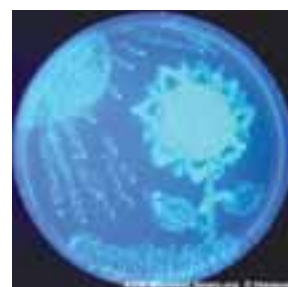


Genetic engineering in Ljubljana, Slovenia

Cracking Tough Bugs Open

Borut Štrukelj and his group have developed a new method for fast transformation of competent bacterial cells. However, this should only be the epilogue for an even more ambitious project: the use of genetically engineered lactic acid bacteria as “living vaccines”.



Could you lead the same lifestyle in the complete absence of modern biotechnology? If your answer is yes, then think again! Compounds from many products we use daily, like fat-degrading enzymes in washing powders, skin-rejuvenating proteins in face creams, alcohol and other industrial chemicals as well as many approved drugs, like insulin and growth hormones, are being produced by genetically engineered microorganisms.

Transformers

The beginning of the biotechnology era was marked in 1972, when California-based scientists Herbert Boyer and Stanley Cohen joined their efforts and succeeded in transferring a plasmid, containing artificially inserted pieces of DNA, into *Escherichia coli*. Three patents followed and from this time on the multibillion-dollar biotechnology industry has flourished.

The building blocks of genetic engineering are simple: you only need a protein-producing factory (bacteria in most cases) and a piece of DNA that encodes the desired protein. To complete this equation, the DNA only has to be inserted into bacteria by a process called transformation.

The most common methods of making bacteria take up plasmids apply either chemicals combined with heat-shock or electric pulses (electroporation) in order to reversibly make the bacteria leaky and, therefore, competent of taking up DNA. However, there's one drawback. Although these methods work perfectly in transferring DNA into *E. coli* and other well-studied species, many other bacteria still resist taking up DNA that way.

Researchers in Borut Štrukelj's group at the University of Ljubljana, Slovenia, have now succeeded in transforming some of these tenacious bugs. At the heart of their new transformation method is the use of natural detergent-like chemicals to introduce plasmid DNA into bacteria (*Biotech-*

nol. Lett., publ. online before print 19 August 2009).

Ironically, these groups of substances weren't appreciably explored for use in bacterial transformation before because a couple of studies had been around showing that some detergents could inhibit bacterial transformation even when present in trace amounts. The new paper by Štrukelj and co., however, demonstrates that some of these chemicals can instead be very successful in making bacteria competent.

“The paper is the result of an interest-

genetically engineered *Clostridium* bacteria could be used to carry therapeutics to the anaerobic centres of tumours.

“Initially, we developed a method of making cells competent by applying ultrasonic pulses but then, unfortunately, we were scooped by a high impact paper,” says Matjaž. That, however, didn't discourage them but made them turn to a different direction and look at natural chemicals that are known to make pores in cellular membranes. “We chose saponins, plant detergents which had already been used for cell permeabilisation experiments, as well as cholates, or bile acid salts, which disturb biological membranes by mechanism that is still not completely understood. We hypothesised that if we were to use low enough concentrations of these, the cells could be permeabilised and still survive,” explains Matjaž.

Resistance is useless

And they were right. “Using these substances in low concentrations, we managed to achieve transformation efficiencies similar to the commonly used heat-shock and electroporation methods. Hence, we were the first

group to prove that surface-active chemicals can make bacterial cells competent without killing them, which was previously thought not to be possible,” Matjaž proudly explains. “Because we tested only a small number of compounds and already got very promising results, we believe that the results can even be improved if we test more similar compounds.” And yet another benefit: the new method is very fast and does not need ice or hot water baths, which makes it suitable for doing field transformations, outside the lab.

Having proven that their principle works, the door is now open for the scientific community and the biotech industry to test and optimise the method for use in different bacterial species as well as trying other compounds with similar properties. “The



Borut Štrukelj (left) and Matjaž Ravnikar (right)

ing undergraduate research project,” recalls Matjaž Ravnikar, the first author of the paper. “The project aimed to develop new approaches for making bacterial cells permeable for DNA molecules and tackle the problem of delivering DNA to difficult-to-transform bacteria.”

