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CRISPR Technology for Next Generation Probiotics

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Next Generation Probiotics and CRISPR/Cas

- Next Generation probiotics (NGP) are non-conventional/native gut microbiota bacteria, identified through the use of new generation sequencing techniques
- NGP have the potential of being treated as therapeutic agents (under live bio-therapeutic products (LBP), mainly for oral or vaginal use)
- Requirements for approval of NGP are much more stringent than traditional probiotics
- NGPs may also be genetically engineered (likely to be grouped under recombinant LBP with additional requirements for approval)
- Clustered Regularly Interspaced Palindromic Repeats (CRISPR) form a part of a natural defense mechanism of bacteria
- CRISPR/Cas is the gene-editing tool used to gene-edit these bacteria
- Combination of CRISPR and Cas proteins enables the cutting of DNA at a specific location.
- When bacteria are attacked by a virus (bacteriophages), they retain a section of the virus's DNA in their own DNA, flanked by CRISPR sequences. This enables the bacteria to remember the virus and counteract - when the virus attacks again.
- The bacteria use a specific CRISPR-associated protein number 9 (CAS9) to cut the virus's DNA, thereby destroying the virus.

https://pubmed.ncbi.nlm.nih.gov/17379808
http://europepmc.org/abstract/med/15791728
http://europepmc.org/abstract/med/11952905
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC213968/pdf/jbacter00202-0107.pdf

CRISPR/Cas-9 and the Nobel Prize

- Serendipity led to the discovery of CRISPR technology in 1987 in Japan
- Francisco Mojica and Ruud Jansen at the University of Alicante in Spain coined the term CRISPR in the year 2002
- In 2007 Philippe Horvath and his colleagues at Danisco, validated Jansen's hypothesis, based on their studies on different strains of *Streptococcus thermophilus* used for yogurt culture
- Their experiments uncovered how CRISPR/Cas system operates in bacteria and its ability to edit genes within the human genomes
- Their work also laid the foundation for the development of CRISPR/Cas-9 as a versatile gene editing tool
- Jennifer Doudna (at the University of California, Berkeley) and Emmanuel Charpentier (at the Max Planck Unit for the Science of Pathogens), were awarded the Nobel prize in 2020 for pioneering the precise CRISPR/Cas-9 genome-editing technology
- These Nobel laureates founded companies (**Mammoth biosciences**, **Caribou Biosciences, Editas Medicine, CRISPR therapeutics)** to leverage this tool for therapeutic applications
- These companies have been profiled in the following slides

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Mammoth Biosciences

About

- **Location: San Francisco Bay Area, Western US**
- **Funding amount: $ 69.5 M in 6 funding rounds**
- **Founded in 2017**
- **Founders:**
  - Ashley Tehranchi
  - Janice Chen
  - Jennifer Doudna (Won the Nobel Prize in 2020)
  - Lucas Harrington
  - Trevor Martin
- **Lead investors:**
  - NFX
  - Mayfield Fund
  - Decheng Capital
  - National Institute of Health

- Mammoth Biosciences is backed by investors including Decheng, Mayfield, NFX and 8VC, Brook Byers, Tim Cook and Jeff Huber.
- The company is leading the development of CRISPR as a medical diagnostic tool. Its diagnostics use CRISPR to find specific genetic sequences in patient samples confirming the presence of a corresponding human disease, bacteria or virus. This process could make disease detection quicker, more accurate and cheaper.
- In January 2021, Mammoth Biosciences announced a co-marketing agreement with Agilent Technologies to support the anticipated launch of a complete CRISPR-based SARS-CoV-2 diagnostic solution comprising Agilent's Bravo automation workstation and Mammoth Biosciences' DETECTR BOOST™ assay
- Mammoth Biosciences also announced that it had secured a subcontract with MRIGlobal, the prime contractor with the Defense Advanced Research Projects Agency (DARPA), to develop CRISPR-based diagnostics and biosurveillance technologies for the U.S. Department of Defense (DOD). The program is titled "Detect It with Gene Editing Technologies" (DIGET) program.
- In November 2020, the company signed agreements with MilliporeSigma and Hamilton Company targeting commercialization of a high-throughput CRISPR-based SARS CoV-2 test. The test leverages Mammoth's DETECTR BOOST platform and will provide a sample-to-answer turnkey solution for commercial laboratories to enable a multi-fold increase in testing capacity. The company will release its first product which is a testing platform known as DETECTR BOOST, during end 2021.

Caribou Biosciences

About

- **Location: Berkeley, California, US**
- **Funding amount: $ 167.5 M in 5 funding rounds**
- **Founded in 2011**
- **Founders:**
  - James Berger
  - Jennifer Doudna
  - Martin Jinek
  - Rachel Haurwitz
- **Lead investors:**
  - Anterra Capital
  - Farallon Capital Management
  - PFM Health Sciences
  - Ridgeback Capital

