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Get Cultured: Eat Bacteria

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Abstract

The Klaenhammer group at North Carolina State University pioneered genomic applications in food microbiology and beneficial lactic acid bacteria used as starter cultures and probiotics. Dr. Todd Klaenhammer was honored to be the first food scientist elected to the National Academy of Sciences (2001). The program was recognized with the highest research awards presented by the American Dairy Science Association (Borden Award 1996), the Institute of Food Technologists (Nicholas Appert Medal, 2007), and the International Dairy Federation (Eli Metchnikoff Award in Biotechnology, 2010) as well as with the Outstanding Achievement Award from the University of Minnesota (2001) and the Oliver Max Gardner Award (2009) for outstanding research across the 16-campus University of North Carolina system. Dr. Klaenhammer is a fellow of the American Association for the Advancement of Science, the American Dairy Science Association, and the Institute of Food Technology. Over his career, six of his PhD graduate students were awarded the annual Kenneth Keller award for the outstanding PhD dissertation that year in the College of Agriculture and Life Sciences. He championed the use of basic microbiology and genomic approaches to set a platform for translational applications of beneficial microbes in foods and their use in food preservation and probiotics and as oral delivery vehicles for vaccines and biotherapeutics. Dr. Klaenhammer was also a founding and co-chief editor of the *Annual Review of Food Science and Technology*.

HISTORY

I was born in 1951 in St. Paul, Minnesota in the working-class neighborhood of Maplewood. Neither of my parents, Robert and Eleanor, went to college, but both were sure that I was going. Every day, they encouraged me to do the best I could and to finish what you start. My father was the fire marshal in Maplewood and at one in the morning on Christmas Eve, 1967, he got a call for a house fire at the far end of the county line. I was 15 years old and heard him leave, knowing he "had to go." Lights and siren running when he dashed off, only to be broadsided by a driver who ran an intersection with a four-way stop sign: DOA. Because he died in the line of duty, throughout my undergraduate tenure at the University of Minnesota I received a social security survivor check to help pay for my college tuition. Over the summer months, I worked the 11 pm–7 am shift at a gas station and the last two years of college delivered mail for the US Postal Service on a walking route in St. Paul, MN. I lived with my mother, Eleanor, and had a wonderful girlfriend (now wife, Amy) all through the late 1960s and early 1970s.

Nearly 100 years ago, the Russian scientist Elie Metchnikoff, then at the Pasteur Institute, proposed that lactic bacteria in fermented milk could promote the development of a healthy intestinal microbiota. Specifically, the Nobel Prize winner developed a theory that lactic acid bacteria (LAB) in the digestive tract could, by preventing putrefaction, prolong life. Since that time, consumption of LAB has increased, primarily via fermented foods (e.g., yogurt, cheese, sausages, vegetables, and breads) and the probiotic microbes of the *Lactobacillus* and *Bifidobacterium* genera. Although the beneficial roles of LAB in food preservation and as probiotics were widely purported, the microbial taxonomy, physiology, structural features, genetics, and metabolic pathways responsible for the activities of these beneficial microbes had yet to be identified in 1973.

My scientific career began by working toward a bachelor's degree in microbiology (in 1973) at the University of Minnesota. During my senior year, I interned as an undergraduate researcher in the laboratory of Dr. Russell Johnson, then working with the spirochetes *Leptospira* and *Treponema*. This is where I learned real aseptic techniques, because these microbes were nasty pathogens and grew so slowly that any mistake led to contamination of the cultures or you. One of the post-doctoral students in that laboratory encouraged me to consider graduate school and meet with this new assistant professor in food science who was doing some landmark genetic work with food microbes, specifically bacterial starter cultures used to make cheese. At our first meeting, Dr. Larry McKay asked if I played softball (apparently an important qualification for graduate school—luckily, I did and the department team needed an infielder). Then he explained that his group had just discovered extrachromosomal DNA elements in dairy LAB used as starter cultures in milk fermentations. He spelled out his overarching research hypothesis to me, which was that inconsistencies in the industrial performance of LAB in food fermentations were due to the genetic instability of plasmids that encoded vitally important fermentation properties, notably lactose fermentations (acid production), proteolytic activity (milk coagulation and flavor development), and antimicrobial activities of bacteriocins (nisin) that killed pathogens. Dr. McKay was the first scientist to bring genetics into solving problems in food microbiology and fermentation; his pioneering work and landmark research program were widely acclaimed internationally (McKay 2015) (**Figure 1**). As a student of Louis Pasteur and his seminal work in fermentation microbiology, I was intrigued by both the genetic approach toward solving the issues of the day in food microbiology and the practical applications of McKay's research. Falling into the right place, at the right time, with an insightful and talented mentor, inspired me to work hard toward master's (1975) and doctoral degrees (1978) in food science, with minors in biochemistry and genetics; plus, some exciting softball games thrown in over five years.

Figure 1

Professor Larry L. McKay with some former alumni from the University of Minnesota graduate program. Left to right: Robert Hutkins (University of Nebraska), Todd Klaenhammer (North Carolina State University), Jeffrey Broadbent (Utah State University), Mary Wagner (Gallo), Jeff Kondo (Utah State University), and Larry L. McKay. Banquet for the Lactic Acid Bacteria Genomics Consortium, October 10, 2002.

In 1978, after obtaining my doctoral degree, I was offered a position in the Department of Food Science at North Carolina State University (NCSU) to work with Dr. Marvin L. Speck, a William Neal Reynolds Distinguished Professor of food science and microbiology. NCSU was one of the top food microbiology programs in the United States. Dr. Speck was known for editing *The Compendium of Methods for the Microbiological Examination of Foods* (Speck 1984). Speck's research focused on preservation methods for concentrated dairy starter cultures and the development of Sweet Acidophilus™ milk, using *Lactobacillus acidophilus* NCFM (North Carolina Food Microbiology). Today, this culture is distributed widely as a probiotic in dietary supplements and dairy foods, such as yogurt. At 26 years old, I arrived at NCSU as an assistant professor, fortunate to drop into an established and nationally renowned food microbiology program with talented colleagues and graduate students who were excited to apply the explosion in genetics and biotechnology to beneficial microbes in food.

