

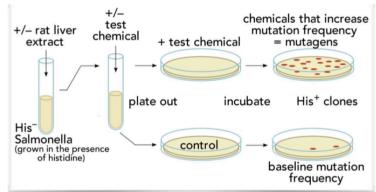


## Past questions?

## Ames test for mutagenic chemicals

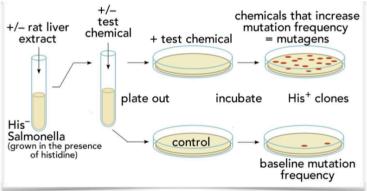
You should know that there are additional mutations in the bacterial strains used in the Ames test. Both of the strains have a defect in the lipopolysaccharide cell wall causing them to be more vulnerable to exogenous mutagens. Additionally, both strains have defective DNA excision-repair mechanisms. These mechanisms would normally correct mutations arising during DNA replication or from exogenous mutagens.

" ... most (as in 99.99%, according to his estimate) of the pesticides in the human diet are those found in the plants themselves. The cruciferous vegetables (broccoli, cabbage, mustard and so on) are particularly rich in compounds that will light up an Ames test. A fine article of his from 1990 (Ang. Chem. Int. Ed., 29, 1197) states that "... it is probably true that almost every plant product in the supermarket contains natural carcinogens.



The Ames Test and the Real World: Derek Lowe July 30, 2002

Ames's point is that the mental  
have between "artificial"  
or "synthetic" chemicals (bad)  
and "natural" ones (good) is nonsense.



The same number of toxic compounds are found in each category, and we're exposed to far more of the latter. Instead of worrying about parts-per-billion of pesticide residues, we should worry about greater public health risks like smoking, alcohol, etc.

The Ames Test and the Real World: [Derek Lowe July 30, 2002](#)

We're back to "the dose makes the poison." The principle applies not only to people who are exposed to huge doses of chemicals, but to unlucky lab rats as well.

Ames has forcefully made the point that testing compounds in animals at or near their maximum tolerated dose (MTD) is a poor measure of their cancer-causing potential. About half the compounds so tested show up as carcinogens, but the dose-response curves aren't linear.

The Ames Test and the Real World: [Derek Lowe July 30, 2002](#)

**Question:** Which is likely to be more toxic, an artificial (synthetic) molecule, or a natural (biological) molecule, and why?

When mutations are induced by radiation....

mutations that produce the phenotype of interest occur together with other mutations throughout the genome.

These must be removed by "back-crossing" to wild type individuals (sufficiently so that recombination can disconnect them).

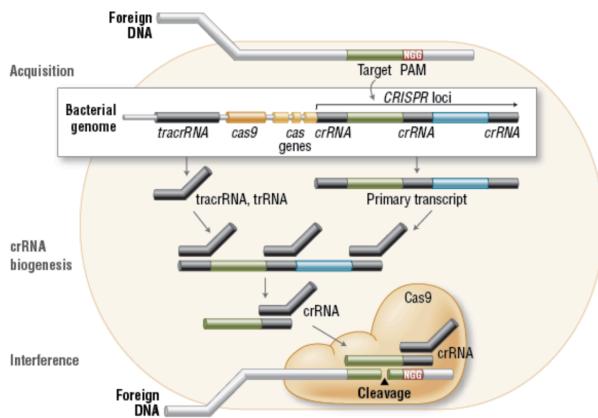
### Rational mutagenesis

What is needed for rationale mutagenesis?