- Caribou Biosciences is a genome editing company that focuses on developing solutions for cellular engineering and analysis based on CRISPR-Cas technology platform. It has four active drug programs in its pipeline. The majority of its drugs are used for broad-based applications, and to treat infectious diseases and Multiple Myeloma.
- In 2021, the company and AbbVie entered into a collaboration and licensing agreement to research and develop chimeric antigen receptor (CAR)-T cell therapeutics. Under this agreement, AbbVie will utilize Caribou's Cas12a CRISPR hybrid RNA-DNA (chRDNA) genome editing and cell therapy technologies to research and develop two new CAR-T cell therapies directed to targets.
- In 2020, it entered into several licensing agreements:
  - MaxCyte Inc, to utilize MaxCyte's Flow Electroporation® technology and ExPERT platform for the advancement of CRISPR gene-edited allogeneic T cell therapy programs.
  - Memorial Sloan Kettering Cancer Center (MSK) under which it can use 'fully human anti-CD371 single-chain variable fragments (scFvs) in the field of allogeneic CD371-targeted cell therapies including CAR, or iPSC-derived cell products.
  - Novome Biotechnologies took assignment to certain microbial intellectual property, and non-exclusively licensed foundational CRISPR-Cas9 intellectual property to expand its therapeutic pipeline and platform capabilities.
  - Caribou Biosciences also announced a sale and assignment agreement under which it would gain access to a ProMab humanized scFv targeting the B Cell Maturation Antigen (BCMA) for use in allogeneic engineered cell therapies. Caribou intends to utilize this scFv in the development of its CB-011 program, an allogeneic CAR-T therapy targeting BCMA-positive tumors including multiple myeloma.
- In 2019, the company and Oxford Nanopore entered into an agreement under which Caribou granted Oxford Nanopore, a non-exclusive license under foundational CRISPR-Cas9 intellectual property for nanopore sequencing.
- In 2017, Caribou Biosciences and DuPont launched a new method for comprehensively mapping CRISPR-Cas9 cleavage sites, SITE-Seq.

Editas Medicine

About

- **Location: Cambridge, Massachusetts, US**
- **Funding amount: $ 656.6 M**
- **Founded in 2013**
- **Founders:**
  - Feng Zhang
  - Jennifer A. Doudna
  - George McDonald Church
  - J. Keith Joung and
  - David R. Liu
- **Lead investors:**
  - Flagship Pioneering
  - Polaris Partners
  - Third Rock Ventures
  - Juno Therapeutics
  - Boris Nikolic

- Editas Medicine is a genome editing company that focuses on translating its genome editing technology into a novel class of human therapeutics that enable precise and corrective molecular modification to treat the underlying cause of a range of diseases at the genetic level. It develops a proprietary genome editing platform based on CRISPR technology.
- It entered into several licensing agreements in the past 3 years:
- Modalis Therapeutics Corp (formerly EdiGene Corp.) has obtained a license to certain intellectual property that is controlled by Editas Medicine. Modalis is utilizing its proprietary epigenetic gene modulation technology, CRISPR-GNDM (Guide Nucleotide Directed Modulation), to treat patients with serious genetic disorders (2020)
- Sandhill Therapeutics, Inc., a cellular immuno-oncology company, for a strategic research collaboration, license, and option agreement to combine their respective genome editing and cell therapy technologies to discover, develop, and manufacture allogeneic engineered natural killer (NK) cells and non-alpha beta T cell medicines for the treatment of cancer. (2020)
- GenEdit acquired worldwide license, with rights to sublicense for CRISPR-Cpf1 based therapies. (2020)
- Editas Medicine will use MaxCyte's Flow Electroporation technology and ExPERT instruments for the advancement of engineered cell medicines, including EDIT-301, an experimental CRISPR medicine designed to durably treat sickle cell disease and beta-thalassemia. (2019)
- Cross-licensing agreement with BlueRock Therapeutics to combine the respective genome editing and cell therapy technologies to discover, develop, and manufacture novel engineered cell medicines.(2019)
- Expanded a licensing agreement with Celgene, originally signed in May 2015, for the development of chimeric antigen receptor and high-affinity T cell receptor therapies for cancer, being developed by utilizing CRISPR Cas9 mediated genome editing technology. (2018)
- Licensing and option agreement with Beam Therapeutics, Inc., for the treatment of human disease, for exclusive rights to certain intellectual property. Beam Therapeutics has licensed exclusive rights to Editas Medicine, certain intellectual property of Harvard university, The Broad Institute, Inc., and Massachusetts General Hospital. (2018)

CRISPR Therapeutics

About

- **Location: Zug, Switzerland**
- **Funding amount: $ 127 M**
- **Founded in 2014**
- **Founders:**
  - Rodger Novak
  - Emmanuelle Charpentier
  - Shaun Patrick Foy
  - Matthew Porteus
  - Daniel Anderson
  - Chad Cowan
  - Craig Mellow
- **Lead investors:**
  - Versant Ventures
  - Celgene
  - SR One
  - Bayer Global Investments
  - Vertex Pharmaceuticals
  - Franklin Templeton Investments
  - New Leaf Venture Partners
  - Bill & Melinda Gates Foundation

- CRISPR Therapeutics AG engages in the development and commercialization of therapies derived from genome-editing technology. Its proprietary platform CRISPR/Cas9-based therapeutics allows for precise and directed changes to genomic DNA. It has R&D operations in Massachusetts, US, and business operations in London, the UK.
- Its major development programs include ex vivo programs involving gene editing of hematopoietic cells; ex vivo programs in immuno-oncology; in vivo programs targeting the liver and additional in vivo programs targeting other organ systems including muscle and lung.
- It has signed several licensing agreements, a few of which are listed below.
- In 2020, the company and Vertex Pharmaceuticals received Regenerative Medicine Advanced Therapy (RMAT) designation to CTX001 from the FDA for the treatment of severe hemoglobinopathies. the company entered into a collaboration with Vertex Pharmaceuticals to develop, manufacture, and commercialize CTX001 in sickle cell disease and beta thalassemia. Bothe entered into an agreement in 2019 for developing novel therapies for the treatment of DMD and DM1. Both had entered into a strategic research collaboration in 2015, focused on the use of CRISPR's gene editing technology, to discover and develop potential new treatments aimed at the underlying genetic causes of human disease.
- In 2019, the company and StrideBio expanded their strategic collaboration to generate engineered AAV capsids.
- In 2018, the company and MaxCyte signed a commercial license agreement for the development of new immuno-oncology therapies. The company collaborated with ViaCyte to develop gene-edited stem cell-derived therapy for diabetes.
- In 2017, the company and Casebia Therapeutics, a JV established by CRISPR and Bayer, signed a joint commercial license agreement with MaxCyte, a US-based global company dedicated to accelerating the discovery, development, manufacturing and commercialization of next-generation cell-based medicines, to develop CRISPR/Cas9-based therapies. The company also entered into an agreement with MaSTherCell SA, to develop and manufacture allogeneic cell therapies.

