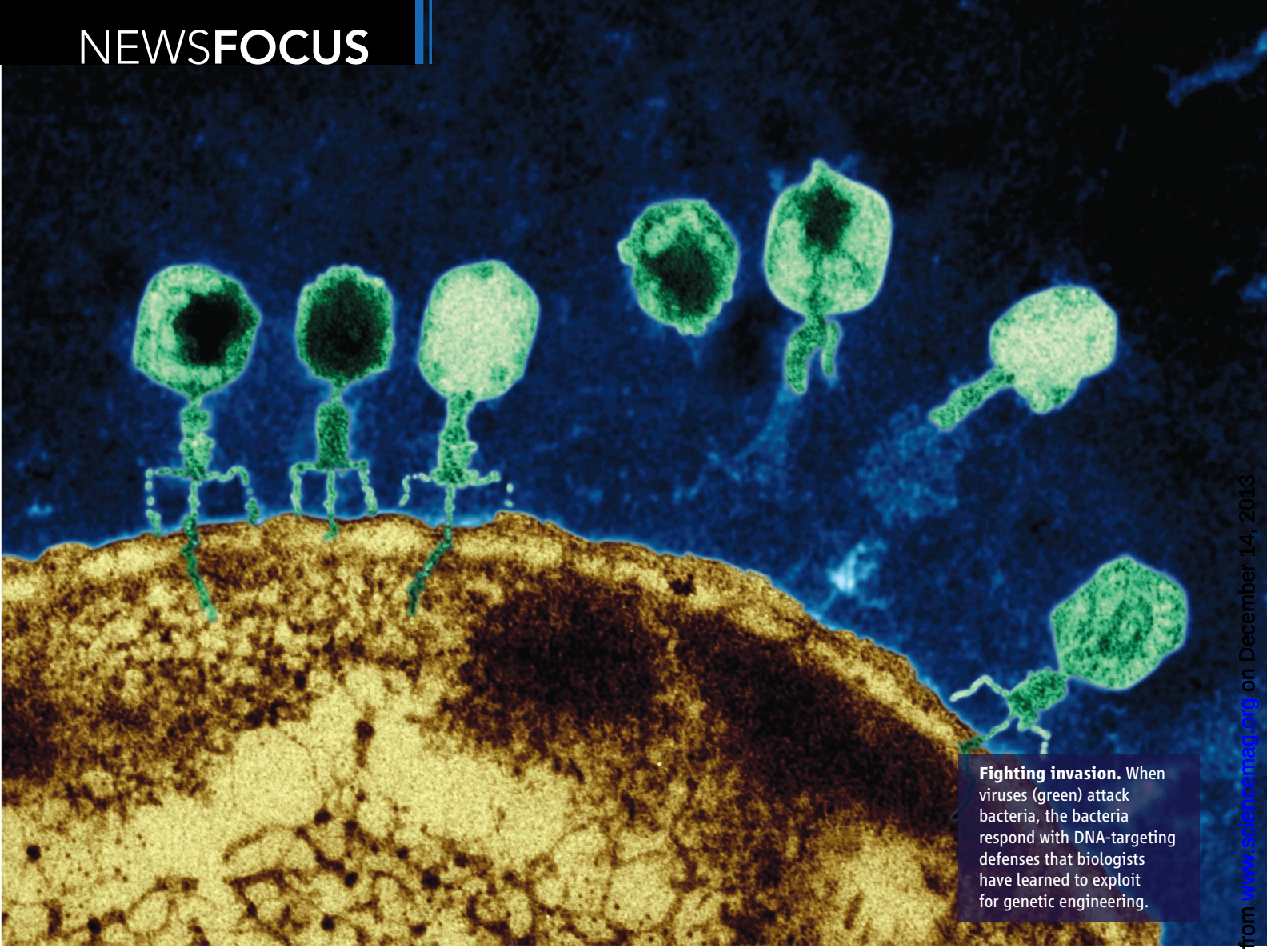

Fighting invasion. When viruses (green) attack bacteria, the bacteria respond with DNA-targeting defenses that biologists have learned to exploit for genetic engineering.

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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. In January, four research teams reported harnessing the system, called CRISPR for peculiar features in the DNA of bacteria that deploy it, to target the destruction of specific genes in human cells. And in the following 8 months, various groups have used it to delete, add, activate, or suppress targeted genes in human cells, mice, rats, zebrafish, bacteria, fruit flies, yeast, nematodes, and crops, demonstrating broad utility for the

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technique. Biologists had recently developed several new ways to precisely manipulate genes, but CRISPR's "efficiency and ease of use trumps just about anything," says George Church of Harvard University, whose lab was among the first to show that the technique worked in human cells.

