Better living through microbes

Genetically engineered bacteria made biotech drugs possible. Now, they are becoming drugs in their own right, Monya Baker reports.

On April 30 this year, two Florida patients received a unique mouth rinse. Using a cotton swab, a dentist painted billions of genetically modified live bacteria of the species *Streptococcus mutans* onto their pearly whites. If all goes as planned, these bacteria, which lack the gene to make enamel-eroding lactic acid, will replace their naturally occurring, acid-making counterparts, which cause tooth decay.

Besides *S. mutans,* a number of strains of live bacteria are being developed as therapies (**Fig. 1**; **Table 1**). A *Lactococcus Lactis* strain, engineered to secrete a therapeutic protein, has already been tested in patients with Crohn disease at the University of Amsterdam; in April, trial results were submitted for publication. Also that month, Osel of Santa Clara, California, began phase 2 trials using a proprietary, naturally occurring strain of live *Lactobacillus crispatus* to treat recurrent urinary tract infection and recurrent bacterial vaginosis. Other academics and companies are exploring genetically modified bacteria against cancer and infectious diseases.

As drugs, bacteria offer several advantages: compared with therapeutic proteins, they are easier to grow, purify and store; although engineering them to express (or not express) a particular gene takes expertise, it needs to be done only once. But along with their convenience and versatility, bacteria reproduce and evolve, making them hard to predict (**Box 1**). That creates tricky regulatory issues, and most investors are keeping their distance. But even without venture backing, the field is progressing steadily through the clinic.

Living therapies

The first dose of engineered *S. mutans* comes two years after the US Food and Drug Administration (FDA) placed Alachua, Florida-based Oragenics' clinical trial on hold for safety concerns. The current seven-day trial requires bacteria with additional engineering. The gene for alanine racemase is deleted, which makes the bacteria dependent on D-alanine for their growth. During the clinical trial, they are supplied with the nutrient in a mouth rinse applied twice-daily. And, just in case the bacteria must be eliminated quickly, all patients enrolled in the trial must wear dentures, which can be removed should problems arise. When the trial is over, patients will rinse their mouths with an antibiotic and be monitored, along with their spouses, for three months.

Oragenics hopes that eventually the bacteria will become a routine part of dentistry, a one-time treatment applied to children's teeth before they start getting cavities. CEO Chuck Soponis acknowledges no one has any specific idea what ill health effects the bacteria might cause. The strain does not make a recombinant protein or displace other species of bacteria living on the tongue and gums. Still, he says, the FDA is right to be cautious. "You're putting a genetically modified bacteria that's going to be in people's mouths for a lifetime, and that hasn't been done before."

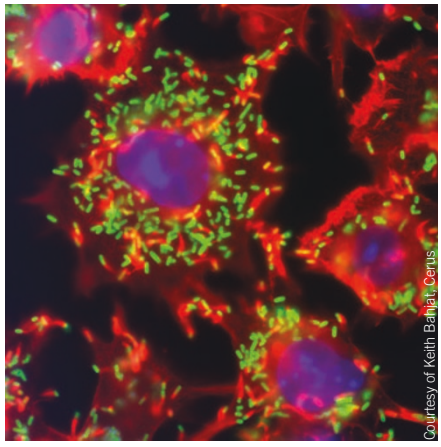

Figure 1 Fluorescent photomicrograph of antigen-presenting cells after engulfing wild-type *L. monocytogenes*. The bacteria (green) escape the engulfing lysosome to propagate within the cytoplasm. This evokes a potent immune response that researchers at Cerus hope to turn against cancer. (Nuclei are stained blue).

Using live bacteria, or probiotics, to promote health is already common in Japan and becoming increasingly so in Europe. In fact, about 30 probiotic prescription products are sold in Japan. Far more common are products sold without prescriptions as so-called functional foods. The Japanese company Yakult Honsha of Tokyo sells 25 million bottles a day of a fermented milk drink, each one boasting that it contains 8 billion live *Lactobacillus casei* strain Shirota, which allegedly make for a more healthful gut flora. However, the efficacy of these over-the-counter products is uncertain, and they can't command the high prices of prescription drugs.