CRISPR Engineered Probiotics - Start-Ups

Industrial research on CRISPR for providing curative benefits through patient immune cells is in progress and, and many of them are in clinical trials stage. However, research on CRISPR engineered probiotics for therapeutic benefits is still in nascent stage.

SNIPR Biome is a preclinical stage start-up based out of Denmark, It focuses on the use of CRISPR and microbiome for providing cumulative health benefits. The company's research focus lies in targeting the endogenous microbiome with CRISPR based vectors to selectively kill the pathogenic strains without harming the beneficial ones. Based on its patent filings, it is predicted that SNIPR Biome will be using the engineered probiotics as an add-on therapy along with standard therapies for a specific disease.

Van Pijkeren Laboratory at the University of Wisconsin-Madison is developing bacteriophage capable of carrying a customized CRISPR message to address antibiotic resistance in pathogens. The probiotics can be used as delivery vehicles for the bacteriophage, which can then be easily lysed by stomach acid and the released phage DNA can target any nearby pathogens (ex. Clostridium difficile), causing them to degrade their own DNA.

Eligo Bioscience, along with its partner GSK, has been working on antimicrobials (Eligobiotics) by using a CRISPR system to genetically disable inflammation-inducing gene in otherwise healthy skin bacteria, killing only those that contain the inflammation-inducing gene. The French company hopes that their Eligobiotic (EB005) will eventually result in the creation of a topical cream that can be applied to acne-irritated skin, penetrating the skin microbiome to deliver bacteria-killing phages to the affected areas. If proven to be safe and effective, it has the potential to address the root cause of acne.

Locus Biosciences, a clinical-stage biotechnology company based out of North Carolina in the US, is in possession of Phase 1b clinical trial results for a drug product (LBP-EC01), which is a CRISPR-Cas3-enhanced bacteriophage (crPhage™) specifically targeting Escherichia coli (E. coli) bacteria that causes urinary tract infections (UTIs). The world's first completed, randomized, placebo-controlled trial of recombinant bacteriophage therapy has met its primary and secondary endpoints and has demonstrated proof of concept.

https://www.nature.com/news/gene-edited-crispr-mushroom-escapes-us-regulation-1.19754
https://www.innovationfiles.org/modified-mushroom-escapes-regulation/
https://news.berkeley.edu/story_jump/crispr-put-to-work-to-save-chocolate-from-devastation/
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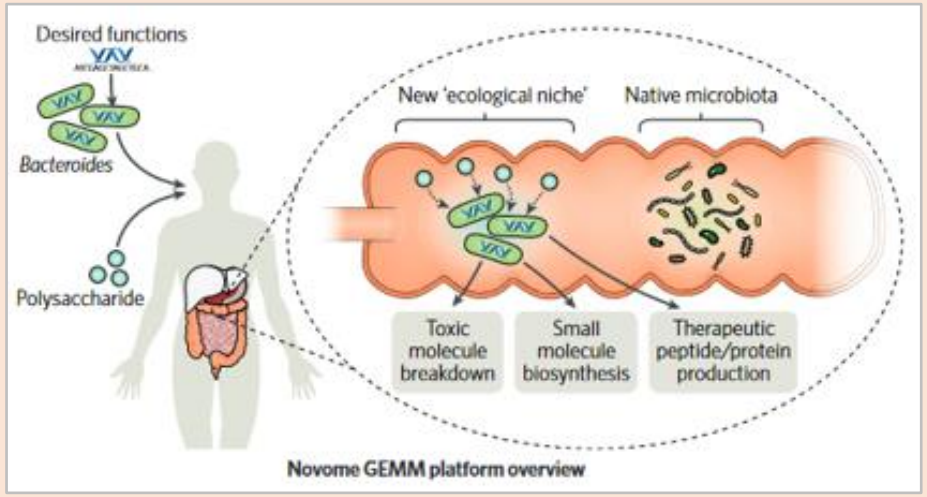
https://www.globenewswire.com/fr/news-release/2021/02/24/2181504/0/en/Locus-Biosciences-completes-first-of-its-kind-controlled-clinical-trial-for-CRISPR-enhanced-bacteriophage-therapy.html

CRISPR Engineered Probiotics - Start-Ups

Novome Biotechnologies, a startup founded in 2016 by scientists from Stanford University and the University of California, Berkeley, has developed a platform for controlled and robust colonization of the human gut with engineered therapeutic bacteria, known as Genetically Engineered Microbial Medicines (GEMMs) platform. The focus of the company is on hyperoxaluria treatment. The clinical proof-of-concept has been demonstrated through Phase 1 trial and the company is keen on expanding its platform to include additional disease indications. To accelerate the process, it has entered into a non-exclusive license agreement with Caribou Biosciences to access its CRISPR-Cas9 intellectual property. Novome scientists are focusing on engineering Bacteroides, which are the native gut bacteria and can effectively compete with other resident microbes to durably colonize the gut. Gene cassette introduced into the Bacteroides' genome by Novome scientists allows the GEMM to metabolize porphyran and create a new ecological niche. Daily oral feeding of encapsulated porphyran maintains both GEMM colonization and expression of desired therapeutic protein which degrades oxalate in the gut before it can be absorbed.



