The Effect of Transformed *Escherichia coli* on the Mouse Intestine Microbiome: the Microbial Metabolic Enhancement Hypothesis

Bryar P. Kader

A Senior Thesis submitted in partial fulfillment
of the requirements for graduation
in the Honors Program
Liberty University
Spring 2016

Acceptance of Senior Honors Thesis

This Senior Honors Thesis is accepted in partial fulfillment of the requirements for graduation from the Honors Program of Liberty University.

_____

Michael Price, Ph.D.
Thesis Chair

_____

Timothy Brophy, Ph.D.
Committee Member

_____

Mark Tinsley, D. Min.
Committee Member

_____

James H. Nutter, D.A.
Honors Director

_____

Date

Abstract

Metabolic disorders affect around thirty-four percent of the population in the United States. Among these disorders is lactose intolerance, which results from diminished production of the human lactase enzyme. This disorder and others like it are genetically determined and cannot be cured. However, the use of transformed bacteria implanted in the colon may provide a means by which the faulty pathway can be bypassed. To test whether transformed bacteria have the capability to aid in the digestion of normally indigestible compounds, a transformed strain of *Escherichia coli* overexpressing the beta-galactosidase enzyme encoded by the *lacZ* gene was colonized in the mouse intestine to enhance lactose digestion. The experiment provides the platform for similar research to be conducted in the future.

The Effect of Transformed *Escherichia coli* on the Mouse Intestine Microbiome: the Microbial Metabolic Enhancement Hypothesis

Lactose Intolerance as a Disorder

Lactose is the primary sugar component of dairy products and is the molecule responsible for the characteristic sweetness in these products. Lactose intolerance is a condition in which an individual is unable to digest the disaccharide lactose when ingested. It results from a body's inability to produce sufficient amounts of the beta-galactosidease enzyme lactase, which degrades lactose sugar into glucose and galactose monosaccharide units. The production of the enzyme in humans occurs in the cells lining the lumen of the small intestine and is secreted in response to the presence of lactose in the gastrointestinal tract (*1*).

Mammals characteristically produce milk from mammary glands present in female members of mammalian species. Mammalian young feed on the milk produced and thus have a higher lactase production rate than that of corresponding adult members of a species. Several mammalian adults lose the ability to produce lactase completely and thus become lactose intolerant as a result of reaching maturity. Humans are one of the few mammalian species that continue to drink milk into adulthood and the only mammalian species to drink the milk of other species. Moreover, consumption of food products high in lactose has become a large part of cultural diets around the world (*1*).

Many individuals mistakenly call lactose intolerance an allergy to lactose when in fact it is not an allergy by the conventional definition. Allergens, or substances that cause an allergic reaction, elicit a response from the immune system that often results in the release of pro-inflammatory signals and increased immune activity. Lactose intolerance

in its common form does not cause these reactions; rather it results in simple stomach cramps and possibly diarrhea due to the indigestion of the sugar by the body. The symptoms of the disorder make sufferers experience discomfort of varying degrees depending on the severity.

The Mammalian Gut Microbial Community

The mammalian colon is home to a number of microorganism species. These microorganisms form a microscopic ecosystem in which species compete for energy sources and colonization space, much like a macroscopic ecosystem. The enterobacterium *Escherichia coli* (*E. coli*) is a part of this ecosystem and fills the ecological niche of a scavenger in the mammalian gut (*2*).

Gram negative enterobacteria like *E. coli* make use of broken down disaccharides and monosaccharides that result from the catabolic action of obligate anaerobic bacteria, such as members of the *Bacteroides* genera, upon polysaccharide chains extending into the mucous layer of the colon. The resulting degradation of polysaccharides results in two mucous layers: one with complete polysaccharide chains extending from host intestinal epithelial cells and the second comprised of a mixture of mono-, di-, and oligosaccharide chains. It is in the partially degraded second layer of carbohydrates above the cellular polysaccharide chains that most enterobacteria reside (*3*).