The ensuing 40 years in North Carolina brought many challenging and exciting events. My personal highlights are the following: I married the right stuff, my wife Amy—anyone meeting Amy suddenly thought better of me. My daughter Ellen arrived in 1986 and is now a practicing physician's assistant in cardiology. Amy and I built a log cabin on Mayo Lake at the North Carolina/Virginia border (1990–1993) with tons of help from many friends. While launching a boat, my Ford truck sunk into that lake on Good Friday morning in 1994. I was sitting in water up to my chest when the truck finally settled on the bottom, but remarkably I drove out and it still ran for another 15 years. We raised and trained seven hunting golden retrievers who all religiously fetched the paper every day and "helped" our unwitting house guests unpack any "open" suitcases to retrieve and display their delicates for all to see. As a final personal highlight, during my phased retirement (2016), I rolled off a 14-foot ledge on the fireplace inside the lake cabin and bounced off the mantle onto a raised stone hearth, landing face down on the wood floor with two retrievers trying to wake me up. I was out for 2 minutes. Luckily, Amy was with me and screamed "don't you move" as she ran off to call EMS, which dispatched both an ambulance and a helicopter. Bummer,

I missed the helo ride, as they could not land anywhere near the cabin. Broken parts were: back, T5 vertebrate, three ribs, right shoulder, and right foot plus two toes. Fully recovered now, I celebrated my second year of "I am still alive" on September 4th, 2018. Golf and racquetball are still on the schedule.

Our research program at NCSU started with an emphasis on basic genetic approaches to solving translational applications of beneficial food microbes in foods. The knowledge and technology explosions at the time (1980s) in microbiology, genomics, and biotechnology were the platforms on which our group targeted five primary areas of study: genetics of LAB, bacteriocins/antimicrobials, mechanisms of phage resistance in starter cultures, mechanisms of probiotic functionality, and, later, the use of LAB as oral delivery vehicles for biotherapeutics.

GENETIC TOOLS FOR LACTIC ACID BACTERIA

Early on in our research program, we embarked on developing genetic tools and plasmid vectors for investigation and manipulation of beneficial LAB, notably lactococci (cheese/milk starter cultures) and probiotic lactobacilli (*L. acidophilus* and *Lactobacillus gasseri*). Essential to those efforts were developing and exploiting natural systems of gene transfer, specifically plasmid conjugation (Klaenhammer & Sanozky 1985), phage transduction (Raya et al. 1989), and DNA transformation by electroporation (Luchansky et al. 1988). Given those important genetic tools, our focus shifted to exploiting new approaches to investigate some of the current questions and issues of the time about the LAB and probiotic microbes.

BACTERIOCINS

Initially, our group looked at the ability of probiotic lactobacilli to produce small antimicrobial peptides, termed bacteriocins. Both *L. acidophilus* and *Lactobacillus johnsonii* produced bacteriocins, designated lactacin A and lactacin F, respectively (Allison et al. 1994, Barefoot & Klaenhammer 1983). The host ranges of these bacteriocins were narrow, inhibiting only closely related species that occupy similar ecological niches; in this case, the gastrointestinal (GI) tract. Most interesting was the discovery and characterization of a two-component peptide system, in which expression of the two lactacin peptides was required for optimal activity (Klaenhammer 1993). Later, some of our efforts were directed to the broad-spectrum bacteriocin nisin. Nisin effectively kills Gram-positive foodborne pathogens (*Staphylococcus aureus*, *Listeria monocytogenes*, and Clostridia) but is ineffective against Gram-negative bacteria. The mechanism of action for nisin was considered to be the cytoplasmic membrane. We hypothesized that the outer membrane of Gram-negative bacteria could be excluding nisin from acting at the cytoplasmic membrane. Using a chelating agent, Stevens et al. (1991) disrupted the outer membrane of *Salmonella* Typhimurium and sequentially treated those cells with nisin. Indeed, we found that nisin was bactericidal to *Salmonella*, and its action against the cytoplasmic membrane appeared to be universal to both Gram-positive and Gram-negative bacteria. This was an exciting and major discovery that expanded the scope and potential applications of nisin in food preservation. This is particularly relevant today, as protein engineering of nisin has significantly elevated its effectiveness against both Gram-positive and Gram-negative bacteria (Field et al. 2012).

RESISTING PHAGE ATTACK

Early on, our long-term research focused on understanding how starter cultures resist attack by bacteriophages in cheese and cultured milk fermentations. Bacterial viruses appear constantly in

milk fermentations, infect and kill the lactic starter culture, and often slow or stop the fermentation. Thus, protecting fermentation bacteria from bacteriophage attack is a chief concern to the dairy and other bioprocessing industries. Between 1980 and 1986, our laboratory discovered that some strains of *Lactococcus lactis* harbored a collection of plasmid-encoded defense systems that prevented phage adsorption, recognized and cleaved incoming phage DNA, and stopped phage DNA replication (Sanders & Klaenhammer 1981, 1983). In some of our earliest work at NCSU (Klaenhammer & Sanozky 1985, Steenson & Klaenhammer 1985), we directed the transfer of plasmids encoding phage defense systems into *L. lactis* strains, using conjugation. This work was expanded in collaboration with Dr. Mary Ellen Sanders at Miles Laboratories (now Danisco-DuPont) and represented the first example in which industrial starter cultures were genetically improved via a natural gene transfer technology (Sanders et al. 1986). It was an exciting period for our research group because these phage-resistant strains were used as starter cultures industrially, and we could follow their performance under the most dynamic and challenging environments for phage evolution and adaptation (Hill et al. 1991).

