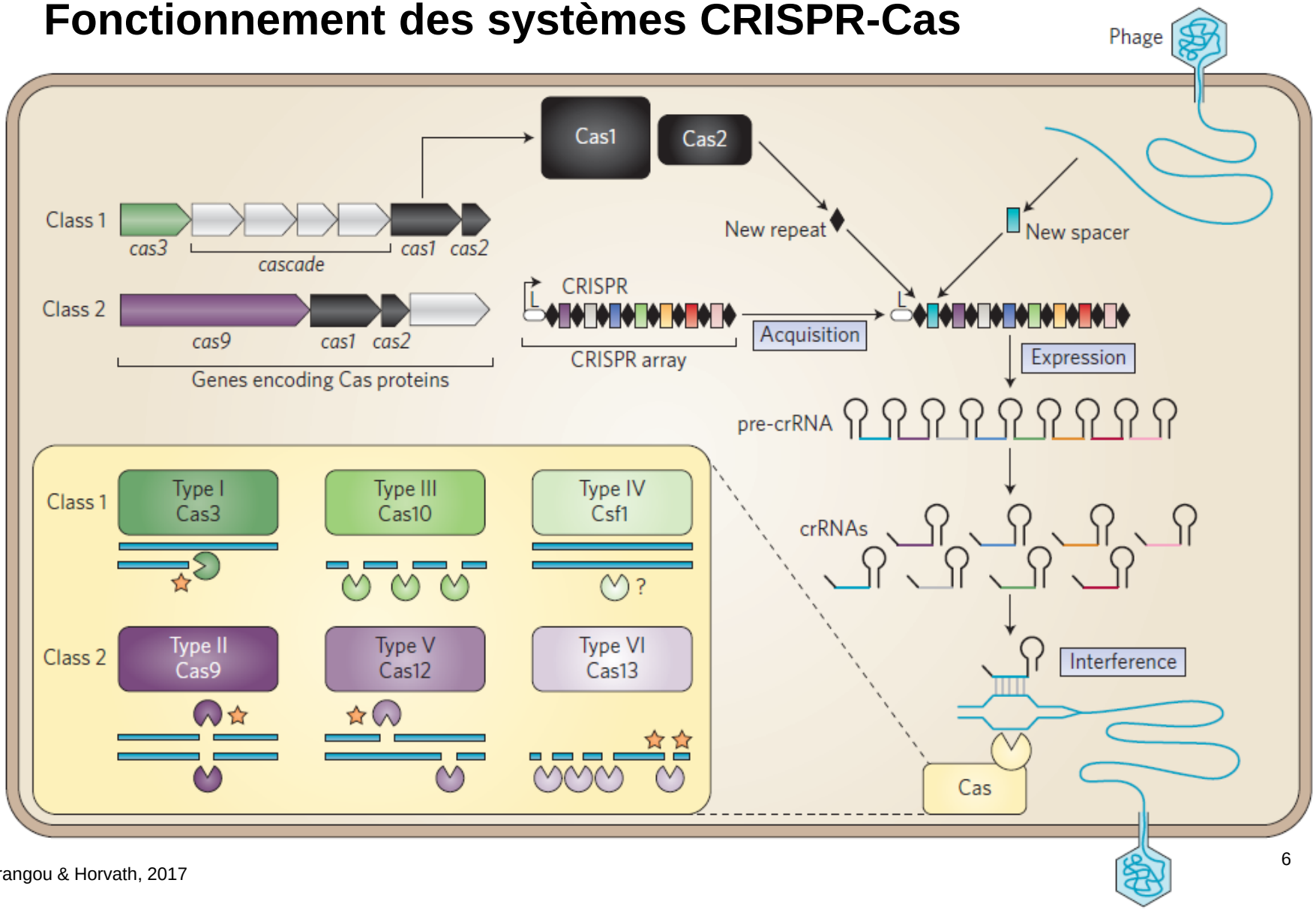
Systèmes CRISPR-Cas

- Très grande diversité de contenu et d'organisation des gènes *cas*
- Classification complexe, révisée plusieurs fois
- Simplifiée en :
  - 2 classes principales
  - 6 types
  - Plusieurs sous-types identifiés par des gènes "signature"
- *cas1–cas2* sont "universels"
- La présence de systèmes CRISPR-Cas est une spécificité de souche