Novome GEMM platform overview

https://www.globenewswire.com/en/news-release/2020/08/12/2077236/0/en/Novome-Biotechnologies-Expands-Therapeutic-Focus-and-Platform-Capabilities-with-Acquisition-of-Preclinical-Projects-and-Intellectual-Property-from-Caribou-Biosciences-and-License-t.html

https://media.nature.com/original/magazine-assets/d43747-020-01160-7/d43747-020-01160-7.pdf

DuPont - a Pioneer in CRISPR Engineered Probiotics

- DuPont, currently a world leader in dietary supplements for health and wellness, acquired Danisco in 2011
- DuPont has emerged to be a pioneer in the CRISPR technology area holding a dominating number of patents
- DuPont has around 6000 phages in its collection which can be used to immunize bacteria cultures
- It holds exclusive licenses through strategic partnerships with various startups
- It is quite likely that DuPont has been commercially producing CRISPRized dairy products
- DuPont is to manufacture a **new strain of *Lactobacillus plantarum* for clinical trials to** study the effect of probiotics on gut microbiome of infants with sepsis
- The study is funded by Bill & Melinda Gates Foundation and is conducted by a Canadian hospital and two institutes in Bangladesh and, was initiated in last quarter of 2020.

https://pubmed.ncbi.nlm.nih.gov/17379808
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Challenges and Future Prospects

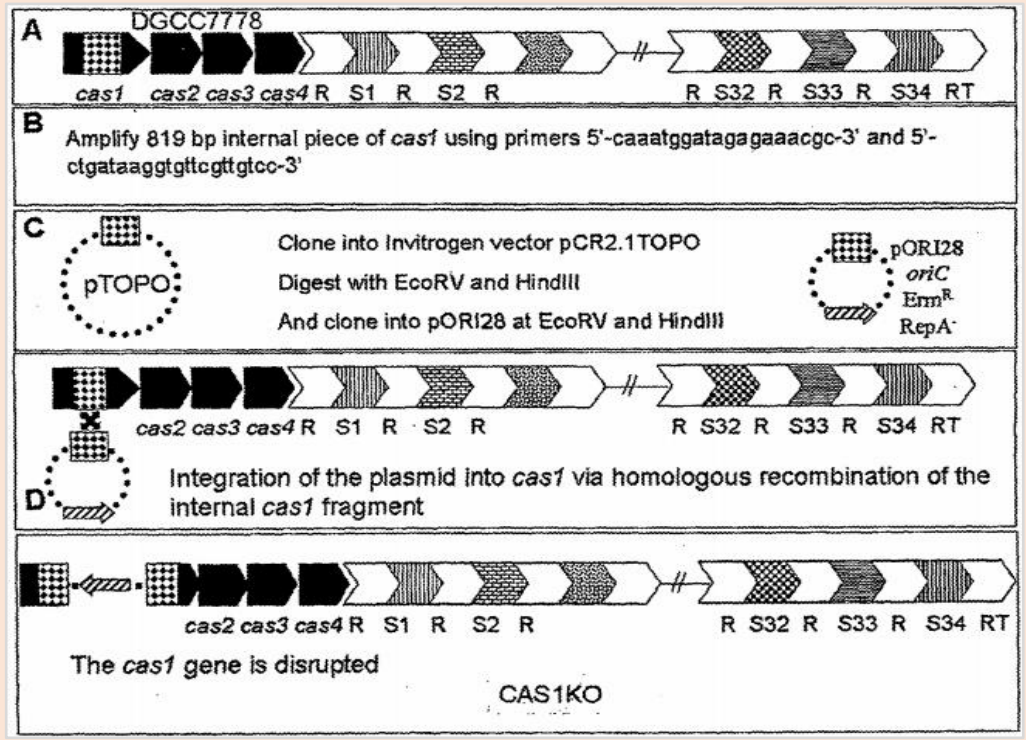
- CRISPR-Cas systems seemingly offer easy portability between species, but the outcome of DNA cleavage by Cas nucleases can vary depending on the DNA repair pathways present in different organisms. This needs to be addressed through the development of specific tools and strategies designed for different species.
- Another challenge is in introducing foreign DNA in microbiome because techniques such as electroporation, conjugation or transduction are not reliable in some bacteria.
- Some bacteria also carry restriction systems to destroy the foreign DNA. Some may not be able to replicate plasmid DNA, or it may not be easy to grow some bacteria in the laboratory.
- Despite the challenges, CRISPR tools provide strategies not only to study the microbial biology, but also to understand their role within the complex communities and drive the development of novel therapies.
- In recent years, the CRISPR-Cas system has been utilized for genome editing in various Lactobacillus species, and more recently, a flexible and universal genome engineering strategy has been developed for both Lactobacilli and Bifidobacterium.
- In near future, one can expect that these developments will be expanded to other beneficial genera as well.
- Once scientists address existing challenges with CRISPR technology, the engineered microbiome can modulate the composition and functional activities of gut microbiome. The manipulation of gut microbiome will result in improved therapeutic and health benefits.
- Excerpts from a few interesting patents have been presented to showcase the future directions in gut microbiome research.

US10640778B2 - Method of Modulating Cell Resistance - DuPont Nutrition Biosciences APS

- DuPont has disclosed DNA constructs (comprising Cas gene which comprises nucleotide sequences of the SEQ ID Nos. selected from the group consisting of SEQ ID Nos. 505-508, 510-516, and 518-521) for modulating resistance in a cell against a target nucleic acid or a transcription product. These DNA constructs can be used for inducing bacteria with phage resistance and also resistance to genomic DNA via plasmid transfer, mobile genetic elements; and for disrupting antibiotic resistance genes and genes encoding virulence factors. These genetically engineered bacteria which are resistant to spoilage by bacteriophages, are useful in and as starter culture or a probiotic culture for food production. These bacteria are also resistant to any plasmid DNA transfer that can confer the probiotic bacteria with undesired virulent factors.