With CRISPR, scientists can create mouse models of human diseases much more quickly than before, study individual genes much faster, and easily change multiple genes in cells at once to study their interactions. This year's CRISPR craze may yet slow down as limitations of the method emerge, but Church and other CRISPR pioneers are already forming companies to harness the technology for treating genetic diseases. "I don't think there's any example of any field moving this fast," says Blake Wiedenheft, a biochemist at Montana State University in Bozeman.

Humble beginnings

The first inkling of this hot new genetic engineering tool came in 1987, when a research team observed an oddly repetitive sequence at one end of a bacterial gene. Few others took much notice. A decade later, though, biologists deciphering microbial genomes often found similar puzzling patterns, in which a sequence of DNA would be followed by nearly the same sequence in reverse, then 30 or so seemingly random bases of "spacer DNA," and then a repeat of the same palindromic sequence, followed by a different spacer DNA. A single microbe could have several such stretches, each with different repeat and intervening sequences. This pattern appears in more than 40% of bacteria and fully 90% of microbes in a different domain, the archaea, and gives CRISPR its name. (It stands for clustered regularly interspaced short palindromic repeats.)

Many researchers assumed that these odd sequences were junk, but in 2005, three bioinformatics groups reported that spacer DNA often matched the sequences of phages, indicating a possible role for CRISPR in microbial immunity. "That was a very key clue," says biochemist Jennifer Doudna of the University of California (UC), Berkeley. It led Eugene Koonin from the National Center for Biotechnology Information in Bethesda, Maryland, and his colleagues to propose that bacteria and archaea take up phage DNA, then preserve it as a template for molecules of RNA that can stop matching foreign DNA in its tracks, much the way eukaryotic cells use a system called RNA interference (RNAi) to destroy RNA.

Enter the Danisco team. In 2007, Rodolphe Barrangou, Philippe Horvath, and others with the company showed that they could alter the resistance of *S. thermophilus* to phage attack by adding or deleting spacer DNA that matched the phage's. At the time, Barrangou, who is now at North Carolina State University in Raleigh, didn't see CRISPR's full potential. "We had no idea that those elements could be readily exploitable for something as attractive as genome editing," he says.

Precise cuts. In just 8 months, CRISPR modifications of DNA resulted in dumpier nematodes (*top*, bottom), zebrafish embryos with an excess of ventral tissue (*middle*, bottom), and fruit flies with dark eyes (*bottom*, right), demonstrating its broad utility for editing genes in animals.

Doudna and Emmanuelle Charpentier, currently of the Helmholtz Centre for Infection Research and Hannover Medical School in Germany, took the next step. They had independently been teasing out the roles of various CRISPR-associated proteins to learn how bacteria deploy the DNA spacers in their immune defenses. But the duo soon joined forces to focus on a CRISPR system that relies on a protein called Cas9, as it was simpler than other CRISPR systems.

When CRISPR goes into action in response to an invading phage, bacteria transcribe the spacers and the palindromic DNA into a long RNA molecule that the cell then cuts into short spacer-derived RNAs called crRNAs. An additional stretch of RNA, called tracrRNA, works with Cas9 to produce the crRNA, Charpentier's group reported in *Nature* in 2011. The group proposed that together, Cas9, tracrRNA, and crRNA somehow attack foreign DNA that matches the crRNA.

The two teams found that the Cas9 protein is a nuclease, an enzyme specialized for cutting DNA, with two active cutting sites, one site for each strand of the DNA's double helix. And in a discovery that foreshadowed CRISPR's broad potential for genome engineering, the team demonstrated that they could disable one or both cutting sites without interfering with the ability of the complex to home in on its target DNA. "The possibility of using a single enzyme by just changing the RNA seemed very simple," Doudna recalls.

Before CRISPR could be put to use, however, Doudna's and Charpentier's teams had to show that they could control where Cas9 went to do its cutting. First, Doudna's postdoc, Martin Jinek, figured out how to combine tracrRNA and spacer RNA into a "single-guide RNA" molecule; then, as a proof of principle, the team last year made several guide RNAs, mixed them with Cas9, and showed in a test tube that the synthetic complexes could find and cut their DNA targets (*Science,* 17 August 2012, p. 816). "That was a milestone paper," Barrangou says.