The starter cultures constructed were remarkably successful in their industrial performance. Subsequently, detailed genetic sequence examination of the key plasmid involved, pTR2030, revealed three distinct phage resistance genetic determinants on the plasmid (**Figure 2**). The first is a restriction–modification system (Hill et al. 1989, O'Sullivan et al. 1995) that recognizes and cuts incoming unmodified DNA (phage DNA destroyed, no progeny phage, microbe survives). The second is an abortive infection system (AbiA) (Hill et al. 1990) that halts any phage DNA replication and triggers suicide of the infected microbe before the phage cycle is complete and thereby stops the release of new progeny phages into the fermentation milieu. The third is another abortive system (Durmaz & Klaenhammer 2006), in which AbiZ activated transcription of the phage's own lysin and holin genes, causing premature cell death via explosive lysis before any progeny phage formed. Thus, pTR2030 encoded two complementary suicide systems that killed the cell and aborted phage development at the point of a seminal infection, preventing release

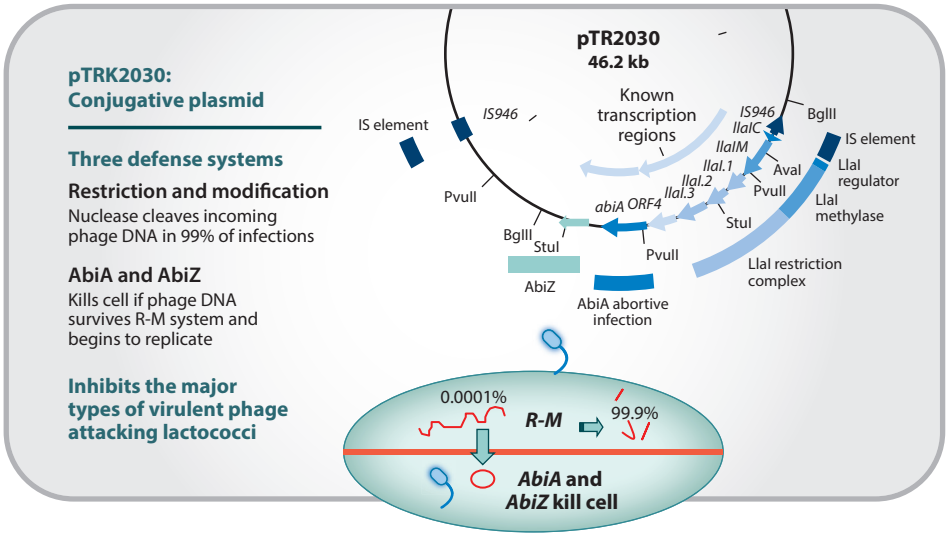

Figure 2

Genetic organization of the three bacteriophage-resistance loci (Abortive infection determinants, AbiA and AbiZ) and the LlaI restriction–modification (R-M) system encoded by the conjugative plasmid, pTR2030.

of new phage into the fermentation vat. Consider that you have 100,000,000 bacterial cells per milliliter in multiple 1,000- to 5,000-liter fermentation vats; when the first phage-infected cell commits suicide, it entombs the virus and thereby stops phage development and proliferation, altruistically saving the rest of the microbes in the population.

The longer-term outcome from these studies was the development of genetic strategies to stack multiple plasmid-encoded defenses within *Lactococcus* starter cultures and rotate these internal defenses to systematically trap the bacteriophages and halt their development and proliferation in dairy fermentation plants (Durmaz & Klaenhammer 1995, Sing & Klaenhammer 1993). These strategies were instrumental in providing fast acid-producing, phage-resistant *L. lactis* starter culture strains for the dairy industry and served as models to other bioprocessing industries that are threatened by bacteriophage attacks.

This body of work was the major platform presented to support my nomination to the National Academy of Sciences (NAS). My election in 2001 was a complete surprise—a miracle—a point stated universally by anyone ever elected. First, the process leading to election is so unpredictable, complicated, and difficult; second, one is humbled by the fact that there are so many deserving individuals who are not elected. Nevertheless, my induction in 2002 (**Figure 3**) importantly positioned me as a member-editor of the *Proceedings of the National Academy of Sciences* (PNAS). In that role, as the first food scientist elected to the NAS, I was able to support the food and beneficial microbe community with access to new opportunities to submit articles, direct an informed peer review process, and promote publications in PNAS. Since then, our group and collaborators have published 16 articles in PNAS, and I have edited more than 35 articles for other authors. Topics covered were exploding issues of the day in food microbiology: multiple genomes of LAB, lactobacilli, and bifidobacteria; cell-surface structures [pili, surface (S)-layers, lipoteichoic acid (LTA)] impacting adherence to host tissues and immunomodulation; bile acid metabolism; prebiotic transport and metabolism; human milk oligosaccharides and multiple reports about bacteriocins produced by LAB. I also had the opportunity to serve on the committee of scientific programs for the NAS (2003–2007), which defines and organizes most of the NAS-sponsored scientific events for the academy each year. I took that opportunity, with my colleague Dr. Jeffrey Gordon (Washington University), to organize an Arthur M. Sackler Colloquia at the

Figure 3

Alumni celebrating my 2001 election to the National Academy of Sciences. Bell Tower at North Carolina State University was lit red that evening.

NAS on "Microbes and Health." The colloquia presented 28 keynote lectures representing international scientists working on GI microbiology, host–microbe interactions, and probiotic microbes. In 2011, twenty-eight papers from the colloquia were published in volume 108 of PNAS. One very fulfilling opportunity of my career with the NAS was to act as an oversight member for the German–United States Frontiers of Science program for young investigators. The program invited young scientists (<40 years) who were leading multidisciplinary areas of science to participate in forums alternating between Germany and the United States each year. In those forums, I had the opportunity to invite and work with a number of young US-based scientists in the area of food microbiology/beneficial microbes. Among those were David Mills (University of California–Davis), Andy Benson (University of Nebraska), Ruth Ley (Cornell University), and Jens Walter (University of Nebraska). Alumni of the Frontiers of Science program are commonly elected to the NAS later in their careers.