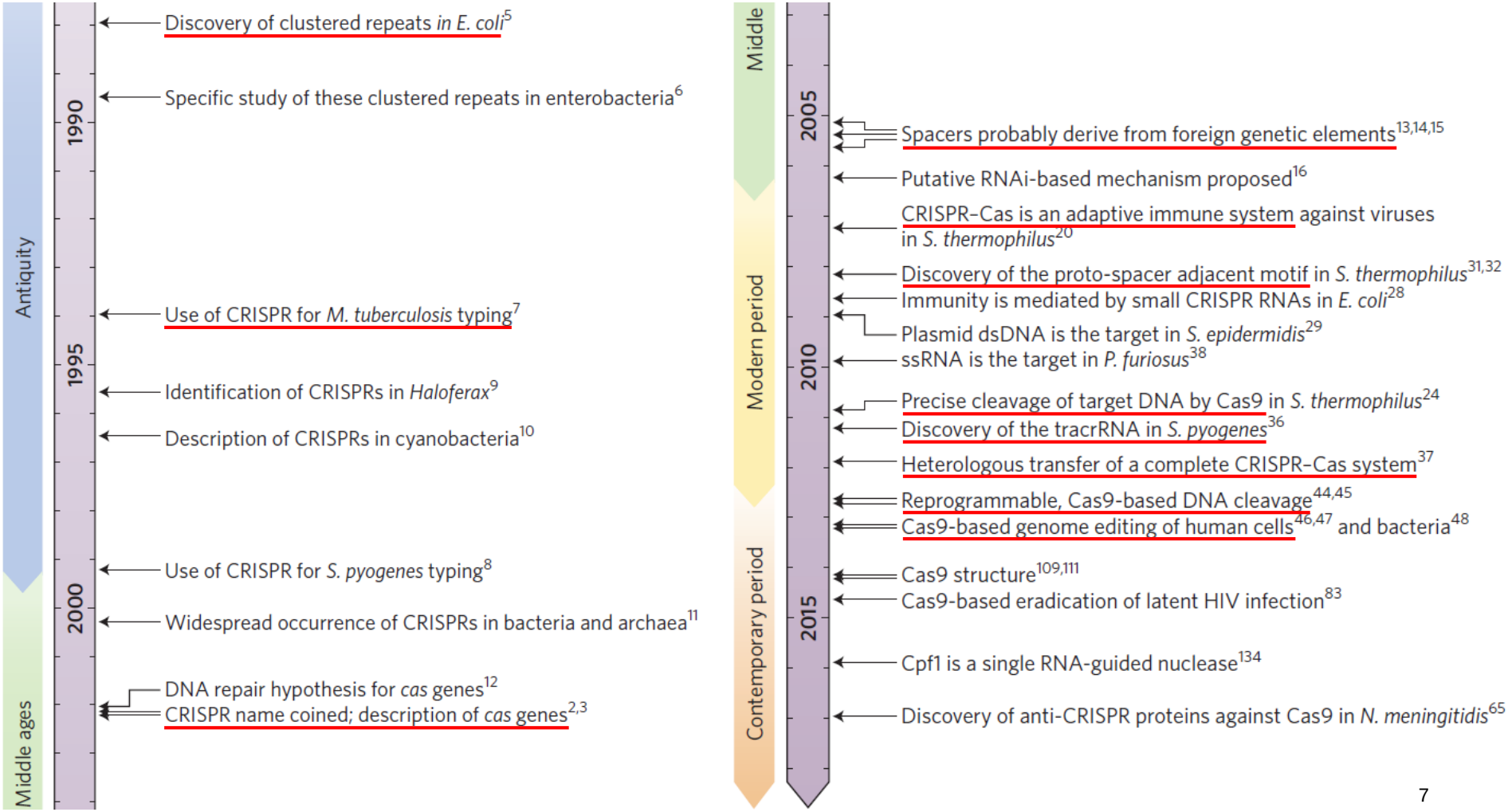
Makarova *et al.*, 2015

Fonctionnement des systèmes CRISPR-Cas



Barrangou & Horvath, 2017

CRISPR-Cas : déjà 30 ans d'histoire !



Barrangou & Horvath, 2017

Découverte fortuite de séquences répétées chez *E. coli*

JOURNAL OF BACTERIOLOGY, Dec. 1987, p. 5429–5433 Vol. 169, No. 12
0021-9193/87/125429-05$02.00/0

Nucleotide Sequence of the *iap* Gene, Responsible for Alkaline Phosphatase Isozyme Conversion in *Escherichia coli*, and Identification of the Gene Product

YOSHIZUMI ISHINO, HIDEO SHINAGAWA, KOZO MAKINO, MITSUKO AMEMURA, AND ATSUO NAKATA*

```
TGAAAATGGGAGGGAGTTCTACCGCAGAGGCGGGGGAACTCCAAGTGATATCCATCATCGCATCCAGTGCGCC (1,451)
    (1,452) CGGTTTATCCCCGCTGATGCGGGGAACACCAGCGTCAGGCGTGAAATCTCACCGTCGTTGC (1,512)
    (1,513) CGGTTTATCCCTGCTGGCGCGGGGAACTCTCGGTTCAGGCGTTGCAAACCTGGCTACCGGG (1,573)
    (1,574) CGGTTTATCCCCGCTAACGCGGGGAACTCGTAGTCCATCATTCCACCTATGTCTGAACTCC (1,634)
    (1,635) CGGTTTATCCCCGCTGGCGCGGGGAACTCG (1,664)

consensus: CGGTTTATCCCCGCT(GG/AA)CGCGGGGAACTC
```

FIG. 5. Comparison of direct-repeat sequences consisting of 61 base pairs in the 3′-end flanking region of *iap*. The 29 highly conserved nucleotides, which contain a dyad symmetry of 14 base pairs (underlined), are shown at the bottom. Homologous nucleotides found in at least two DNA segments are shown in boldface type. The second translational termination codon is boxed. The nucleotide numbers are in parentheses.

Génotypage de bactéries pathogènes

Molecular Microbiology (1993) **10**(5), 1057–1065

Nature of DNA polymorphism in the direct repeat cluster of *Mycobacterium tuberculosis*; application for strain differentiation by a novel typing method

Peter M. A. Groenen,[1,†] Annelies E. Bunschoten,[1]
Dick van Soolingen[2] and Jan D. A. van Embden[1]*

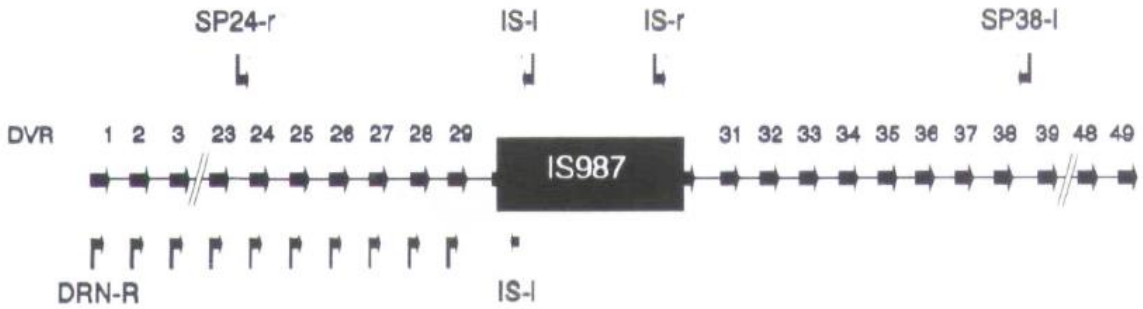


Fig. 1. Schematic representation of the DR region of *M. bovis* BCG. The IS*6110*-like element IS*987* resides in DR 30 (Hermans *et al.*, 1991). The primers used for PCR are drawn as shaded arrows, with M13 tags depicted as vertical lines. The black arrows represent the 36 bp DRs, intervened by spacers with sizes ranging from 35 to 41 bp. N represents either one of the four nucleotides used in individual DR-spacer driver primers.

CRISPR, une nouvelle famille de séquences répétées d'ADN

OMICS A Journal of Integrative Biology Volume 6, Number 1, 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]

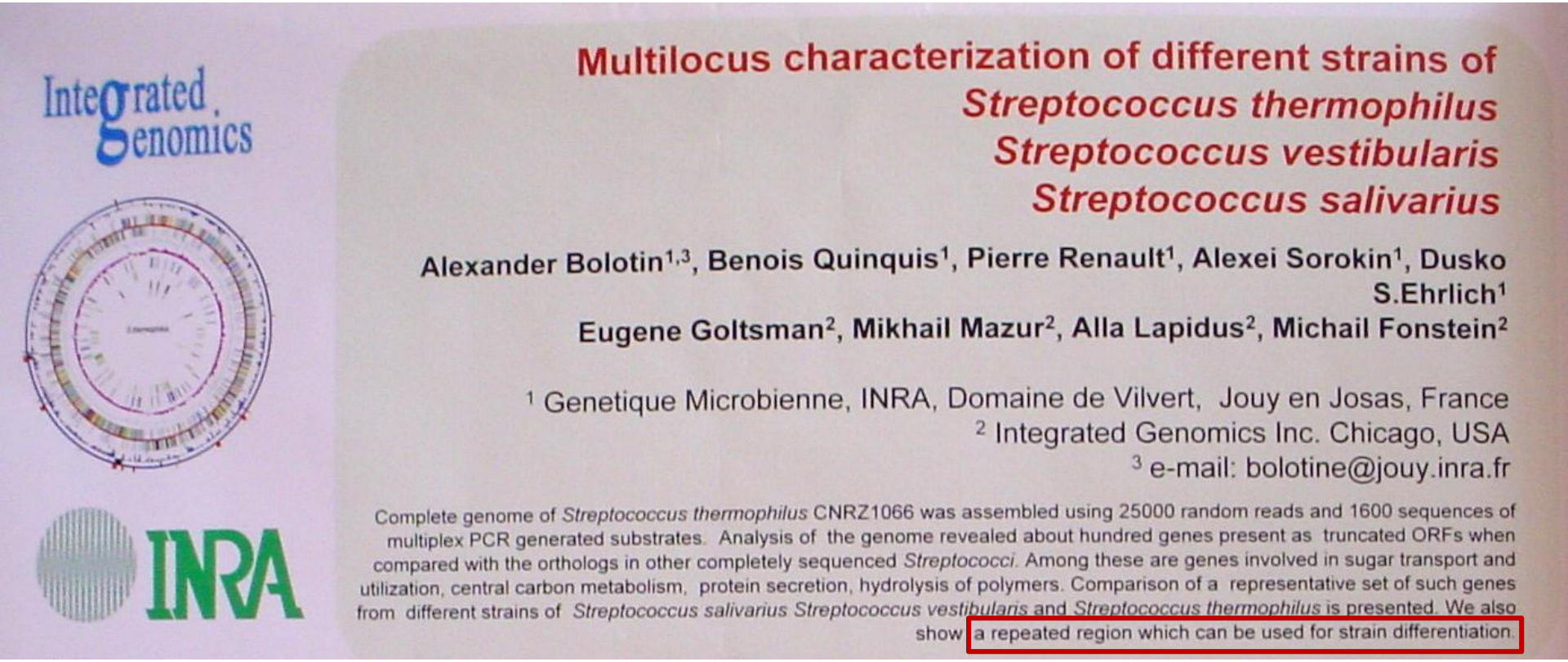
Identification of genes that are associated with DNA repeats in prokaryotes