- The recombinant nucleic acid sequence comprises Cas gene and two CRISPR repeats together with CRISPR spacer, wherein the CRISPR spacer is heterologous to Cas gene and/or CRISPR repeats to modulate resistance against a target nucleic acid or transcription product . The CRISPR repeats are derived from the same cell which is being subjected to alteration.

- Cas genes/proteins and the CRISPR repeats may be of same origin, but the spacer may be obtained from a bacterial cell which is resistant to a target nucleic acid. The CRISPR spacer may be a synthetic nucleic acid sequence derived from bacteriophage DNA or any of plasmid DNA, mobile genetic element, transposable element, insertion sequence or an antibiotic resistance gene. The target nucleic acid may be derived from a nucleic acid encoding a virulence factor (such as toxin, an internalin and a hemolysin).



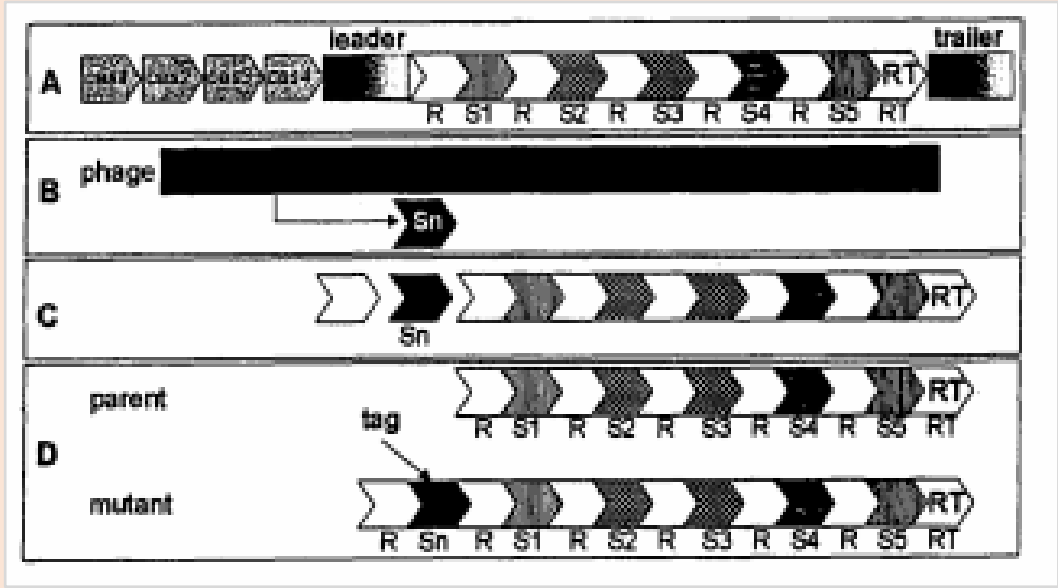
Bacteria genome alteration using the novel DNA constructs: Streptococcus thermophilus DGCC7778 CRISPR mutant resistant to phage 858 has lost the resistance due to disruption of cas1 protein

US9399801B2 - Tagged Microorganisms and Methods of Tagging – DuPont Nutrition Biosciences APS

- DuPont has disclosed methods for identifying microorganisms using CRISPR loci tags especially in bacteria of Streptococcus genera. The method for generating a CRISPR variant comprising a tag, includes the steps of: (a) exposing a parent bacterium comprising a CRISPR locus and a Cas gene bacteriophage to produce a culture of bacteriophage resistant variant bacteria comprising a modified CRISPR locus with an additional repeat-spacer unit, naturally inserted therein. The spacer provides a tag and has a length between 20 bp and 58 bp and has 100% identity to a nucleotide sequence in the genome of said bacteriophage (b) selecting said bacteriophage resistant variant bacteria by comparing with parent bacteria and then isolating and/or cloning and/or sequencing the additional repeat-spacer unit.

- The tagged bacteria may be produced using recombinant DNA techniques that are known in the art.

- These tagged bacteria find application in and as starter cultures extensively used in the food industry in the manufacture of fermented products including milk products (e.g., yogurt and cheese), meat products, bakery products, wine, and vegetable products. The starter culture is a lactic acid bacteria species, with strains of Bifidobacterium, Brevibacterium, or Propionibacterium. Suitable starter cultures of the lactic acid bacteria group include, strains of Lactococcus, Streptococcus, Lactobacillus Enterococcus, Pediococcus, Leuconostoc, and Oenococcus. In addition, probiotic strains such as Bifidobacterium lactis, Lactobacillus acidophilus, Lactobacillus casei find use in flavor enhancement and health benefits.



Tagging sequence and a CRISPR repeat are integrated at one end of the CRISPR locus. Panel A shows a CRISPR locus and elements, including repeats (R), spacers (S), the upstream leader and downstream trailer, with the terminal repeat (RT) adjacent to the trailer, and cas genes in the vicinity. Panel B shows a phage sequence in black, with a fragment of the sequence (Sn) being used as an additional spacer (i.e., tagging sequence). Panel C shows the insertion of a new spacer (Sn) (i.e., tagging sequence) at one end of the CRISPR locus (close to the leader in this example at the 5' end of the CRISPR locus), between two repeats. Panel D provides a comparison of the CRISPR locus content between the parent and the mutant bacterium (i.e., tagged bacterium), with a new spacer (Sn) (i.e., tagging sequence). The new spacer (Sn) constitutes the tagging sequence which is specific for the mutant bacterium (i.e., tagged bacterium).