This precision targeting drives the growing interest in CRISPR. Genetic engineers have long been able to add and delete genes in a number of organisms. But they couldn't dictate where those genes would insert into the genome or control where gene deletions occurred. Then, a decade ago, researchers developed zinc finger nucleases, synthetic proteins that have DNA-binding domains that enable them to home in and break DNA at specific spots. A welcome addition to the genetic engineering toolbox, zinc fingers even spawned a company that is testing a zinc finger to treat people infected with HIV (*Science,* 23 December 2005,

CREDITS (TOP TO BOTTOM): FRIEDLAND *ET AL.*, *NATURE METHODS* **10** (JUNE 2013); ANDREW GONZALES/JOANNA YEH; SCOTT GRATZ/UNIVERSITY OF WISCONSIN, MADISON

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p. 1894). More recently, synthetic nucleases called TALENs have proved an easier way to target specific DNA and were predicted to surpass zinc fingers (*Science,* 14 December 2012, p. 1408).

Now, CRISPR systems have stormed onto the scene, promising to even outcompete TALENs. Unlike the CRISPR system, which uses RNA as its DNA-homing mechanism, zinc finger and TALEN technologies both depend on custom-making new proteins for each DNA target. The CRISPR system's "guide RNAs" are much easier to make than proteins, Barrangou says. "Within a couple weeks you can generate very tangible results that using alternative methods would take months."

Harnessing CRISPR

Speed is not its only advantage. Church's group had been pushing the use of TALENs in human cells, but when he learned of Doudna and Charpentier's results, he and his colleagues made guide RNA against genes they had already targeted with TALENs. In three human cell types, the CRISPR system was more efficient than TALENs at cutting the DNA target, and it worked on more genes than TALENs did (*Science,* 15 February, p. 823). To demonstrate the ease of the CRISPR system, Church's team synthesized a library of tens of thousands of guide RNA sequences, capable of targeting 90% of human genes. "You can pepper the genome with every imaginable CRISPR," he says.

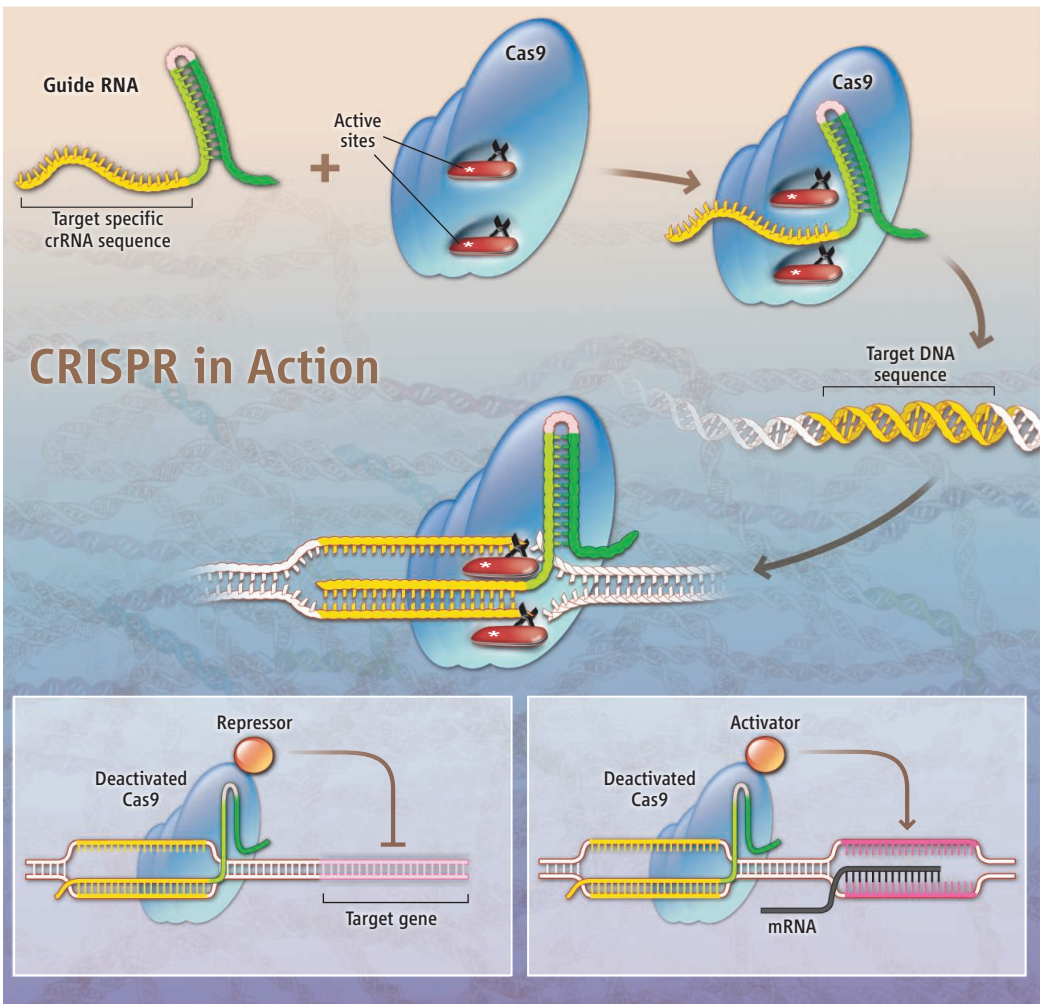
That makes it possible to alter virtually any gene with Cas9, exploiting its DNA-cutting ability to either disable the gene or cut it apart, allowing substitute DNA to be inserted. In an independent paper that appeared at the same time as Church's, Feng Zhang, a synthetic biologist at the Broad Institute in Cambridge, Massachusetts, and his colleagues showed that CRISPR can target and cut two genes at once in human cells (*Science,* 15 February, p. 819). And working with developmental biologist Rudolf Jaenisch at the Whitehead Institute for Biomedical Research in Cambridge, Zhang has since disrupted five genes at once in mouse embryonic stem (ES) cells.