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

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

Présence de CRISPR dans la bactérie du yaourt

- Poster présenté au *7th Symposium on Lactic Acid Bacteria* (Pays-Bas, septembre 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.

2005 : émergence de l'hypothèse anti-virale

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

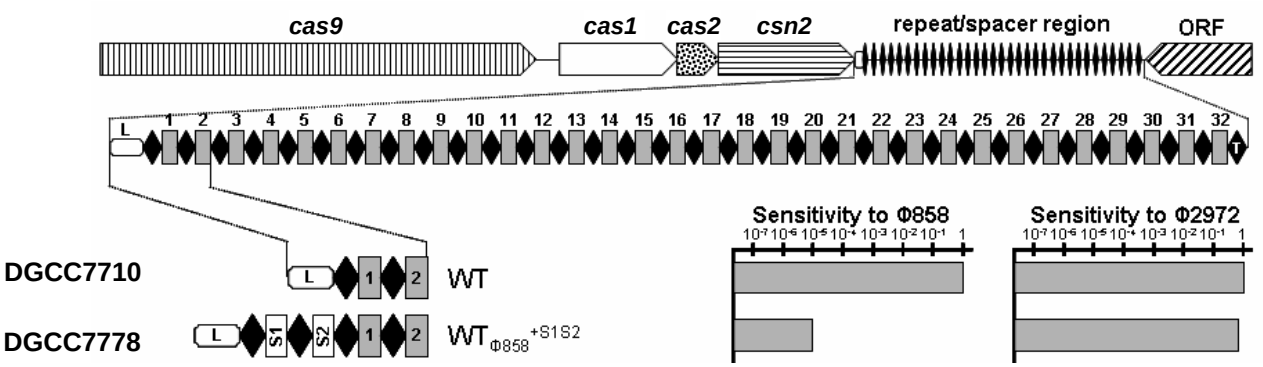
1ère démonstration du rôle biologique de CRISPR-Cas

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



Découverte du PAM (*proto-spacer adjacent motif*)

JOURNAL OF BACTERIOLOGY, Feb. 2008, p. 1390–1400 Vol. 190, No. 4
0021-9193/08/$08.00+0 doi:10.1128/JB.01412-07

Phage Response to CRISPR-Encoded Resistance in *Streptococcus thermophilus*▽

Hélène Deveau,[1] Rodolphe Barrangou,[2] Josiane E. Garneau,[1] Jessica Labonté,[1] Christophe Fremaux,[3] Patrick Boyaval,[3] Dennis A. Romero,[2] Philippe Horvath,[3] and Sylvain Moineau[1]*

Received 31 August 2007/Accepted 21 November 2007

DGCC7710$_{\Phi 2972}^{+S4}$	CTCAGTCGTTACTGGTGAACCAGTTTCAAT
Phage 2972	CTCAGTCGTTACTGGTGAACCAGTTTCAATTGAGAAAAA
Phage 2972.S4A	CTCAGTCGTTACTGGTGAACCAGTTTCAATTGA**A**AAAAA
Phage 2972.S4B	CTCAGTCGTTACTGGTGAACCAGTTTC**G**ATTGAGAAAAA
Phage 2972.S4C	CTCAGTCGTTACTGGTGAACCAGTTTCAATTGAGA**G**AAA
Phage 2972.S4D	CTCAGTCGTTACTGGTGAACC**G**GTTTCAATTGA**A**AAAAA

Coupure précise de l'ADN cible par Cas9

The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA

Josiane E. Garneau[1], Marie-Ève Dupuis[1], Manuela Villion[1], Dennis A. Romero[2], Rodolphe Barrangou[2], Patrick Boyaval[3], Christophe Fremaux[3], Philippe Horvath[3], Alfonso H. Magadán[1] & Sylvain Moineau[1]

4 NOVEMBER 2010 | VOL 468 | NATURE | 67

Table 2 | Cleavage sites within the proto-spacers in bacteriophage 2972 or plasmid pNT1

BIM or PIM	Spacer (size)	Homology (position)	Strand	Proto-spacer* (5′ to 3′)	PAM†
DGCC7710$_{\Phi 2972}^{+S4}$	S4 (30 nt)	Bacteriophage 2972 (31582)	+	CTCAGTCGTT↓ACTGGTGAACCAGTTTC↓AAT	TGAGAAA
DGCC7710$_{\Phi 2972}^{+S4}{}_{\Phi 858}^{+S32}$	S4 (30 nt)	Bacteriophage 2972 (31582)	+	CTCAGTCGTT↓ACTGGTGAACCAGTTTC↓AAT	TGAGAAA
DGCC7710$_{\Phi 2972}^{+S4}{}_{\Phi 858}^{+S32}$	S32 (30 nt)	Bacteriophage 2972 (33044)	+	ATTGTCTATTA↓CGACAACATGGAAGAT↓GAT	GTAGAAA
DGCC7710$_{\Phi 2972}^{+S40}$	S40 (29 nt)	Bacteriophage 2972 (31583)	+	TCAGTCGTT↓ACTGGTGAACCAGTTTC↓AAT	TGAGAAA
DGCC7710$_{\Phi 2972}^{+S7}$	S7 (30 nt)	Bacteriophage 2972 (10299)	−	AAGCAAGTTGATATATTTCTCTTTCTT↓TAT	TAAGAAA
DGCC7710$_{\Phi 2972}^{+S41}$	S41 (30 nt)	Bacteriophage 2972 (31518)	−	TTCCCTTCGATAATGGCAAGACCGAAA↓CGT	TCAGAAA
DGCC7710$_{\Phi 2972}^{+S42}$	S42 (30 nt)	Bacteriophage 2972 (31084)	−	ATATTCATATTCCCTGCTCATGTTTGA↓TAG	CAAGAAT
DGCC7710$_{pNT1}^{+S46}$	S46 (30 nt)	Plasmid pNT1 (528)	−	TTTCCCAATCTTCTGGAATTGAATCGG↓GAT	AGAGTAG

BIM, bacteriophage-insensitive mutant; nt, nucleotide; PAM, proto-spacer adjacent motif; PIM, plasmid-interfering mutant.