Among various strains of *E. coli*, and extending to other bacterial populations in the gut, there exists a competition for the limited sugar sources available. Moreover, only the cell populations that maintain the ability to either make metabolic use of a carbon source that other populations cannot use or inherently have better molecular mechanisms for enhanced colonizing ability will persist in the intestine and act as a native flora

species. The former describes what is known as the nutrient niche hypothesis. Only cells that can position themselves in an open space in the ecosystem of the colon will persist and proliferate to form a stable population (*3*).

When niches open up in the gut microbiome that pathogenic or even native bacteria have the opportunity to cause disease in the host organism. It is when a microbe that inhabits a specific niche in the gut is either inadvertently eradicated from the ecosystem or is out-competed by a different species or strain that colonization by a pathogen can occur. Often the virulence of the given pathogen is at least in part due to its attachment to the apical surface of host epithelial cells. Colonization at the apical surface in turn results in inflammation and, depending on severity, may lead to serious symptoms such as diarrhea and intestinal bleeding (*4*).

Lactic Acid Bacteria and the Intestine

Lactic acid bacteria are defined as bacterial species that produce lactic acid as a byproduct of carbon metabolism. Members of this group are known for their consumption of lactose as an energy source. Most belong to the class of bacteria known as Firmicutes, which are characterized by gram positive cell wall structure and endospore formation.

Lactic acid bacteria have been used throughout time as fermenting agents in the production of dairy products such as yogurt and kefir and fermented vegetable foods like sauerkraut and kimchi. Consumption of fermented foods has been linked to intestinal health, as the lactic acid bacteria contained within promote better digestion of certain energy sources (*5*).

A genera of lactic acid bacteria known as *Lactobacillus* is actually part of the normal flora in the human body. In the early stages of life, infants have a higher amount of *Lactobacillus* bacteria residing in the colon than adults. This could be attributed to the need of infants to obtain virtually all of their nutrition from the milk of the mother, which contains large amounts of lactose sugar. Hence, with a higher percentage of lactic acid bacteria in the colon, it may be assumed that there is a higher amount of lactose catabolism carried out by such bacteria. The phenomenon may point to the gut microbiome playing an important role in lactose degradation in the early stages of life and possibly into adulthood as well (*6*).

Physiologically, most people from the country of Indonesia are lactose intolerant; the gastrointestinal tracts of these individuals do not produce lactase. However, such individuals are able to consume large amounts of dairy products without experiencing the symptoms of lactose intolerance (*5*). The lack of symptoms is counterintuitive until one realizes that large amounts of yogurt containing live lactic acid bacteria cultures are also consumed with the dairy products. The bacteria, many of which are *Lactobacilli*, thus consume the lactose in the dairy products and prevent the symptoms from occurring. This is the concept behind the use of probiotics, most of which contain several species of *Lactobacillus* and *Bifidobacterium,* another genera of lactic acid bacteria (*7*).

These bacterial species may provide aid to lactose intolerance sufferers in the digestion of lactose. However, most, if not all, lactic acid bacteria are poor colonizers of the mature adult gastrointestinal tract. Due to the nutrient niche factor, there are no open ecological niches for these species to inhabit, thus they are either out-competed and die shortly after ingestion or are shed in feces. To provide a permanent solution to lactose

intolerance, a bacterial species that colonizes the adult mammalian gut well must be genetically transformed to digest lactose with a fast enough rate to degrade the sugar before symptoms can manifest.

The Microbial Metabolic Enhancement Hypothesis

Genetic transformation of microorganisms is not a novel idea. Scientists have been using genetically modified microorganisms along with a plethora of other similarly modified organisms to express desired traits for decades. For instance, it is now quite common for diabetic patients to receive human insulin produced by recombinant *E. coli* cells expressing the human INS gene (*8*). Recombinant cells like those producing insulin are thus used for the production of desired biomolecules for research and/or medical applications.