Such work lays the foundation for generating mutant mice, a key tool for biomedical research. One approach would be to add the altered mouse ES cells to a developing embryo and breed the resulting animals. But Zhang has demonstrated a faster option. His team found it could simply inject fertilized mouse eggs, or zygotes, with Cas9 messenger RNA and two guide RNAs and, with 80% efficiency, knock out two genes. They could also perform more delicate genomic surgery on the embryos by shackling Cas9, so that it nicks target DNA instead of cutting it. In this way, they could introduce a new part of a gene through a process called homology-directed repair, they reported in the 2 May issue of *Cell*.

Developing a new mouse model for a disease now entails careful breeding of multiple generations and can take a year; with Zhang's CRISPR technique, a new mouse model could be ready for testing in a matter of weeks. And Zhang thinks the approach is not limited to mice. "As long as you can manipulate the embryo and then reimplant it, then you will be able to do it" in larger animals, perhaps even primates.

Doudna's group and a Korean team reported using CRISPR to cut DNA in human cells 3 weeks after Zhang's and Church's papers went online, and, at the same time, another group revealed they had used CRISPR to make mutant zebrafish. This cascade of papers has had a synergistic effect, commanding the attention of a broad swath of the biology community. "If a single paper comes out, it gets some attention, but when six papers come out all together, that's when people say, 'I have to do this,' " says Charles Gersbach, a biomedical engineer at Duke University in Durham, North Carolina.

Once she saw Doudna and Charpentier's paper a year ago, Gao Caixia became one of the early converts. Her group at the Chinese



DNA surgeon. With just a guide RNA and a protein called Cas9, researchers first showed that the CRISPR system can home in on and cut specific DNA, knocking out a gene or enabling part of it to be replaced by substitute DNA. More recently, Cas9 modifications have made possible the repression (*lower left*) or activation (*lower right*) of specific genes.

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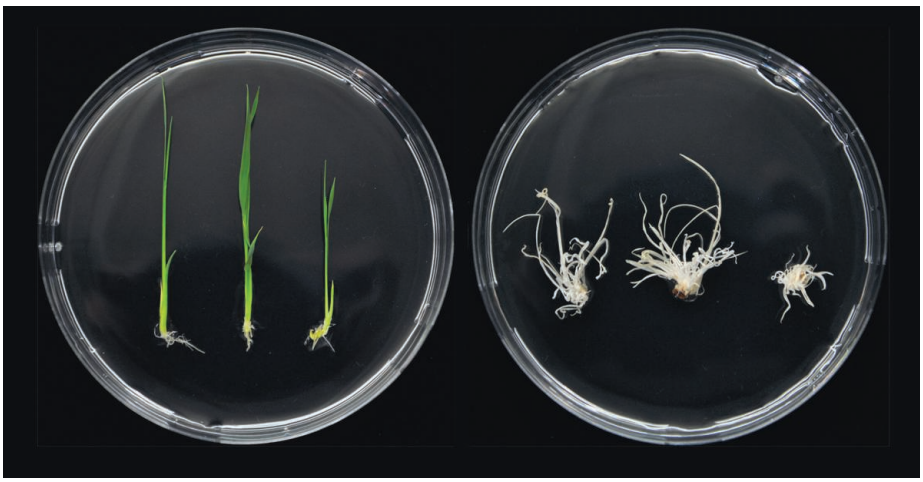
Academy of Sciences' Institute of Genetics and Developmental Biology in Beijing had been using zinc finger and TALENs technology on rice and wheat. Using CRISPR, they have now disabled four rice genes, suggesting that the technique could be used to engineer this crucial food crop. In wheat, they knocked out a gene that, when disabled, may lead to plants resistant to powdery mildew. In a measure of the excitement that CRISPR has generated, the team's report in the August issue of *Nature Biotechnology* was accompanied by four other papers describing CRISPR successes in plants and in rats.