* ↓ Indicates the cleavage site

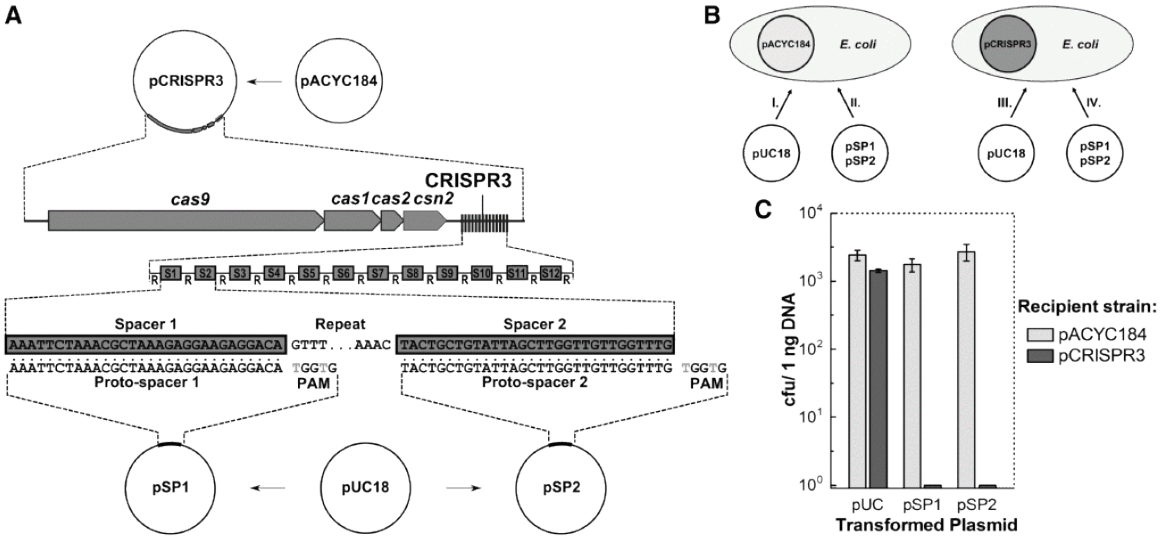
† The mismatches with the consensus proto-spacer adjacent motif (NNAGAAW) are underlined.

Transfert hétérologue d'un système CRISPR-Cas

Published online 3 August 2011 *Nucleic Acids Research, 2011, Vol. 39, No. 21* **9275–9282**
doi:10.1093/nar/gkr606

The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*

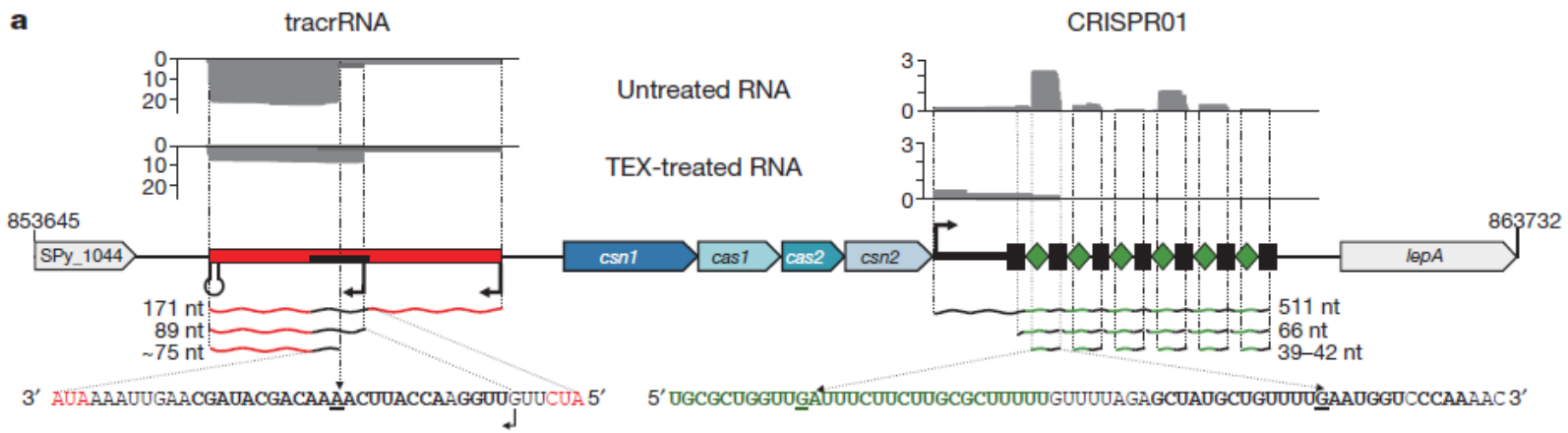
Rimantas Sapranauskas[1], Giedrius Gasiunas[1], Christophe Fremaux[2], Rodolphe Barrangou[3], Philippe Horvath[2] and Virginijus Siksnys[1,*]

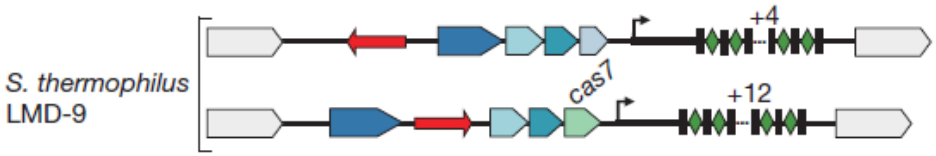


Découverte du tracrRNA, le "chaînon manquant"

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

2012 : invention de l'ARN guide (single guide-RNA)

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.

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[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.
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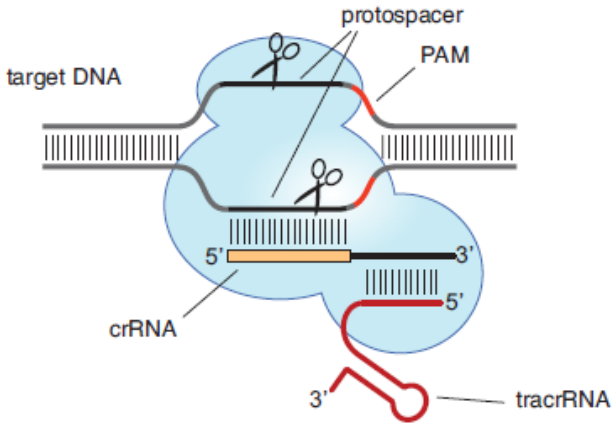
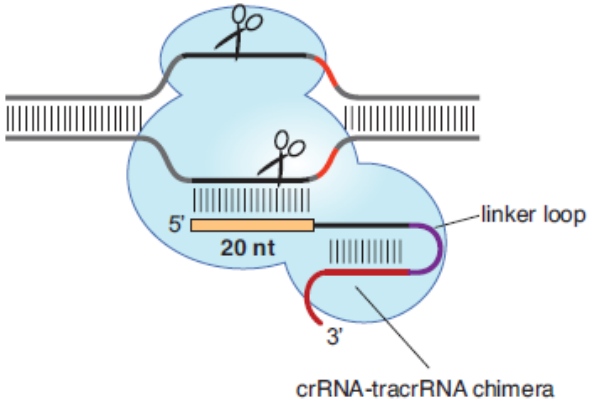
Read full article: scim.ag/1piiXv7