There are plenty of examples. Most *Bacillus* species, for instance, are completely resistant to traditional transformation protocols. Included are plant-infecting species, which thus could be commercially interesting because they might be used as vehicles to genetically alter plants and crops.

Another example of hard-to-transform bacteria comes from the genus *Clostridium*. Their preference to grow in an oxygen-free environment also offers a range of opportunities for biotech applications. For example,

organism we used for the proof of principle was *E. coli* but the wider appeal of the method lies in the option of transforming bacteria which are resistant to transformation by the commonly used methods," Matjaž points out.

Matjaž is a PhD student in the Department of Pharmaceutical Biology at the Faculty of Pharmacy, which is led by Borut Štrukelj, one of the pioneers of genetic engineering in Slovenia. Besides his group's excellent publication record, he has received many awards, holds a world patent on a genetically modified, virus-resistant potato and is a committee member at the European Medicines Agency based in London. His department is divided into three parts: the biotechnology group, where Matjaž works, is developing novel biological drugs; the plant pharmacognosic group uses analytical methods to identify bioactive plant compounds; and the cell biology group uses tissue culture models to study different aspects of cancer biology, detection and drug delivery. "Our department is very diverse," says Matjaž. "That opens lots of opportunities for interesting connections between projects. We are a big team, which makes things easier."

Your good morning vaccine

Matjaž's main PhD project aims to use lactic acid bacteria as a tool to develop novel genetically engineered vaccines. Due to their popularity, their wide use and the lack of public stigma in contrast to many other genetically engineered organisms, lactic acid bacteria are a very popular organism in biotechnology.

Precisely, Matjaž has been using phage display techniques to identify surface proteins that are specifically present in certain human pathogens. He then wants to select pathogen-specific antigens and introduce them into lactic acid bacteria. These antigens are recognised by the immune system and antibodies against them are created. That way, the lactic acid bacteria could act as a "living vaccine" that may be taken orally.

There are many advantages of such "living vaccines" over the traditional ones. First

of all, one could forget the fear of needles as they would be as easy to apply as drinking our morning yoghurt. More importantly, however, it has been proven that lactic acid bacteria are quite resistant to gastric acid and that the majority of them survive the path to the intestine. Conveniently, the intestine is rich in immune system cells that trigger the antibody production against foreign antigens. Another great property of lactic acid bacteria is their ability to adhere to the intestinal walls and remain there for some time. This may produce better immune responses against pathogens, whose antigens they carry.

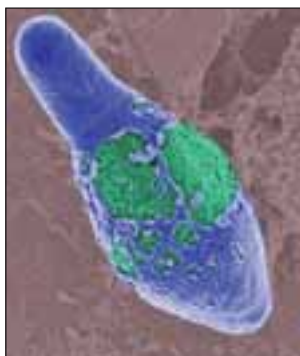
Commercial potentials

Besides exploring lactic acid bacteria for use as vaccines, Borut Štrukelj's group has also been studying the possibilities of genetically engineering them to produce novel probiotics. In a recent study, which has been done in collaboration with the Ljubljana-based Jožef Štefan Institute, they expressed the sweet protein brazzein from a West African climbing plant in *E. coli* and lactic acid bacteria (*Appl. Microbiol. Biotechnol.* 73(1): 158-65). Brazzein is much sweeter than sucrose; therefore, probiotic yoghurts with brazzein-expressing lactic-acid bacteria would be sweet without the need to add sugar – and thus healthier and suitable for diabetics.

Because their work carries a high commercial potential, part of Matjaž's thesis work will soon be filed in a patent application. Filing a patent is still a route that most research groups in Slovenia don't often take. The main reasons are the lack of financial and administrative support for the application and maintenance of the patents, and the Slovenian rating system used for evaluating grant applications, which heavily favours journal publications over patents. Matjaž is optimistic though, saying that the future looks brighter as technology transfer is increasingly becoming a hot topic, and more and more EU funding is being attracted.

"What I enjoy the most about my work is the freedom of pursuing my own ideas and being able to work with interesting people," says Matjaž. And what does he consider to be the biggest challenge of his future scientific career? "To create better, more effective and affordable medicines." Cracking the difficult bugs will certainly help.

IRENA HRELJAC



Clostridium tetani, one of the resistance fighters that may be broken by Ravnikar *et al.*