MECHANISMS OF PROBIOTIC FUNCTIONALITY

In 2001, our group at NCSU was working to sequence the genomes of *L. acidophilus* and *L. gasseri*, two probiotic microbes used widely in the United States and internationally. We believed that the genome sequences of these human commensal microbes would provide insights into the mechanisms by which probiotic cultures survive and positively act within the GI tract. In efforts led by Eric Altermann et al. (2005) and Andrea Azcarate-Peril et al. (2008) at NCSU, the genome sequences were determined, annotated, and published (**Figure 4**). For historical comparison, the cost then was approximately $2,000,000 to fully sequence, curate, and hand-annotate one genome, whereas today's cost for a draft genome is around $500 with automated annotation. At that time, numerous genome sequencing efforts were underway internationally to sequence LAB and probiotic microbes (Claesson et al. 2006, Kankainen et al. 2009, Kleerebezem et al. 2003, Pridmore et al. 2004, Schell et al. 2002, van de Guchte et al. 2006), including a multi-institutional effort in the United States by the Lactic Acid Bacteria Genomics Consortium (Makarova et al. 2006).

GENOME SEQUENCING AND ANNOTATION

The genomes of these organisms were small (~2–3 Mb) and lacked many important biosynthetic pathways, consistent with their auxotrophic nature and fastidious nutritional growth requirements. Accordingly, the microbes encoded many transporters, permeases, peptidases, and glycosylases for internalization and catabolism of sugars and amino acids, features likely reflecting the organism's adaptation to the nutrient-rich environment of the upper human GI tract. Our group went on to identify numerous genetic regions within *L. acidophilus* and *L. gasseri* genomes that contributed to the organism's survival and interactions within the GI tract. For example, the microbes encoded a variety of cell-surface proteins and structural components (e.g., S-layer proteins, mucus-binding proteins, machinery for synthesis of LTA) implicated in adherence to mucus and epithelial cells and important for signaling to immune and dendritic cells (DCs) of the intestinal mucosa (Johnson & Klaenhammer 2014, Klaenhammer et al. 2002, Lightfoot et al. 2015). Moreover, we were able to identify numerous regulatory regions encoding systems vital to the microbes' ability to transport and metabolize carbohydrates, lactose, prebiotics, and glycogen (Goh & Klaenhammer 2014, 2015) and sense and adapt to changes in the environment, notably, acid (Kullen & Klaenhammer 1999) and bile tolerance (Pfeiler & Klaenhammer 2009).

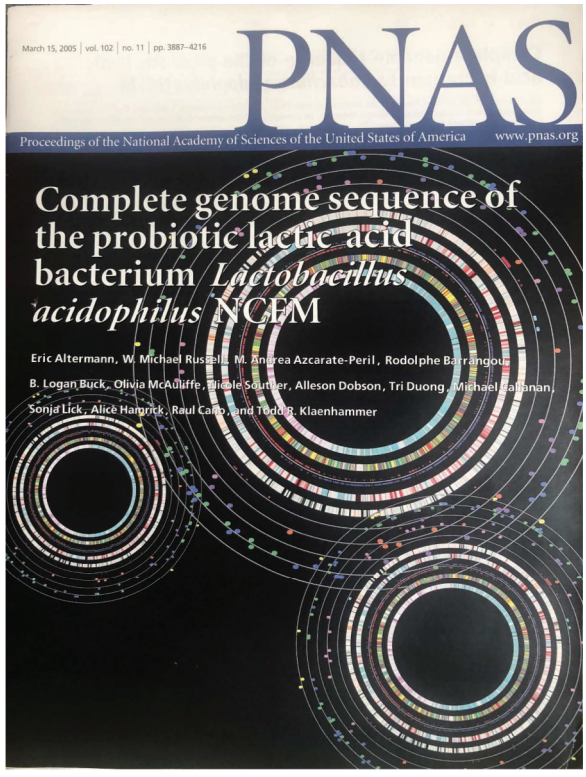

Figure 4

Cover of volume 102, issue 11 of the *Proceedings of the National Academy of Sciences*, depicting the publication of the complete and annotated genome sequence of *Lactobacillus acidophilus* NCFM. Copyright (2005) National Academy of Sciences, U.S.A.

FUNCTIONAL GENOMICS

In the era of molecular biology exploding around *Escherichia coli* and *Bacillus subtilis*, research on LAB was paralyzing from 1985 through the 1990s because we did not have the tools to transform or genetically manipulate these beneficial and probiotic microbes. Over the ensuing years, our group and others systematically developed genetic tools for the modification of LAB and intestinal *Lactobacillus* species. A plasmid-based integration system for inactivation of targeted chromosomal genes in probiotic lactobacilli was finally developed (Russell & Klaenhammer 2001). Together with the appearance of complete genome sequences in the 2000s, this targeting integration tool was used to systematically knock out genes and investigate metabolic pathways, identify cell-surface proteins that mediate attachment to intestinal epithelial cells, and ultimately construct beneficial lactobacilli that may be used to orally deliver biotherapeutics (O'Flaherty & Klaenhammer 2016). Taking this functional platform one step further, in vivo mouse models were developed by Dr. Jun Goh and Dr. Sarah O'Flaherty and exploited to evaluate the impact of gene deletions on the GI-tract survival, persistence, and competitiveness of probiotic lactobacilli. Goh & Klaenhammer (2014) established the significance of glycogen biosynthesis on the competitive retention of *L. acidophilus* in the mouse intestinal tract, demonstrating for the first time that the ability to synthesize

intracellular glycogen contributes to gut fitness and retention among probiotic microorganisms. In a second study, in vivo competitive co-colonization of *L. acidophilus* NCFM against its sortase (Δ*srtA*) mutant showed that sortase-dependent cell-surface proteins contribute to gut retention of probiotic microbes in the mouse GI tract (Call et al. 2015).