Cas9 programmed by crRNA:tracrRNA duplex

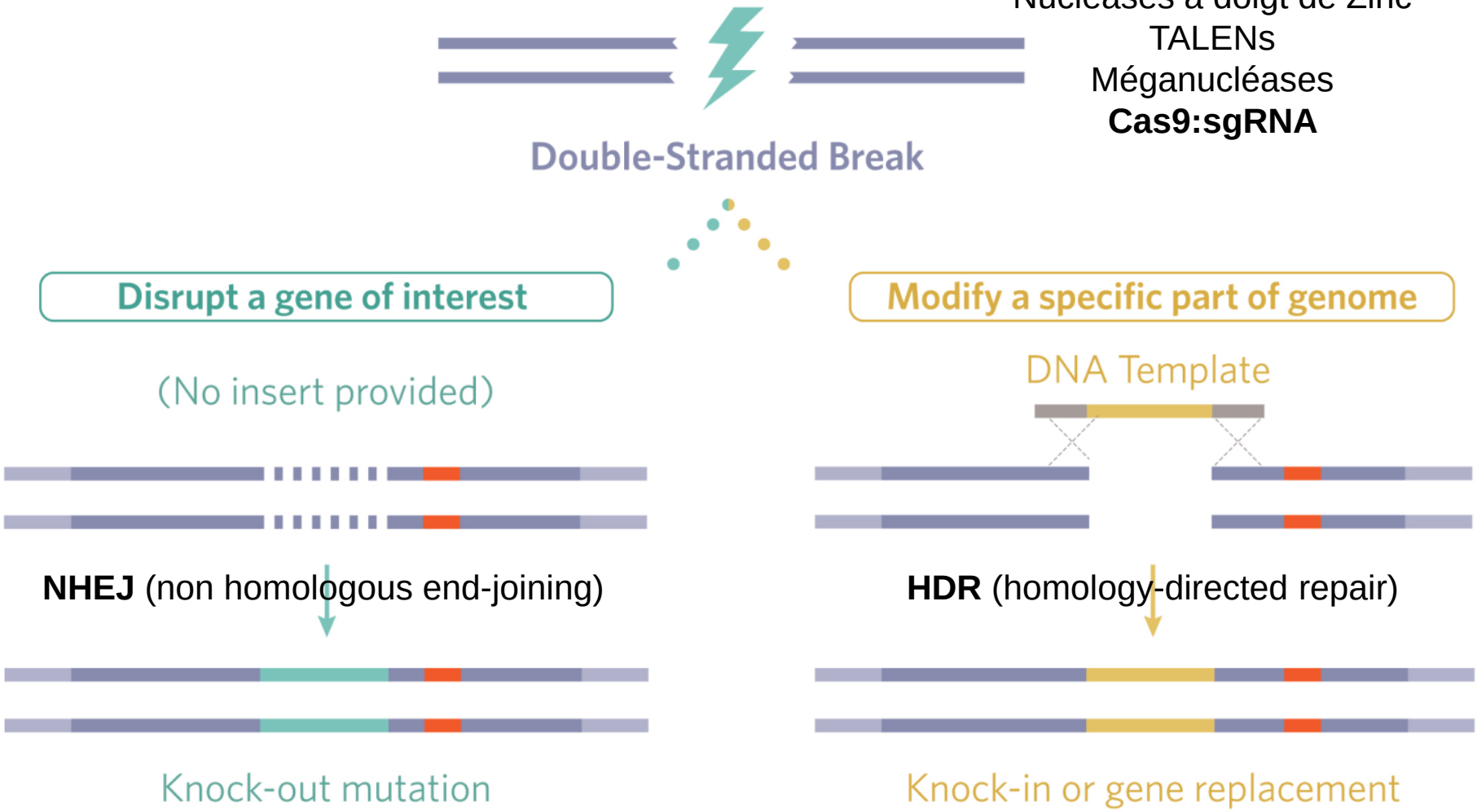


Cas9 programmed by single chimeric RNA



Principe du *genome editing*

Nucléases à doigt de Zinc
TALENs
Méganucléases
Cas9:sgRNA



2013 : premières utilisations de Cas9 chez des mammifères

Multiplex genome engineering using CRISPR/Cas systems

Le Cong,[1,2*] F. Ann Ran,[1,4,*] 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

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[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.

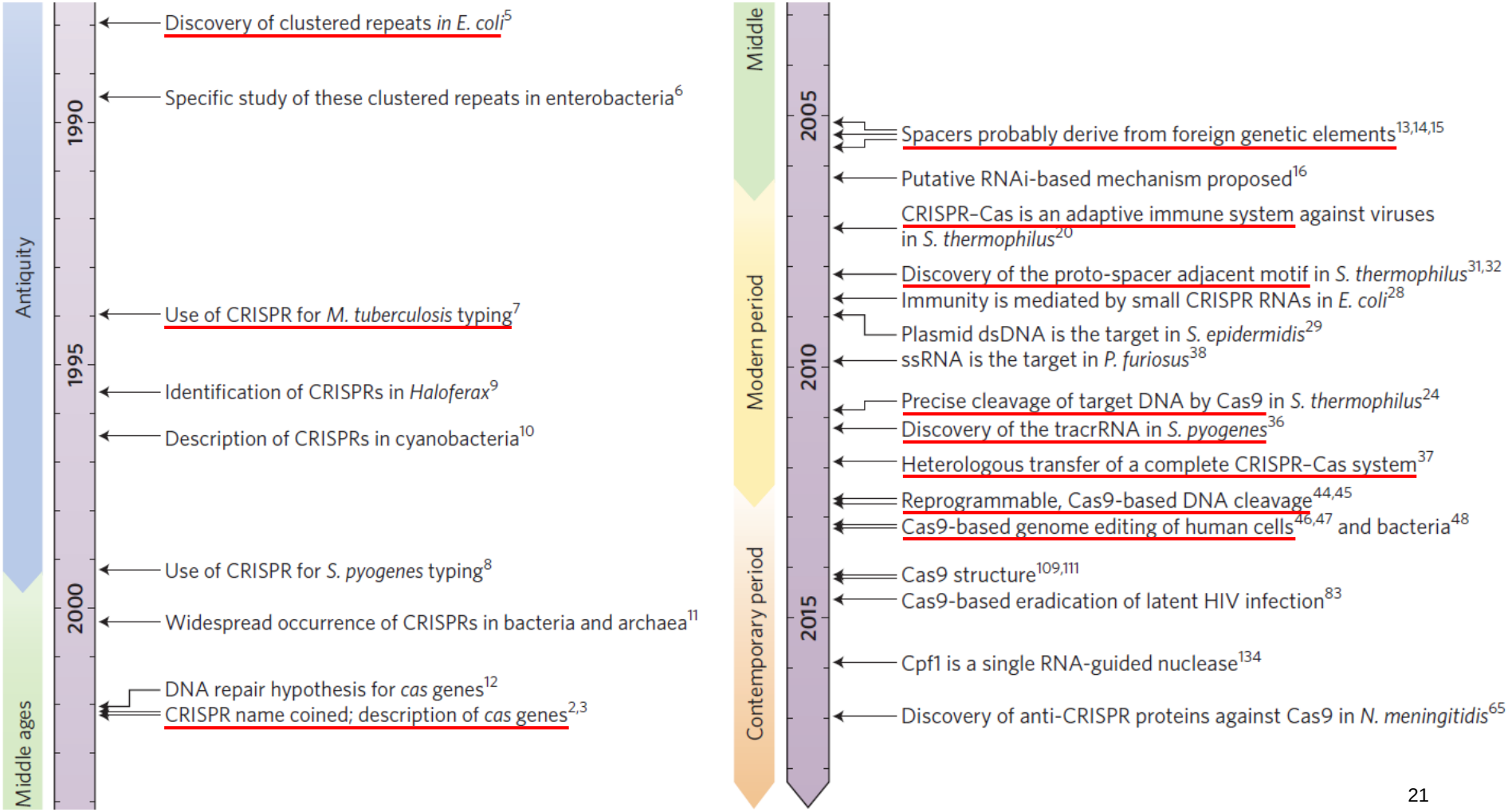
RNA-guided human genome engineering via Cas9