CRISPRed rice. Earlier this month, researchers showed CRISPR works in plants, such as rice, where the knocked-out gene resulted in dwarf albino individuals (*right*).

The cost of admission is low: Free software exists to design guide RNA to target any desired gene, and a repository called Addgene, based in Cambridge, offers academics the DNA to make their own CRISPR system for $65. Since the beginning of the year, Addgene—to which 11 teams have contributed CRISPR-enabling DNA sequences—has distributed 5000 CRISPR constructs, and in a single July week the repository received 100 orders for a new construct. "They are kind of crazy hot," says Joanne Kamens, Addgene's executive director.

Fine-tuning gene activity

The initial CRISPR genome-editing papers all relied on DNA cutting, but other applications quickly appeared. Working with Doudna, Lei S. Qi from UC San Francisco and his colleagues introduced "CRISPRi," which, like RNAi, turns off genes in a reversible fashion and should be useful for studies of gene function. They modified Cas9 so it and the associated guide RNA would still home in on a target but would not cut DNA once there. In bacteria, the presence of Cas9 alone is enough to block transcription, but for mammalian applications, Qi and colleagues add to it a section of protein that represses gene activity. Its guide RNA is designed to home in on regulatory DNA, called promoters, which immediately precede the gene target.

Last month, that team and three other groups used a Cas9 to ferry a synthetic transcription factor—a protein fragment that turns on genes—enabling them to activate specific human genes. Just using one CRISPR construct had a weak effect, but all four teams found a way to amplify it. By targeting multiple CRISPR constructs to slightly different spots on the gene's promoter, says Gersbach, one of the team leaders, "we saw a huge synergistic effect."

> "I don't think there's any example of any field moving this fast."
>
> —Blake Wiedenheft, Montana State University

In the 25 July issue of *Nature Methods*, he reported activating genes tied to human diseases, including those involved in muscle differentiation, controlling cancer and inflammation, and producing fetal hemoglobin. Two other teams also targeted biomedically important genes. CRISPR control of such genes could treat diseases ranging from sickle cell anemia to arthritis, Gersbach suggests.

CRISPR technology may yet have limitations. It's unclear, for example, how specific the guide RNAs are for just the genes they are supposed to target. "Our initial data suggest that there can be significant off-target effects," says J. Keith Joung from the Massachusetts General Hospital in Boston, who back in January demonstrated that CRISPR would alter genes in zebrafish embryos and has used CRISPR to turn on genes. His work shows that nontarget DNA resembling the guide RNA can become cut, activated, or deactivated.

Joung's group showed that a guide RNA can target DNA that differs from the intended target sequence in up to five of its bases. Zhang has gotten more reassuring results but says that "the specificity is still something we have to work on," especially as more people begin to think about delivering CRISPR systems as treatments for human diseases. "To really make the technology safe, we really have to make sure it goes where we want it to go to and nowhere else."

Researchers must also get the CRISPR components to the right place. "Delivery is an enormous challenge and will be cell type and organism specific," Joung notes. With zebrafish, his team injects guide RNA and messenger RNA for Cas9 directly into embryos; with mammalian cells, they use DNA constructs. How CRISPR might be delivered into adult animals, or to treat disease in people, is just now being considered.

Ultimately, CRISPR may take a place beside zinc fingers and TALENs, with the choice of editing tool depending on the particular application. But for now, researchers are dazzled by the ease by which they can make and test different CRISPR variants and by the technology's unexplored potential. Charpentier and others are looking at the versions of Cas9 in other bacteria that might work better than the one now being used. Microbiologists have harnessed the CRISPR system to vaccinate bacteria against the spread of antibiotic resistance genes. Church, Doudna, Charpentier, and others are forming CRISPR-related companies to begin exploring human therapeutic applications, including gene therapy.

And there's more that can be done, Barrangou says. "The only limitation today is people's ability to think of creative ways to harness [CRISPR]."

Not bad for a system that started with sickly bacteria.

–ELIZABETH PENNISI

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