PREBIOTIC TRANSPORT AND METABOLISM

Our research group sought to characterize the metabolic pathways and enzymes responsible for the transport and catabolism of complex sugars in lactobacilli. We described gene loci in *L. acidophilus* involved in transport and catabolism of fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS), which can promote competition of beneficial microorganisms in the human GI tract (Andersen et al. 2011, Barrangou et al. 2003, Goh & Klaenhammer 2015). FOS are one of several nondigestible prebiotics that selectively stimulate the growth and/or activity of probiotic strains residing in the host GI tract. A wide range of FOS are present in the human diet in sources such as wheat, onions, artichoke, banana, and asparagus. Although FOS have been shown to increase the population of bifidobacteria and lactobacilli within the GI tract, little had been known about the metabolic pathways and enzymes responsible for the transport and catabolism of such complex sugars in lactobacilli. A locus within the *L. acidophilus* NCFM genome sequence was identified in silico, indicating the presence of a gene cluster encoding proteins potentially involved in prebiotic transport and hydrolysis. Our research group computationally identified this specific cluster and then employed a functional genomic approach, using gene knockouts, to determine how FOS were metabolized by *L. acidophilus* (Barrangou et al. 2003). Using a membrane-associated ABC transporter, the organism first internalizes larger oligosaccharides and then proceeds to catabolize the molecule via an intracellular fructosidase into monosaccharides for glycolysis. We hypothesized from these transporter results that the ability to capture oligosaccharides would provide a competitive edge to this organism during competition for sugars in the human GI tract.

GOS are milk-based, smaller prebiotics (typically a chain of 3–4 galactose moieties terminated by a glucose). Historically investigated as human breast milk oligosaccharides, GOS are known to stimulate the growth of bifidobacteria in breastfed infants, facilitate the establishment of the microbiota in the GI tract, and promote proper development of the mucosal immune system. Bifidobacteria and some lactobacilli (notably *L. acidophilus)* ferment GOS. On the basis of the genome sequence of NCFM, our group functionally investigated the transport and catabolism of GOS by selectively knocking out predicted transporters and galactosidases suspected to be involved. Initially, we assumed that an ABC transporter (like FOS) would be involved in the transport of this complex carbohydrate. To our surprise, Andersen et al. (2011), with our Danish collaborators, discovered that the lactose operon encoded the genes for the transport (LacS permease) and catabolism (B-galactosidase) of GOS. This explained why some commensal/probiotic human lactobacilli, notably *L. gasseri*, do not ferment GOS, as they rely on a phospho-enol-pyruvate phosphotransferase system to transport lactose and a phospho-β-galactosidase to hydrolyze the internalized lactose 6-P. This was a circle-of-life moment for me, as I first learned about lactose metabolism in cheese starter cultures from Dr. Larry McKay, who taught all his graduate students about phospho-β-galactosidase, the alternative lactose operon encoded by plasmids in lactococci.

In this effort, we had an unusual advantage because I was working on another project with Ritter Pharmaceuticals to feed GOS to lactose-intolerant (i.e., lactase deficient) human subjects. The underlying hypothesis was that feeding GOS would increase the abundance of

lactose-fermenting microbes in the GI tract, notably bifidobacteria and lactobacilli. This adaptation of the GI microbiota might increase the level of microbial lactases, compete for lactose, and reduce the level of free lactose flowing through the GI tract to undesirable microbes that ferment it to produce gas and bloating in lactose-intolerant individuals. The problem of the day was that most of the available GOS were contaminated at 50% with free galactose, lactose, and glucose, compromising previous studies reporting on the prebiotic impact of GOS. Ritter Pharmaceuticals, Inc. developed a high-purity GOS (>95%) and kindly provided that material for our studies on microbes that would, or would not, ferment GOS. This highly purified GOS was used in our metabolic/genomic studies on *L. acidophilus* and was further used in both a human clinical trial (Savaiano et al. 2013) and a microbiome study of subjects fed this GOS over 30 days. Clinically, the lactose-intolerant subjects fed GOS showed a reduction in abdominal pain and improved overall tolerance to lactose. The microbiome study (Azcarate-Peril et al. 2017) indicated a definitive change in the fecal microbiome of lactose-intolerant individuals that were responsive to dietary adaptation to GOS, notably increasing the abundance of lactose-metabolizing bacteria and bifidobacteria. For the first time, microbiome changes to a prebiotic correlated with a clinical outcome, in this case tolerance toward lactose.

SOUTHEAST DAIRY FOODS RESEARCH CENTER

Our interest in the GOS study was piqued by my extensive work with the National Dairy Research Centers as director of the Southeast Dairy Foods Research Center (SDFRC). The potential ability to adapt the microbiome of lactose-intolerant individuals toward lactose tolerance was a powerful approach to increasing milk utilization in the United States. I am hopeful that the above prebiotic approach to expanding lactose tolerance will be investigated further and practically realized in the years ahead.

In my 22-year role (1993–2015) as the SDFRC director (**Figure 5**), I was fortunate to work closely with the National Dairy Council, Dairy Management, Inc. (DMI), and the directors of the five other centers located across the United States. Working with Joe O'Donnell (California DFRC), Rusty Bishop (Wisconsin Center for Dairy Research), Joe Warthesen and Lloyd Metzger (Minnesota DFRC), Don McMahon & Marie Walsh (Western DFRC), and Dave Barbano (Cornell DFRC) was among the most gratifying experiences of my career, both professionally and personally. The collective efforts of these individuals promoted and managed a nationally integrated program for product and processing research that developed the technological innovations that continue to drive the dairy industries of today. The infusion of funds from the National Dairy Council and DMI into the DFRC research centers was a platform from which many scientists flourished. Notably, I am very proud that E. Allen Foegeding (whey protein functionality) and MaryAnne Drake (sensory of dairy products/ingredients), both William Neal Reynolds Distinguished Professors at NCSU, built nationally and internationally acclaimed research programs that remain core pillars of the SDFRC and the national DFRC programs.