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

CRISPR-Cas : déjà 30 ans d'histoire !



Barrangou & Horvath, 2017

Certains phages possèdent des systèmes anti-CRISPR

An anti-CRISPR from a virulent streptococcal phage inhibits *Streptococcus pyogenes* Cas9

Alexander P. Hynes[1], Geneviève M. Rousseau[1], Marie-Laurence Lemay[1], Philippe Horvath[2], Dennis A. Romero[3], Christophe Fremaux[2] and Sylvain Moineau[1,4*]

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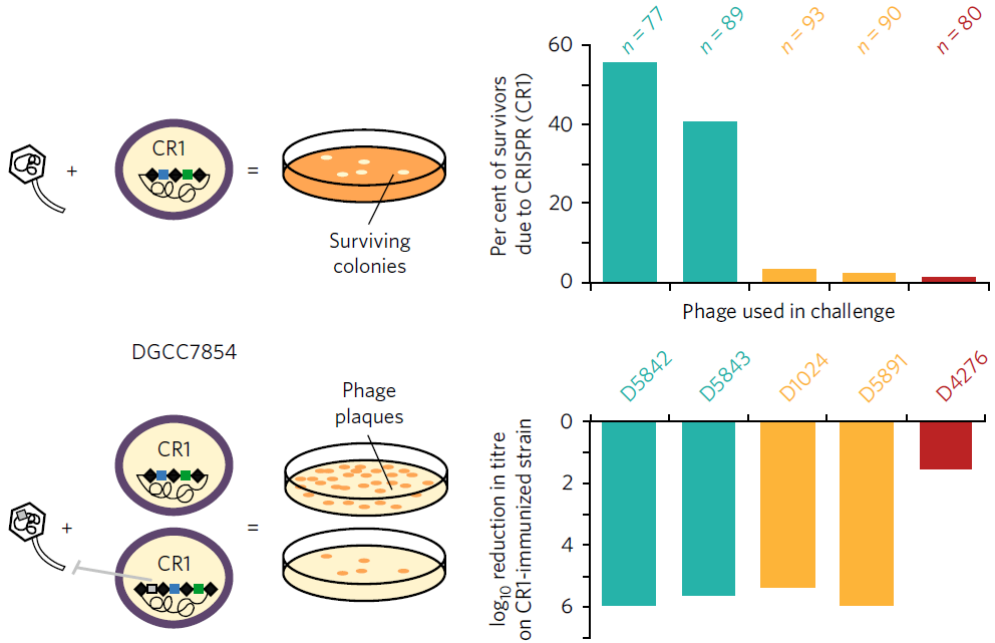
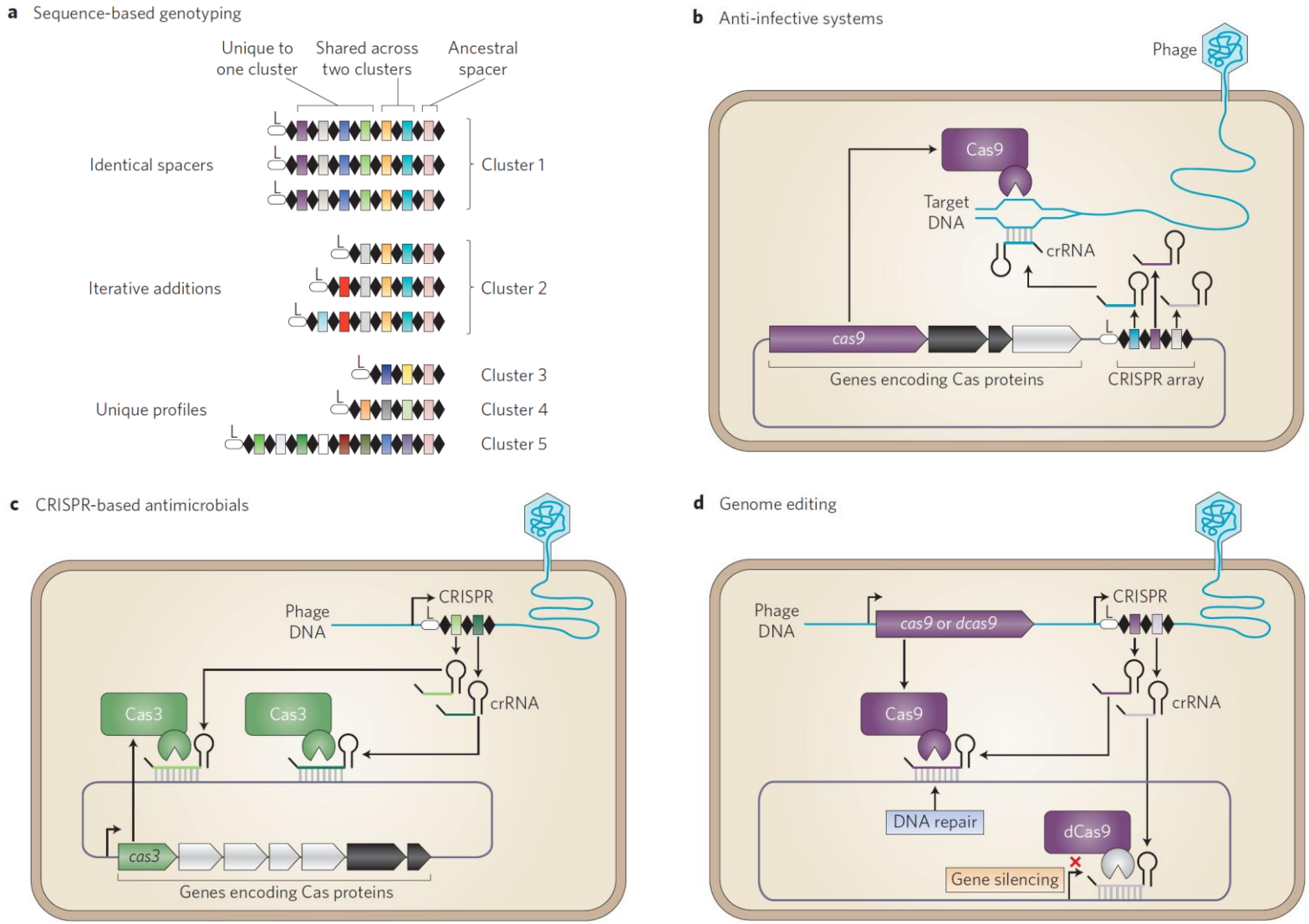