US9951341B2 - Lactococcus CRISPR-Cas Sequences - DuPont Nutrition Biosciences APS

- DuPont has disclosed a method for transformation of host cell in order to confer it resistance to a target nucleic acid which is of phage origin. At least one Lactococcus Cas gene encoding a Cas protein, a spacer flanked by two of Lactococcus CRISPR repeats, wherein the spacer is homologous to a target nucleic, altogether form a functional combination of the recombinant nucleic acid sequence used for the transformation of the host cells. Host cells could be from species such as Bifidobacterium, Brevibacterium, Propionibacterium, Lactococcus, Streptococcusa, Enterococcus, Pediococcus, Leuconostoc and Oenococcus.

- This invention finds application particularly in food and fermentation industry where current strategies such as mixed strain cultures used to minimize bacteriophage infection often lead to the failure of bacterial cultures upon sub-culturing. The repeated sub-culturing of mixed strain cultures leads to unpredictable changes in the distribution of individual strains and eventually leads often to undesired strain dominance. This in turn may lead to increased susceptibility to phage attack and risk of fermentation failures.

- The present invention provides a method for selection of strains suitable to fulfil the needs of phage defense rotation strategies. It also provides methods and compositions suitable to customize strains having lysotypes that are adapted to a particular phage environment. The invention provides methods and compositions suitable for directing the evolution of a given strain to various lysotypes, in order to produce strains that differ from each other only by their spectrum of phage sensitivity (lysotype). This difference of lysotype is a function of the CRISPR-Cas system. Lysotypes are strains which have identical metabolism (e.g., of carbon, nitrogen, etc.) and thus identical functionalities (e.g., acidification, flavor, texture, etc.) but the difference is obtained through the "modulation" of phage resistance. The methods and compositions are provided to produce starter cultures with strictly identical industrial functionalities to be used in rotation dairy fermentation in order to combat sequential phage attacks.

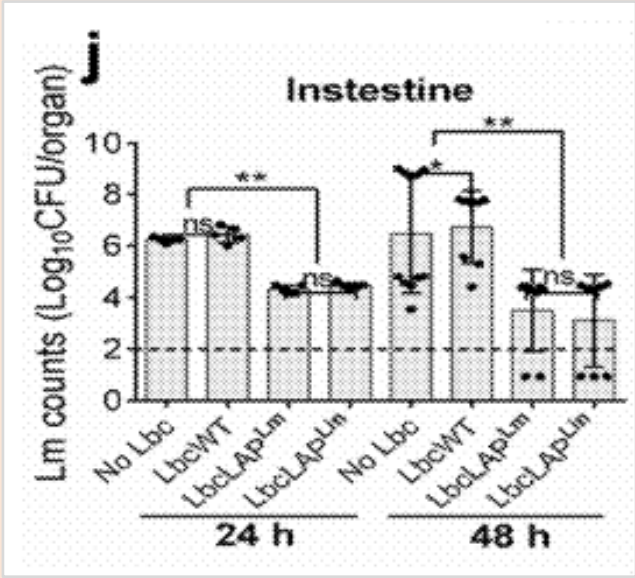
- To address the issues with undesired strain dominance, CRISPR-escape phage mutants were designed. The method for controlling undesired bacterial (eg. lactococcal) populations in a product, includes exposing the fermentation medium to compositions comprising CRISPR-escape phage mutant.

- A bacterial culture (such as a starter culture, a probiotic culture, a dietary supplement culture or other useful cultures) comprising a variant bacterial cell (suitably a lactococcal cell) comprising a Lactococcus CRISPR spacer or a Lactococcus CRISPR repeat or a Lactococcus CRISPR array or a vector are useful in production of food product or feed product or, personal care product, or health care product, or veterinary product or dietary supplements.

US20200000876A1 – Bioengineered Lactobacillus Probiotics and the Uses Thereof - Purdue Research Foundation

- Purdue Research Foundation has disclosed the use of reengineered bacteria expressing Listeria adhesion protein (LAP) for improving the health of humans and animals. These Next Generation Bioengineered Probiotics (NGBPs) are also used in treating or preventing intestinal inflammatory conditions. An animal feed supplement and a meat production method involving addition of reengineered bacteria thus reducing/eliminating the use of antibiotics in the meat-animal feed is disclosed.

- Listeria monocytogenes, a foodborne opportunistic pathogen responsible for severe systemic infection (listeriosis) in humans, uses Listeria adhesion protein (LAP) to cross the intestinal epithelium by inducing epithelial barrier dysfunction. The LAP from L. monocytogenes bears high sequence similarity to the LAP from L. innocua (non-pathogen). The current investigation showed that Lactobacillus casei expressing LAP from L. innocua, supplied to mice in drinking water for 10 days, and subsequently challenged with L. monocytogenes was able to protect mice from listeriosis. This probiotic also significantly reduced L. monocytogenes burden in the extra-intestinal tissues, modulated proinflammatory cytokines levels, dampened NF-kB activity, and improved epithelial innate defense and barrier function to protect mice from the infection.



Bioengineered Probiotics feeding in mice significantly reduced L.monocytogenes colonization in the mice intestine
Lbc – Lactobacilli, Wild type Lbc - LbcWT and bioengineered Lbc strains with LAP from L. monocytogenes/ L. innocua (LbcLAPLin; LbcLAPLm)

- LAP binds to a mammalian cell receptor, Heatshockprotein60 (Hsp60) which is involved in both chaperoning and immune system functions. At low levels, Hsp60 is anti-inflammatory, but at higher concentrations, it can take on pro-inflammatory roles especially in inflammatory disease conditions such as Crohn's Disease (CD), Inflammatory Bowel Disease (IBD), and Ulcerative Colitis (UC), thereby leading to gut barrier disruption.