NEW HORIZONS FOR TRADITIONAL BENEFICIAL MICROBES

For more than 30 years, our research program employed genetic approaches for the investigation, improvement, and diversification of LAB that are used as starter cultures in food/dairy fermentations and as probiotics. These efforts led to the development of genomic information and genetic tools that could be used to manipulate probiotic lactobacilli, microbes with a long history of safe use as oral probiotics. *L. acidophilus* NCFM was first introduced in Sweet Acidophilus™ milk in the

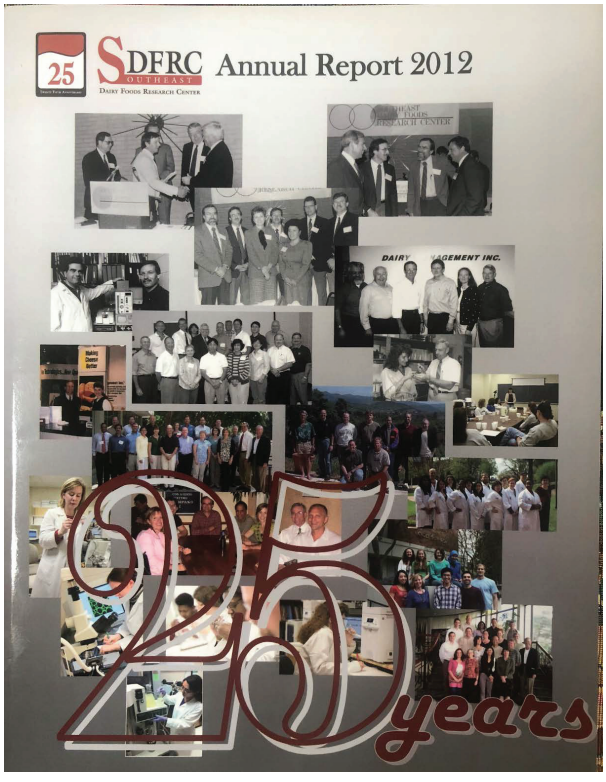

Figure 5

Cover of the 2012 Southeast Dairy Foods Research Center Annual Report.

mid-1970s and remains one of the most widely distributed probiotic microbes in dietary supplements and fermented milks/yogurts today. Safe oral delivery and transit through the stomach and GI tract opened the exciting possibility of using safe *L. acidophilus* and other LAB as live bacterial vehicles to express and deliver biotherapeutic compounds (e.g., vaccines, enzymes) to the intestinal tract or regulate intestinal immune responses (DC signaling and inflammation pathways). This approach taken by our group recognizes that consumption of a probiotic or fermented product delivers billions of bacteria through the stomach and into the intestinal tract. Microbes delivered at levels of 10^9–10^{10} CFU/g can readily overwhelm the normal resident populations in the small intestine, typically estimated at $\sim 10^4$–10^6/cm.

Our research group considered the outer cell surface of lactobacilli to be important for eliciting many predicted probiotic functionalities; for example, survival through the stomach, attachment to mucin and the intestinal epithelium, and interaction with immune cells of the intestinal mucosa. We systematically began to inactivate or delete the genes encoding various cell-surface components of *L. acidophilus* NCFM, specifically, surface layer proteins (SlpA, SlpB, SlpX) (Goh et al. 2009, Konstantinov et al. 2008), selected S-layer-associated proteins (Johnson et al. 2013, 2017), sortase-dependent surface proteins (Call et al. 2015), mucin and fibronectin-binding proteins (Buck et al. 2005, Hymes et al. 2016), and LTA (Mohamadzadeh et al. 2011, Selle et al. 2017). Critical to many of these efforts was the development of a new genetic tool by Dr. Jun Goh (Goh et al. 2009) that provided a positive counter-selection system for the detection of successful gene

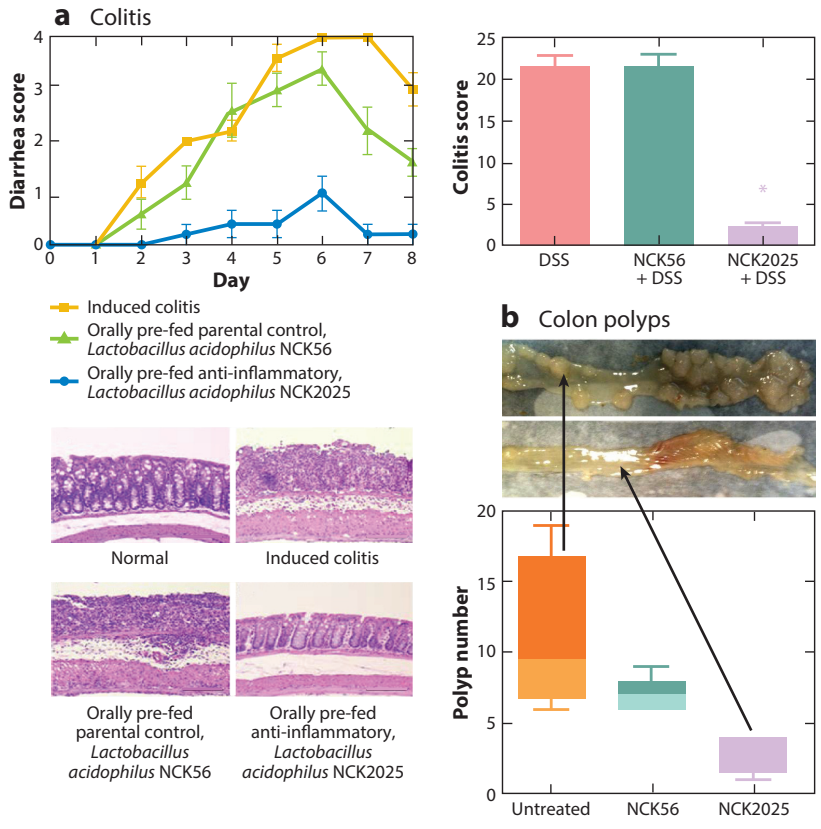

Figure 6

Results showing effects of lipotechoic acid (LTA)-deficient *Lactobacillus acidophilus* on colitis and colon cancer in mouse models (Khazaie et al. 2012, Mohamadzadeh et al. 2011).

deletion events in *Lactobacillus* genomes. Using a functional genomics approach, we compared the parent strain to the various mutants in attempts to identify functional roles for these cell-surface components. Over a decade of work, gene linkages were defined for a number of important probiotic phenotypes, including auto-aggregation, altered cellular morphologies, adhesion to mucin and fibronectin, improved epithelial barrier cell integrity, binding to DCs, and immunomodulatory signaling, including anti-inflammatory responses effecting colitis and colon cancer in mouse models (**Figure 6**).