Applications bactériennes des systèmes CRISPR-Cas



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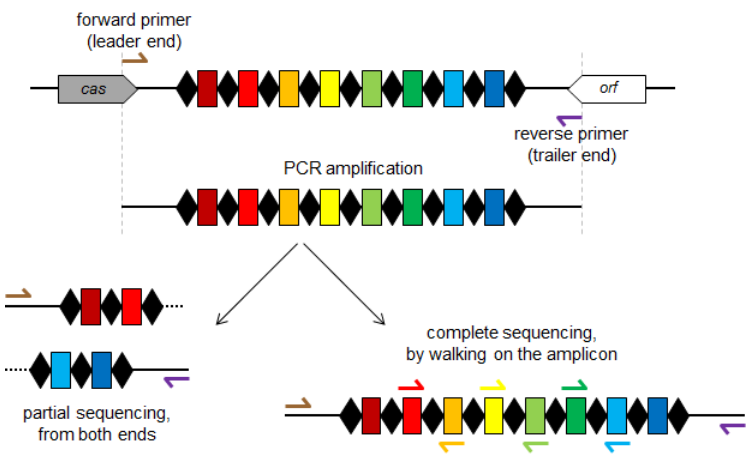
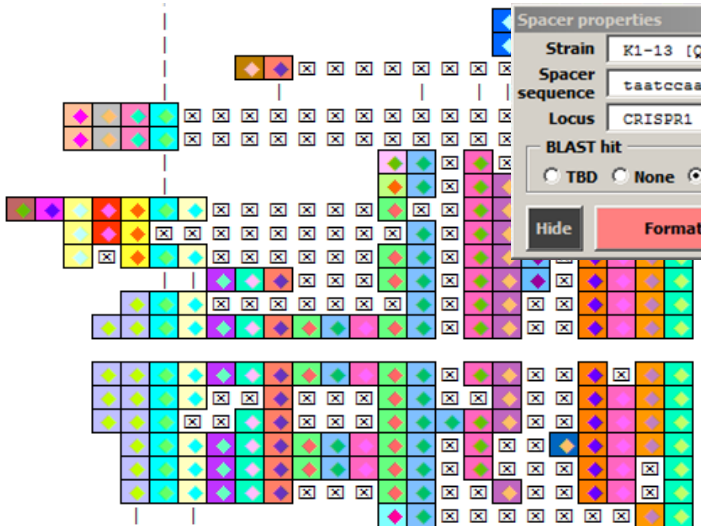
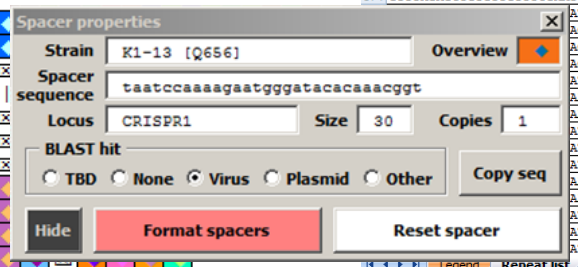
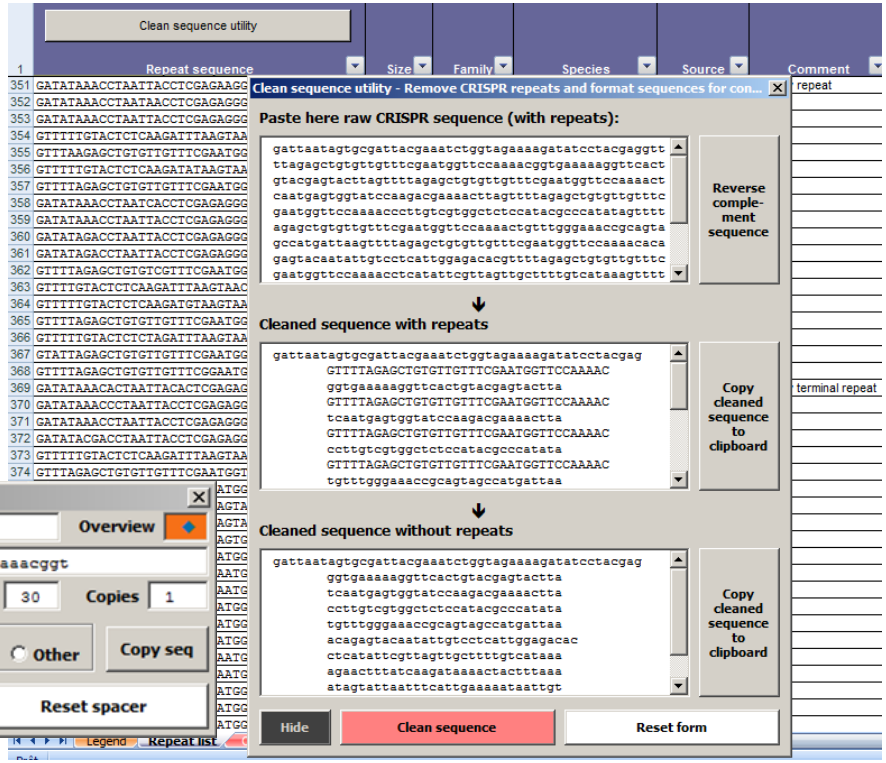
Génotypage bactérien