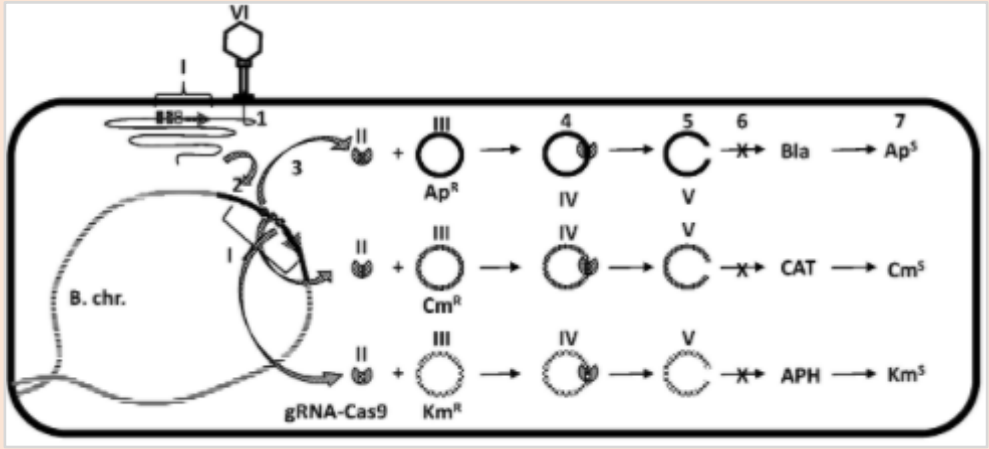
- Therefore, the introduction of NGBPs into food/feed can improve the human/animal's ability to combat intestinal infections without the use of antibiotics. It also improves the quality of meat from animals such as swine which often encounter various stressful situations including excessive heat as well as food and water deprivation during transportation.

EP3132034B1 - Therapeutic - Nemesis Bioscience Ltd.

- Nemesis Bioscience has disclosed a delivery vehicle for introducing a polynucleotide into an antibiotic-resistant microorganism for inactivation of DNA carrying antibiotic resistance gene/s. Delivery vehicle is comprised of recombinant polynucleotide consisting of CRISPR array nucleic acid sequence having or transcribing multiple RNA guide molecules, wherein each RNA guide molecule: (i) is transcribed from its own promoter sequence (ii) mediates the binding of a CRISPR associated (Cas) DNA-binding polypeptide to the antibiotic resistance gene(s); and (iii) has a spacer sequence complementary to a target DNA sequence of the antibiotic resistance gene. The delivery vehicle may also comprise a nucleotide sequence encoding recombinase catalytic domain, which prevents direct killing of the microorganism due to the generation of the double strand break.

- This delivery vehicle could be any of a conjugative plasmid, plasmid replicon, nucleotide vector, linear double-stranded DNA, non-virulent bacteriophage, or a lysogenic bacteriophage (depicted in the adjacent figure).

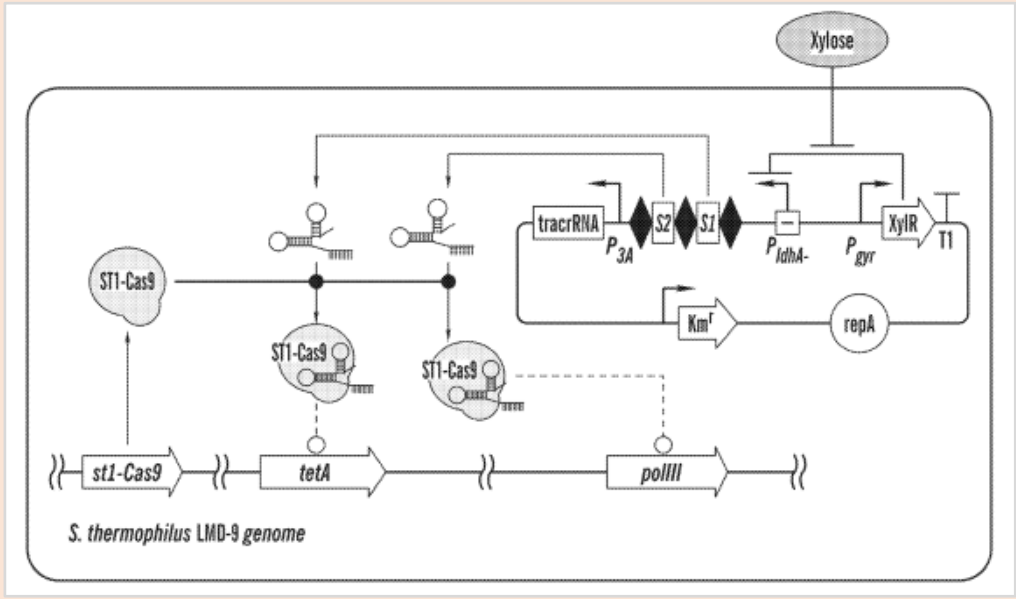
- The invention finds its application in the area of infectious disease treatment by re-sensitizing the microorganisms to the existing antibiotics, which otherwise were considered to be less effective due to prevalence of antibiotic-resistant microorganisms. It also has applications in probiotic compositions. When administered probiotically as a stabilized culture (e.g. Lactobacillus spp) carrying the plasmids in order to transmit the same to gut flora, it generates a prophylactic protection against future infection with antibiotic resistant bacterial pathogens.