Investors hang back

In general, companies seeking funding for live bacteria therapies will have an even harder time finding funding than other biotechnology startups, according to Irena Melnikova, research manager at consulting firm Life Sciences Insights of Framingham, Massachusetts. "This is something very novel," she says, "and when you start talking about putting live bacteria in humans, there's a negative first reaction."

In fact, neither Oragenics nor Osel are accessing traditional venture capital (VC) sources to fund their programs. Oragenics went straight to the public markets, listing the company on the Toronto Venture Exchange in June 2003, and moving it to the American Stock Exchange in May last year. Though Osel began in 1998 with money from the founders and has received funding from government agencies including the National Institutes of Health, it has relied mainly on private investors who are not venture capitalists, according to the company's president Ralph Levy. "The VC community does not have a model which our product fits into," he says. "Small molecules and antibodies, they've seen that; live bacteria they don't understand."

Oragenics is also pursuing an antibiotic and an over-the-counter probiotic, both stemming from research by the company's CSO and cofounder Jeff Hillman. The probiotic is a proprietary mixture of three naturally occurring bacteria expected to prevent periodontal disease when taken daily. Oragenics plans to partner with larger oral care companies and get the product on the market in Europe and Asia sometime next year and use revenues from probiotics sales to partly finance development of its genetically modified bacteria, which will be marketed as a prescription drug administered by dentists.

Osel's Levy is quick to distance himself from the nonprescription marketplace. He doesn't even like the term 'probiotic.' "The easy route is just manufacturing it and putting it in a store. My market is the physician who's going to write a script to treat a patient," he says. Levy thinks venture capitalists will be more forthcoming with funding once phase 2 results are available. "When we have the data, they'll be where I need them to be."

Producing drugs *in situ*

As an academic, Lothar Steidler, a molecular biologist at Cork University in Ireland, bypassed many corporate headaches while still moving a live bacteria therapy into clinical trials. The work began about ten years ago when Steidler, then at

Table 1 Selected therapies with living bacteria

Company	Bacteriotherapy (modification)	Indication	Status
Cerus Corporation Incorporated 1991	*Listeria monocytogenes* (CRS-100) (genes for virulence factors ActA and Internalin B deleted)	Liver-metastasized colorectal cancer	Investigational new drug application planned by end of 2005
Flanders Interuniversity Institute for Biotechnology (Patent holder)	*Lactococcus lactis* (gene for interleukin-10 inserted in place of gene for thymidylate synthase)	Inflammatory bowel disease	Phase 1 trials completed
Osel Founded 1998	*Lactobacillus crispatus* (naturally occurring)	Recurrent urinary tract infection and recurrent bacterial vaginosis	Phase 2 trial
	Clostridium butyricum (naturally occurring)	*Clostridium difficile*–associated disease (CDAD) and antibiotic-associated diarrhea	Marketed in Japan by Miyarisan; phase 2 (US) completed
Oragenics Founded 1996	*Streptococcus mutans* (gene for lactate dehydrogenase deleted from proprietary, naturally occurring, antibiotic-producing strain)	Tooth decay	Phase 1 trial
	Streptococcus rattus; *Streptococcus oralis* and *Streptococcus uberis* (naturally occurring)	Periodontal health	Will be marketed as a supplement in Europe and Asia
Vion Founded 1995	*Salmonella typhimurium* (TAPET) (genes for msbB and purI deleted)	Solid tumors	Phase 1 trials
	S. typhimurium (TAPET-CD) (*E. coli* gene for cytosine deaminase inserted in place of msbB gene necessary for endotoxic activity)	Solid tumors	Phase 1 trials