## CRISPR READING

Figure 1. Cas9 *in vivo*: Bacterial Adaptive Immunity



## CRISPR CAS TIME LINE LINK (BROAD SITE)

### **Discovery of CRISPR and its function**

1993 - 2005 — Francisco Mojica, University of Alicante, Spain

Francisco Mojica was the first researcher to characterize what is now called a CRISPR locus, reported in 1993. He worked on them throughout the 1990s, and in 2000, he recognized that what had been reported as disparate repeat sequences actually shared a common set of features, now known to be hallmarks of CRISPR sequences (he coined the term CRISPR through correspondence with Ruud Jansen, who first used the term in print in 2002). In 2005 he reported that these sequences matched snippets from the genomes of bacteriophage (Mojica et al., 2005). This finding led him to hypothesize, correctly, that CRISPR is an adaptive immune system. Another group, working independently, published similar findings around this same time (Pourcel et al., 2005).

Long stretches of short tandem repeats are present in the largest replicons of the Archaea *Haloferax mediterranei* and *Haloferax volcanii* and could be involved in replicon partitioning. Mojica et al., 1995.

## CRISPR CAS TIME LINE LINK (BROAD SITE)

### **Discovery of Cas9 and PAM**

May, 2005 — Alexander Bolotin, French National Institute for Agricultural Research (INRA)

Bolotin was studying the bacteria *Streptococcus thermophilus*, which had just been sequenced, revealing an unusual CRISPR locus (Bolotin et al., 2005). Although the CRISPR array was similar to previously reported systems, it lacked some of the known cas genes and instead contained novel cas genes, including one encoding a large protein they predicted to have nuclease activity, which is now known as Cas9. Furthermore, they noted that the spacers, which have homology to viral genes, all share a common sequence at one end. This sequence, the protospacer adjacent motif (PAM), is required for target recognition.

## CRISPR CAS TIME LINE LINK (BROAD SITE)

### **Hypothetical scheme of adaptive immunity**

March, 2006 — Eugene Koonin, US National Center for Biotechnology Information, NIH

Koonin was studying clusters of orthologous groups of proteins by computational analysis and proposed a hypothetical scheme for CRISPR cascades as bacterial immune system based on inserts homologous to phage DNA in the natural spacer array, abandoning previous hypothesis that the Cas proteins might comprise a novel DNA repair system. (Makarova et al., 2006)

## CRISPR CAS TIME LINE LINK (BROAD SITE)

### **Experimental demonstration of adaptive immunity**

March, 2007 — Philippe Horvath, Danisco France SAS

*S. thermophilus* is widely used in the dairy industry to make yogurt and cheese, and scientists at Danisco wanted to explore how it responds to phage attack, a common problem in industrial yogurt making. Horvath and colleagues showed experimentally that CRISPR systems are indeed an adaptive immune system: they integrate new phage DNA into the CRISPR array, which allows them to fight off the next wave of attacking phage (Barrangou et al., 2007). Furthermore, they showed that Cas9 is likely the only protein required for interference, the process by which the CRISPR system inactivates invading phage, details of which were not yet known.

## CRISPR CAS TIME LINE LINK (BROAD SITE)

### **Spacer sequences are transcribed into guide RNAs**

August, 2008 — John van der Oost, University of Wageningen, Netherlands

Scientists soon began to fill in some of the details on exactly how CRISPR-Cas systems “interfere” with invading phage. The first piece of critical information came from John van der Oost and colleagues who showed that in *E-scherichia coli*, spacer sequences, which are derived from phage, are transcribed into small RNAs, termed CRISPR RNAs (crRNAs), that guide Cas proteins to the target DNA (Brouns et al., 2008).

## CRISPR CAS TIME LINE LINK (BROAD SITE)

### **CRISPR acts on DNA targets**

*December, 2008 — Luciano Marraffini and Erik Sontheimer, Northwestern University, Illinois*

The next key piece in understanding the mechanism of interference came from Marraffini and Sontheimer, who elegantly demonstrated that the target molecule is DNA, not RNA (Marraffini and Sontheimer, 2008). This was somewhat surprising, as many people had considered CRISPR to be a parallel to eukaryotic RNAi silencing mechanisms, which target RNA. Marraffini and Sontheimer explicitly noted in their paper that this system could be a powerful tool if it could be transferred to non-bacterial systems. [It should be noted, however, that a different type of CRISPR system can target RNA (Hale et al., 2009)].