In the later years, our group focused on the expression and in vivo delivery of vaccines via *L. acidophilus* and *L. gasseri*. In this format, food-grade lactobacilli serve to, first, express, encapsulate, and protect the vaccines during transit through the stomach and, second, to deliver and release the vaccine into the GI tract. Wells & Mercenier (2008) had pioneered the use of LAB for oral delivery of vaccines to the GI tract. Early results showed that vaccine efficacy was possible but was only moderately effective with ~30%–60% effectiveness. Later, we noted the discovery of DC-targeting peptides (Curiel et al. 2004) and, in collaboration with Dr. Mansour Mohamadzadeh, we began to build vaccines linked at the C-terminus with the DC-targeting peptides. The NCSU group successfully designed and constructed effective vaccines for both anthrax and botulism

(Mohamadzadeh et al. 2009, 2010; O'Flaherty & Klaenhammer 2016; Sahay et al. 2018) that were protective in mouse models (anthrax with 100% survival; botulism toxin with 70% survival). The efficacy of these strategies relied on two critical elements: high gene expression (from plasmid or genome locations) and stable chromosomal genetic cassettes. Achieving this goal was made possible by the efforts of Dr. Tri Duong, who constructed expression vectors (Duong et al. 2011), and Dr. Grace Douglas (Douglas & Klaenhammer 2011), who identified high RNA expression regions in the *L. acidophilus* genome, not surprisingly mostly in genes involved in glycolysis. Dr. Sarah O'Flaherty (O'Flaherty & Klaenhammer 2016) built on those results to construct a stable genome integration cassette for the botulism toxin heavy chain (Hc) of BoNT/A with a targeting DC peptide (DCpep). This cassette was positioned into a high expression region of *L. acidophilus* as a secretory immunogenic protein. The construct was genetically stable in vivo and protected mice from oral BoNT/A (Sahay et al. 2018).

Expanding on these platforms in collaboration with Dr. Gregg Dean (NCSU Veterinary Medicine), Kajikawa et al. (2010) used the surface layer protein SlpA as an epitope-displaying scaffold for oral vaccine delivery. The high expression levels of S-layer proteins and their cell-surface location were exploited to construct a genetically modified *L. acidophilus* expressing the membrane proximal external region (MPER) from human immunodeficiency virus type 1 (HIV-1). Intragastric immunization of mice with the recombinants induced MPER-specific and S-layer-protein-specific antibodies in serum and mucosal secretions in a mouse model.

Bacillus thuringiensis crystal (Cry) proteins (Bt toxins) are lethal to nematodes. In collaboration with Dr. Raffi Aroian (UC-San Diego), we sought to construct a LAB that would express Bt toxin for oral delivery to nematodes (bacteria are a primary diet for the worms). Durmaz et al. (2015) cloned the genes encoding two Cry proteins into *L. lactis* via a high-copy-number vector with a strong constitutive promoter. In vivo challenges of *Caenorhabditis elegans* worms demonstrated that the Cry proteins expressed intracellularly by *L. lactis* were biologically active. In the future, anti-helminthics may be delivered orally in food-grade microbes.

Our group at NCSU successfully developed multiple strategies for high gene expression from plasmid or chromosomal locations and protein expression for intracellular, secreted, or surface display formats. In vivo, the vaccines delivered orally via LAB potentiated effective mucosal immune responses against deadly microbial pathogens, toxins, and viruses. It is exciting that the potential use of LAB for safe, oral delivery of vaccines and biotherapeutics is now on the horizon.

CRISPR

The discovery of CRISPR (clustered regularly interspaced short palindromic repeats) was published in *Science* (Barrangou et al. 2007) by a group of research scientists at Danisco studying the bacteriophages attacking *Streptococcus thermophilus*, a yogurt and Italian cheese starter culture. By sequencing both the genomes of the starter cultures and phages attacking those cultures in the fermentation plants, the group realized that the repeated sequences in the genomes were spacers of phage sequences that presented a historical record of viruses infecting the starter cultures. Upstream of this array of repeated sequences were four genes encoding CRISPR-associated proteins (Cas). The group then used a gene knockout/integration system developed by Mike Russell in our NCSU laboratory (Russell & Klaenhammer 2001) to inactivate two of the *cas* genes, resulting in a complete loss of phage resistance. The experiment showed that adaptive immunity to bacteriophage was acquired by banking phage sequences in the genome array, and the mechanics were driven by the *cas* gene complex. The Barrangou et al. (2007) paper predicted that the *cas* genes

Figure 7

NCSU Klaenhammer alumni attending the 7th International Symposium on Lactic Acid Bacteria, 2002.

were responsible for phage DNA recognition, cleavage by a nuclease, and banking a historical record of phages infecting that strain over time.

This discovery has received international acclaim and fueled the explosion of CRISPR and Cas technologies for precise-targeting genome editing in bacteria, plants, and animals. I am extremely proud of the fact that four members of the Danisco team and authors on the original paper are NCSU/TRKLAB alumni: Professor Sylvain Moineau, Dr. Dennis Romero, Dr. Christophe Fremaux, and Dr. Rodolphe Barrangou (all shown in **Figures 7** and **8**, with other alumni from our laboratory attending the seventh and ninth LAB symposia). Interestingly enough, as a PhD student in our group, Rodolphe was a co-author on the PNAS genome paper for *L. acidophilus* (Altermann et al. 2005). In that paper, we reported an array of repeated sequences in the genome but did not realize at the time this was a CRISPR array. Dr. Barrangou joined NCSU as a faculty member in 2015 and is now a full professor leading the research and teaching efforts on LAB, starter cultures, probiotics, and CRISPR. At the end of my career, I was extremely fortunate to work with Rodolphe to co-direct some of my last graduate students on CRISPR-related projects (Selle et al. 2015, Stout et al. 2017). The circle of life comes back again with some of my final work before my retirement ending with questions about the mechanisms of bacteriophage resistance and CRISPR in LAB starter cultures and probiotics.