Belgium's Ghent University, was looking for an inexpensive source of cytokines—soluble protein regulators of the immune system. Steidler and his colleagues acquired all sorts of expression systems to make cytokines in-house, and routinely scanned the literature for more. "We bumped into *Lactococcus* because I met someone at a conference and he had a nice poster," says Steidler, who eventually created *Lactococcus* that secreted human interleukins 2, 6 and 10 (IL-2, IL-6, IL-10) as well as trefoil factors. As a production system the bug was disappointing; its output was a hundredth to a thousandth that of *Escherichia coli*, says Steidler. "But what immediately struck our minds was that the product was soluble and was fully biologically active." Most cytokines do not require glycosylation to be active, and so don't need to be made in eukaryotic cells. In *Lactococcus* cultures, soluble cytokines could be recovered from the supernatant with its secretion leader clipped off correctly. In other words, says Steidler, "The material you got for IL-10 was indistinguishable from the eukaryotic product." Furthermore, people have safely eaten *Lactococcus* in cheese and yogurt for thousands of years. Perhaps, Steidler's team thought, patients could safely consume it as a drug.

The technology seemed especially applicable to inflammatory bowel disease, in which the immune system erroneously attacks the gut, causing chronic discomfort and frequent diarrhea. IL-10 tends to quiet the immune system, but administering the cytokine orally is problematic, as its activity is rapidly destroyed by acid in the stomach. Given intravenously, the cytokine spreads through the entire body, where it can actually rouse the immune system. But *Lactococcus* taken by mouth could travel through the stomach and secrete IL-10 exactly where it was needed, along the intestinal wall. "We're still surprised at the simplicity," says Steidler. In two separate mouse models of colitis, treatment with the bacteria either prevented disease onset or dramatically reduced its severity[1]. The mice suffered no obvious adverse effects.

In an unusual move, the University of Amsterdam approached Steidler about putting the bacteria into clinical trials. But the therapy was not ready for people yet. "You can't just release genetically modified bacteria in the environment without precaution," says Steidler. The research team needed a way to prevent the engineered bacteria from growing outside the body, so they decided to insert

Box 1 Proceed with caution

Only one thing is certain about using live bacteria as medicine: "There will be setbacks." That's according to Theodore Friedman, former chair of the NIH's Recombinant DNA Advisory Committee, which weighs in on whether trials using genetically modified bacteria should be allowed. Nonetheless, he says, current work represents the "opening edge" of "studies that will become more and more interesting."

Human trials with genetically engineered bacteria and viruses as vaccines against infectious diseases have been going on for at least a decade. The decision to test live bacteria is a balancing act between benefits to patients and risks to patients and the environment, says Friedman. Many of these therapies work by inducing an infection, and people can die of bacteremia.

That means that researchers must understand the bacteria very well. Where do they grow? How does the body respond? And most importantly, can the bacteria be quickly eliminated? The stability of genetic modifications and how they could change the bacteria's life cycle must also be factored in.

As for release to the environment, "Clearly you need to find out if the bacteria accumulates and if the bacteria will get out, and there are ways of preventing that," says Friedman. "For cancer patients, the use may outweigh the potential for spread." (The use of bacteria in clinical trials is governed by the FDA's Center for Biologics Evaluation and Research, which did not respond to repeated requests for information on how it weighed patient and environmental safety issues when approving clinical trials.)

IL-10 into the locus normally occupied by the gene for thymidylate synthase[2], which is essential for DNA synthesis, and hence required for growth.

The modified *Lactococcus* can readily absorb thymidine from the nutrient-rich gut, but quickly depletes any available in the environment; its viability decreases about a millionfold within 60 hours after the source of thymidine is removed.

To obtain permission to run trials in Holland, explains Steidler, researchers must publish trial plans in newspapers and the Royal National Library in The Hague. That way the general public can question trials before they start. However, no one raised concerns during that process, he says. Although that may seem surprising, given widespread objection to genetically modified food in Europe, attitudes toward transgenic plants do not predict attitudes toward transgenic therapies.

The technology is currently covered under 45 patents owned by Flanders Interuniversity Institute for Biotechnology (VIB) of Zwijnaarde, Belgium, which is working with Steidler to commercialize the technology. VIB director Rudy Dekeyser says the group is looking both at corporate partners to collaborate on clinical development, as well as venture capitalists to start a new company.

But even if the technology finds corporate backing, that doesn't mean that other companies developing live bacteria will necessarily have an easier time, says IDC's Melnikova, "It's always hard to be the first, but each individual therapy will be considered on its individual merits."