CRISPR/Cas9-mediated bacterial immunization against antibiotic resistant genes on a plasmid: **I,** CRISPR/Cas locus, where boxes denote different spacer sequences targeting different antibiotic resistance genes; **II,** gRNA-Cas9 where boxes denote different gRNAs targeting each antibiotic resistance gene; **III,** plasmid harbouring antibiotic resistance gene; **IV,** target recognition and positioning of Cas9; **V,** cleaved plasmid, **VI:** bacteriophage. **Ap^R** = ampicillin resistant, **Cm^R** = chloramphenicol resistant, **Km^R** = kanamycin resistant. **Ap^S** = ampicillin sensitive, **Cm^S** = chloramphenicol sensitive, **Km^S =** kanamycin sensitive. **Bla=** beta-lactamase, **CAT** = chloramphenicol acetyl transferase, **APH** = aminoglycoside phosphotransferase.
1. Injection - CRISPR/Cas9 is injected into the bacterial cell along with phage DNA by bacteria-specific phage infection. **2. Lysogenisation** - Phage DNA is lysogenized and integrated into the bacterial host chromosome (**B. chr.**). **3. crRNA biogenesis and assembly of Cas9** - Pre-crRNA is transcribed and hybridized with tracrRNA and processed to make mature crRNA:tracrRNA (an RNA guide molecule, or "gRNA"), which is assembled with Cas9. **4. Recognition** - These gRNA-Cas9 complexes recognize target DNA on the plasmid. **5. Cleavage** - The gRNA-Cas9 complexes cleave DNA at the sites recognized by crRNAs. **6. Inactivation** - This leads to inactivation of the production of antibiotic resistant enzymes; 7. **Sensitive** - Thus, the bacterial cell becomes sensitive to various antibiotics

US9701964C1 - Altering Microbial Populations and Modifying Microbiota - SNIPR TECHNOLOGIES

- SNIPR Technologies has disclosed a method of modifying a mixed population of microbiota bacteria by selectively targeting a sub-population using host modifying (HM) crRNAs. These (HM) crRNAs are expressed within the targeted bacteria (host), after being transfected with a vector or mobile genetic elements (MGEs) comprising engineered nucleic acid sequence consisting of (i) a nucleic acid sequence comprising spacer and repeat sequences encoding said HM-crRNA; (ii) a nucleic acid sequence encoding a sequence of said HM-crRNA. The target bacteria has an endogenous Cas nuclease, to form a HM-CRISPR/Cas system, which modifies the host target sequences in host cells, whereby host cells are killed or the host cell population growth is reduced. HM-system also comprises a tracrRNA sequence which hybridize to repeats in the immature crRNAs to form pre-crRNAs in the host cells. The target bacteria may be a gram positive bacteria, Firmicutes such as Streptococcus thermophilus.



A schematic of the xylose-inducible CRISPR device: Upon induction of xylose the CRISPR array targeting both polIII and tetA on the S. thermophilus LMD-9 genome are expressed. Together with the constitutively expressed tracrRNA a complex is formed with Cas9. This complex will introduce a double stranded break in the tetA and polIII genes in the S. thermophilus LMD-9 genome resulting in limited cell viability.

- The invention will help increase the beneficial bacteria and reduce pathogenic bacteria of the gut. The gut is well populated by two distinct phyla, Bacteroidetes and Firmicutes. Bacteroidetes have an essential role in preventing infection with Clostridium difficile by stimulating Paneth cells which produce antibacterial peptides. This invention is directed towards reducing the Firmicutes population against the Bacteroidetes.

- Streptococcus thermophilus ST1-CRISPR array was designed to contain only the CRISPR array repeats and spacers under a xylose inducible promoter, followed by the corresponding tracrRNA under a strong Streptococcus promoter. The tracrRNA plays a role in the maturation of crRNA and it is processed by host RNase III, forming a complex with crRNA. This complex acts as a guide for the endonuclease ST1-Cas9. After transcription of the synthetic array from the xylose inducible promoter, the endogenous Cas9 and RNAses will process it into a functional gRNA. The gRNA/Cas9 complex will cause a double stranded break at the target location. The design of the array used 2 specific target sequences (DNA polymerase III subunit alpha and an antibiotic resistance gene (tetA-like gene)) which are high on GC content and a reduced portion of the tracrRNA.

US10760075B2 - Treating and Preventing Microbial Infections - SNIPR TECHNOLOGIES

- SNIPR Technologies has disclosed a method of treating an infection that is associated with acute septicemia caused by pathogenic bacteria in subjects receiving treatment for diseases such as cancer, organ transplant, cardiovascular issues etc. A programmed Cas nuclease (endogenous Cas nuclease of the pathogenic bacteria) in a conjugative plasmid vector which is delivered from carrier bacteria, preferably Lactobacillus, is administered as the treatment for the septicemia. The invention will help increase the beneficial bacteria and reducing pathogenic bacteria of the gut. A CRISPR/Cas system comprising the nuclease is a Cas nuclease (ex. a Cas 3 or 9) and the system comprises guide RNAs or DNA encoding guide RNAs, wherein each guide RNA is capable of programming the Cas nuclease to cut a target site comprised by the genomes of the microbes.

- The invention involves use of programmable nuclease or vector carrying it (which is a plasmid, cosmid, virus, a virion, phage, phagemid or prophage) for cutting of microbe genomes. The targeted cutting provides selective microbe killing or reduction of growth or proliferation to treat or prevent an infection, which is contrary to broad-spectrum microbial killing using conventional antibiotics. Thereby, selective killing is advantageous to leave beneficial microbes untargeted by the treatment, which will be beneficial to the patient

- The effect of treatment with a programmed nuclease as compared to traditional antibiotic treatment for septicimia was several 1000 folds (ex. 3 or 4 logs) within a timespan of 3 hours after the first exposure of bacteria. The programmed endogenous Cas cuts the genomes of the bacteria to kill the bacteria or reduce growth, thus treating the infection, and enabling a less frequent dosing which is convenient for the healthcare practitioner and patient, while providing economical therapy.