IN CLOSING

The community of scientists working with LAB and beneficial microbes has spanned centuries, with the most concentrated and collaborative efforts occurring over the past 50 years. An

Figure 8

NCSU Klaenhammer alumni attending the 9th International Symposium on Lactic Acid Bacteria, 2008.

abbreviated history of the scientists and international efforts (Asia, Europe, and the United States) that exploded this field in recent decades were reviewed in an article entitled "An Incredible Scientific Journey: The Evolutionary Tale of the Lactic Acid Bacteria" (Klaenhammer & de Vos 2011). It was a privilege, honor, and pleasure for me to work with the many LAB scientists all over the world who positively supported each other as colleagues and friends and defined this incredible journey forward over four decades.

Over and above the excitement of research and teaching, I am very proud of our graduate students and postdoctoral fellows who continued to bring excellence to the NCSU program over many years. From 1983 through 2000, six PhD students were recognized with the highly competitive Kenneth R. Keller Award, given annually to the student with the most outstanding doctoral dissertation in the College of Agriculture and Life Sciences. These PhD students (year they received the award) were Dr. Mary Ellen Sanders (1983), Dr. Larry Steenson (1986), Dr. Peter Muriana (1990), Dr. Dennis Romero (1991), Dr. Polly Dinsmore/Courtney (1996), and Dr. Shirley Walker (1999). There is no greater experience than working with talented young people and being part of their journey, accomplishments, and success. Our group was fortunate to host so many creative and talented students, postdoctoral researchers, visiting scientists, and research staff over nearly four decades (**Figure 9**). I am eternally grateful to all for sharing their experiences with me and our group as one step in their career paths.

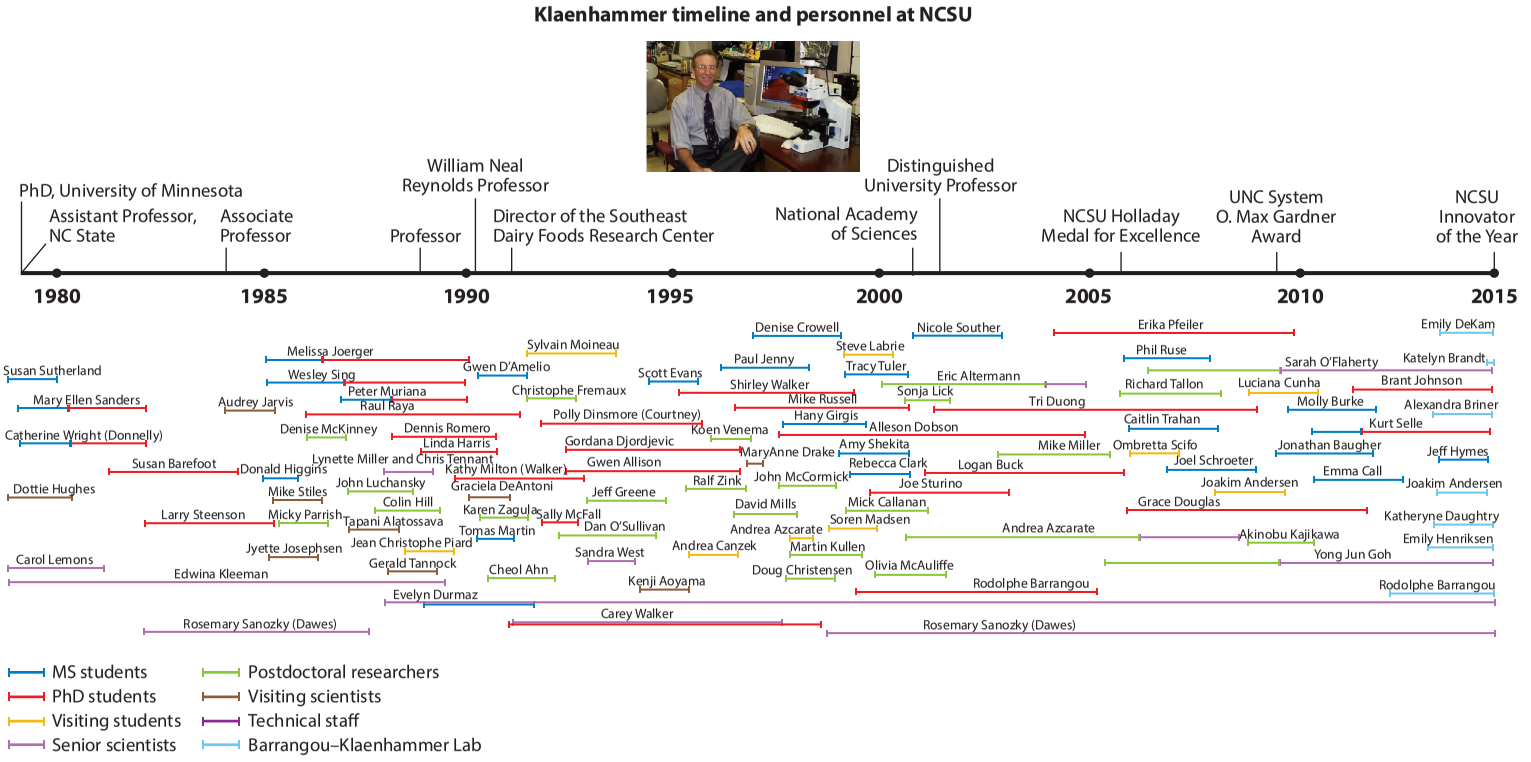

Figure 9

Klaenhammer laboratory timelines for students, postdoctoral fellows, and research staff scientists at NC State University.

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