Bacteria take on cancer

Several groups are looking for ways to use microbes against cancer, and in fact, the naturally occurring bacteria, bacillus Calmette-Guerin, is standard therapy for a type of bladder cancer. Still most work in this area uses viruses rather than bacteria. Viruses had a head start because they can target specific cells, according to David Bermudes, director of microbiology at Vion in New Haven, Connecticut. Still, he says, bacteria offer several practical advantages. "A virus depends on the cell to deliver its payload. Bacteria are self-contained factories." Moreover, bacteria can carry more genetic material, be controlled with antibiotics and "are a joy to manufacture compared with viruses."

Vion has already taken a cancer-fighting bacteria through three phase 1 trials. It chose bacteria for their ability to grow preferentially in tumors, an advantage that molecular biologists were slow to recognize, says Bermudes. The company's genetically modified *Salmonella typhimurium* makes cancer drugs potent at the site of the tumor, allowing a less toxic prodrug to be administered. As it grows in tumors, the engineered *S. typhimurium* expresses an *E. coli* enzyme for cytidine deaminase, which converts nontoxic 5-fluorocytosine (5-FC) to the anticancer drug 5-fluorouracil (5-FU)[3]. In mouse studies, the treatment, called TAPET-CD, slowed tumor growth by as much as 95%.

In mice, the bacterium also has some ability to target tumor cells even without cytidine deaminase. Vion tested versions of *S. typhimurium* lacking the enzyme in phase 1 trials and found that although the bacteria found their way to tumors, the therapy had no antitumor activity. "It was a commercial failure, but a scientific success because you've shown that you can make a bacteria that's safe for systemic administration that targets tumors," says Bermudes. Human trials with TAPET-CD showed conversion of 5-FC to 5-FU in two of three patients, but were stopped for what Bermudes calls "nonmedical reasons." Vion is focusing efforts on a small molecule anticancer drug now in phase 2 trials.

Cerus of Concord, California, looked at both viral and bacterial vectors before deciding to engineer *Listeria monocytogenes* as a cancer vaccine. As one of the deadliest food-borne bacteria, it seems a surprising choice for a vaccine vector, but microbiologists have a long history of finding ways to attenuate pathogens, and *L. monocytogenes* had key advantages, according to Tom Dubensky, head of vaccine development at Cerus. The bacteria is easy to grow and elicits a potent immune response. Also, unlike many other microbes, it is not neutralized by antibodies, so repeated vaccinations are more likely to be effective. In fact, a clinical trial of 20 healthy volunteers showed that a genetically attenuated strain of *L. monocytogenes* could still prompt an immune response without serious side effects.

Wild-type *L. monocytogenes* enters liver cells and spreads from cell to cell by sending bacteria-filled protrusions into neighboring cells, thus avoiding the immune system. Cerus's engineered bacteria lack this ability, but they can still enter antigen-presenting cells and stimulate an immune response[4] (**Fig. 1**). Although Cerus's first-generation *L. monocytogenes* works by boosting only innate immunity, researchers have also created a way to stimulate adaptive immunity. "We spent a lot of time learning how to program *L. monocytogenes* to secrete antigen from the bacterium within the antigen-presenting cell," recalls Dubensky. The next-generation of *L. monocytogenes* vectors make mesothelin, a tumor marker present in many ovarian and prostate cancers; Cerus has a joint project with MedImmune of Gaithersburg, Maryland, using its proprietary EphsA2 antigen as well. Cerus hopes to file an investigational new drug application by the end of this year for its first-generation *L. monocytogenes* against colorectal cancer that has metastasized to the liver. Dubensky expects close scrutiny from the FDA but thinks that for cancer patients that have not benefited from other therapies, the risk-benefit calculations favor experimental therapies. "While there is risk to being the first use in man, these are people for whom there are no other options."