## CRISPR CAS TIME LINE LINK (BROAD SITE)

### **Cas9 cleaves target DNA**

*December, 2010 — Sylvain Moineau, University of Laval, Quebec City, Canada*

Moineau and colleagues demonstrated that CRISPR-Cas9 creates double-stranded breaks in target DNA at precise positions, 3 nucleotides upstream of the PAM (Garneau et al., 2010). They also confirmed that Cas9 is the only protein required for cleavage in the CRISPR-Cas9 system. This is a distinguishing feature of Type II CRISPR systems, in which interference is mediated by a single large protein (here Cas9) in conjunction with crRNAs.

## CRISPR CAS TIME LINE LINK (BROAD SITE)

### **Discovery of tracrRNA for Cas9 system**

*March, 2011 — Emmanuelle Charpentier, Umea University, Sweden and University of Vienna, Austria*

The final piece to the puzzle in the mechanism of natural CRISPR-Cas9-guided interference came from the group of Emmanuelle Charpentier. They performed small RNA sequencing on *Streptococcus pyogenes*, which has a Cas9-containing CRISPR-Cas system. They discovered that in addition to the crRNA, a second small RNA exists, which they called trans-activating CRISPR RNA (tracrRNA) (Deltcheva et al., 2011). They showed that tracrRNA forms a duplex with crRNA, and that it is this duplex that guides Cas9 to its targets.

## [CRISPR CAS TIME LINE LINK \(BROAD SITE\)](#)

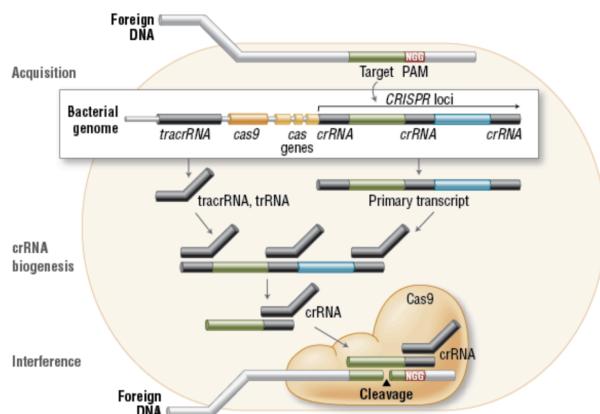
### **CRISPR systems can function heterologously in other species**

July, 2011 — Virginijus Siksnys, Vilnius University, Lithuania

Siksnys and colleagues cloned the entire CRISPR-Cas locus from *S. thermophilus* (a Type II system) and expressed it in *E. coli* (which does not contain a Type II system), where they demonstrated that it was capable of providing plasmid resistance (Sapranauskas et al., 2011). This suggested that CRISPR systems are self-contained units and verified that all of the required components of the Type II system were known.

### **CRISPR READING**

Figure 1. Cas9 *in vivo*: Bacterial Adaptive Immunity



## What is CRISPR?





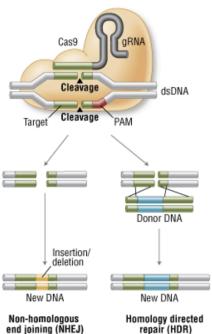
what does it mean to "knock out" a gene?

what type of mutation would "deactivate" Cas9's nuclease activity?

#### Choosing a Target Sequence for CRISPR/Cas9 Gene Editing

CRISPR/Cas9 gene targeting requires a custom single guide RNA (sgRNA) that contains a targeting sequence (crRNA sequence) and a Cas9 nuclease-recruiting sequence (tracrRNA). The crRNA region (shown in red below) is a 20-nucleotide sequence that is homologous to a region in your gene of interest and will direct Cas9 nuclease activity.

#### A. Genome Engineering With Cas9 Nuclease





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