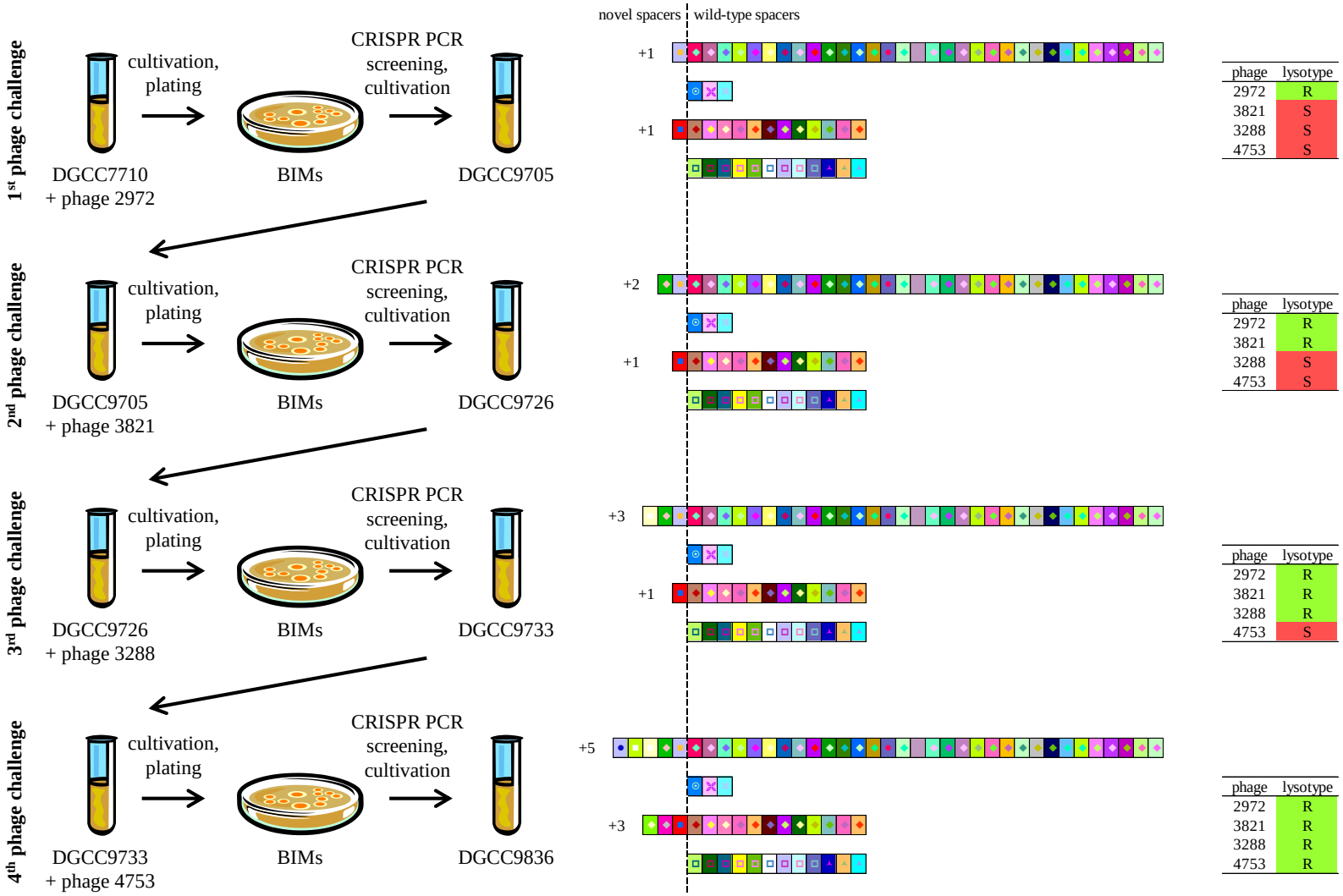






Streptococcus thermophilus, *Lactococcus lactis*, *Lactobacillus* spp., *Bifidobacterium lactis*, *Escherichia coli*, *Salmonella enterica*, *Streptococcus agalactiae*, *Clostridium difficile*…

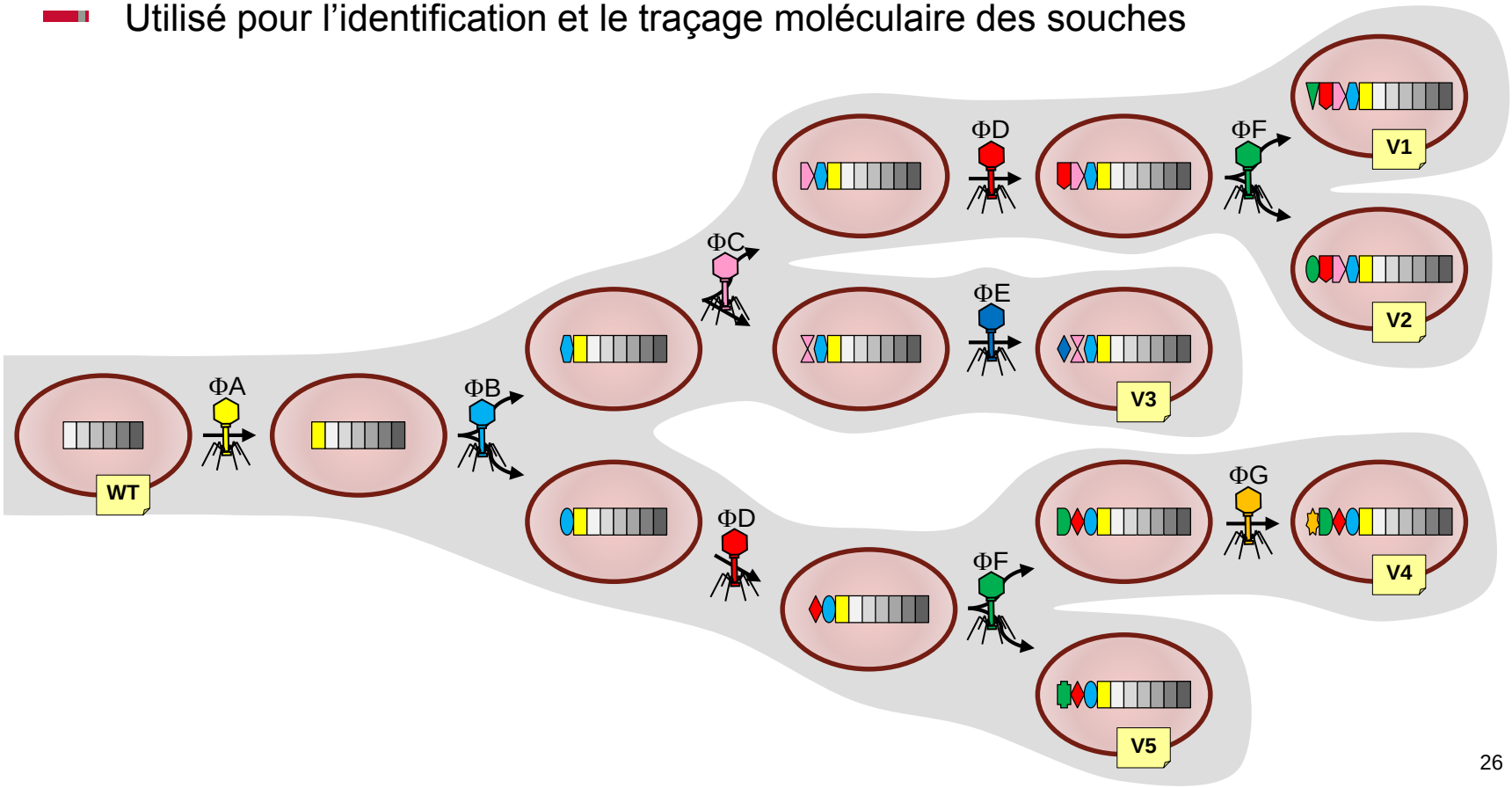
"CRISPérisation" : un processus naturel de vaccination



Barrangou *et al.*, 2013

Marquage génétique naturel

- Les spacers nouvellement acquis constituent une signature génétique
- Les combinaisons de spacers acquis indépendamment sont uniques
- Utilisé pour l'identification et le traçage moléculaire des souches



Horvath *et al*., 2012

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