Bert Vogelstein, an oncologist at Johns Hopkins University in Baltimore, uses a version of *Clostridium novyi* that lacks the gene to make α-toxin. This spore-forming bacteria germinates in oxygen-poor tumors and turns them to mush within18 hours. Ruptured cells from the dying tumor prompt an immune response that trains the immune system to attack remaining cancer cells[5]. The therapy has been tried in mice, rats, and rabbits; in one in three animals the tumor is destroyed without additional drugs. Odds of vanquishing the tumor improve if anticancer drugs are given as well. Vogelstein thinks the therapy is promising, but is adamant that it is not ready to be tested in humans. Toxicities so far seem acceptable, he says, but the immune response elicited can be so strong that it harms the host.

Cautious yet optimistic

Whether liquifying tumors or pushing out harmful bacteria, live-bacteria therapies can exploit mechanisms impossible for small molecule and protein drugs, and can even be engineered to deliver drugs where the body needs them. But for all this, they present unpredictable and unquantifiable risks. As Vogelstein says, "The agent is new and it's self-replicating. The dose that we give and the dose that we get are not the same." Vion's Bermudes says skepticism is warranted, but so is hope, particularly for patients who have no other options. "Ten years ago, everybody said antibody therapies don't work. Where are the products? Now there are almost twenty products on the market."

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1. Steidler, L. *et al.* Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. *Science* **289**, 1352–1355 (2000).
2. Steidler, L. *et al.* Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nat. Biotechnol.* **21**, 1785–1789 (2003).
3. Nemunaitis, J. *et al.* Pilot trial of genetically modified, attenuated *Salmonella* expressing the *E. coli* cytosine deaminase gene in refractory cancer patients. *Cancer Gene Ther.* **10**, 737–744 (2003).
4. Brockstedt, D.G. *et al. L. monocytogenes*-based cancer vaccines that segregate immunogenicity from toxicity. *Proc. Natl. Acad. Sci. USA* **101**, 13832–13837 (2004).
5. Agrawal, N. *et al.* Bacteriolytic therapy can generate a potent immune response against experimental tumors. *Proc. Natl. Acad. Sci. USA* **101**, 15172–15177 (2004).

Erratum: Better living through microbes

Monya Baker
Nat. Biotechnol. **23, 645–647 (2005)**

On page 646, **Table 1**, last column, the status of the Osel bacteriotherapy *Clostridium butyricum* was given as "Phase 2 trial." It should have read, "Phase 2/3 trial (US) planned."
On page 647, column 2, last paragraph, line 16, "…a tumor marker present in many ovarian and prostate cancers…" should have read "a tumor marker present in many ovarian and pancreatic cancers…." Prostate cancers should have been pancreatic cancers. In the last line of the same paragraph, "EphsA2 antigen" should have been "EphA2 antigen."

Erratum: Leaders and laggards in the stem cell enterprise

Stephan Herrera
Nat. Biotechnol. **23, 775–777 (2005)**

On page 776, column 3, paragraph 3, line 8, one of the recipients of the Starr Foundation gift for stem cell research was reported to be Rochester University; it should have read Rockefeller University.

Corrigendum: The origins of new drugs

Robert Kneller
Nat. Biotechnol. **23, 529–530 (2005)**
On page 528, an imaging agent, Prussian Blue, was mistakenly included in the NME totals for 2003. Thus, in **Table 1** and corresponding places in the text, the total number of NMEs approved in 2003 should be reduced from 21 to 20 and the total number of new drugs from 27 to 26. The totals for the 6-year period should be similarly reduced from 145 to 144 for total NMEs and from 171 to 170 for total drugs. Also, the NME Somavert originated in Ohio University, which then licensed rights to Genentech. Thus, the number of university inventions should be increased from 3 to 4 for 2003, and from 20 to 21 for the entire 6-year period.

Corrigendum: National origins of new drugs

Robert Kneller
Nat. Biotechnol. **23, 655–656 (2005)**

On page 655, in calculating the nonpharma portion of NMEs in **Table 1**, errors arose because in the case of two of the drugs that were attributed to multiple countries, a fractional value for one country was not included in the nonpharma totals. Thus, the US NME nonpharma contribution, reported as 32.8 (56%), should have read 33.0 (56%); the Swiss NME nonpharma contribution, reported as 1.3 (13%), should have read 1.0 (10%); and the total nonpharma contribution, reported as 47.8 (33%), should have read 47.7 (33%).