Such recombinant cells have only occasionally been cultured *in vivo* for the purpose of treating disease or deficiency. They are typically cultured *in vitro* and their subsequent products harvested from culture media. However, the transformation of microorganisms native to the human microbiome could result in a potential source of novel metabolic activity occurring within the human host for the purpose of filling a void left by a metabolic deficiency in the host. Strategic genetic transformation of native microbes in the human body could result in novel metabolic pathways in the microbes themselves. These novel pathways could then be used to degrade or generate biomolecules in a patient that lacks the metabolic capability to do so on their own. Colonization of the microbe at a target site could fulfill the metabolic need if corresponding residential strains are either out-competed or eliminated with antibiotics.

This proposed novel use of transformed microbes to fill a metabolic deficit will be henceforth referred to as the microbial metabolic enhancement hypothesis (MME).

Colonization of Transformed *E. coli* in the Streptomycin Treated Gut

This study aims to determine if a strain of *Escherichia coli* transformed with an enhanced ability to catabolize lactose can colonize in a streptomycin-treated mouse intestine and possibly ease the symptoms of lactose intolerance in the murine host. Mice, like most small mammals, are completely lactose intolerant into adulthood, despite the commonly held belief that mice enjoy cheese regularly. As a result, they serve as an excellent model for testing the efficacy of a lactose intolerance treatment.

Streptomycin is an antibiotic that specifically targets the gram negative Enterobacteria, including *E. coli*. The streptomycin-treated gut is therefore devoid of most Enterobacteria (*9*). The only Enterobacteria that survive are those that have spontaneous antibiotic resistance to streptomycin. Several strains of *E. coli* resistant to the antibiotic have been isolated in research settings. One sub-strain of the *E. coli* K12 strain MG1655 has been isolated for streptomycin resistance and is thus able to be used in the streptomycin-treated gut. The nutrient niche factor would not impede the colonization of the strain, as the niches normally inhabited by native Enterobacteria would be left open subsequent to streptomycin treatment. The streptomycin-resistant mutant (MG1655 Str^r) was thus determined to be the perfect strain to use for transformation (*10*).

Initially, a wild type strain of MG1655 (MG1655 Str^r Nal^r) was co-colonized with a knockout mutant strain lacking the *lacZ* gene (MG1655 *ΔlacZ)* - the gene encoding the enzyme beta-galactosidease - in a streptomycin treated mouse (*2, 11*). The mouse host

was given drinking water with fifteen percent lactose to simulate a lactose rich diet. A second co-colonization was done using a derived strain of MG1655 that has been complemented for the *lacZ* gene. This restored strain was made using a plasmid vector under lac operon control from an *E. coli* strain from the ASKA collection of overexpressing *E. coli* mutants (*12*). The first co-colonization serves to determine the importance of lactose to *E. coli* MG1655 in the colon in regards to competitive ability. The second allows for the establishment of a mutant strain expressing lactase activity that can be controlled and regulated in the mouse intestine and allows for the mutant strain's competition with the wild type strain to be studied.

Literature Review: Potential Application

Background on Metabolic Disorders

Metabolic disorders are classified as illnesses that are genetically determined and prevent proper catabolism or anabolism of a given molecule or class of molecules. Sufferers of these disorders are therefore unable to process the substrate correctly, typically resulting in a detrimental buildup of the molecule in the body. The buildup then interferes with other biological functions and results in the symptoms of the disease. Current treatments for such disorders are quite limited in scope, and there is no cure for these illnesses to date. Often times, the subject is required to modify his or her own dietary intake of food to accommodate the body's inability to metabolize these harmful substances. Such modifications can make the intake of vital nutrients difficult if their sources coincide with the sources of the metabolically toxic molecules.

Some potential treatments, such as enzyme, drug, and gene therapy, have been explored at various lengths. However, each method has its own disadvantages. Enzyme

therapy allows for the direct injection of functional catabolic proteins into the blood or to the site of metabolic buildup. The enzymes fill the void created by the absence of the same or similar enzymes that would be present in a phenotypically normal person. Drug therapy involves using molecules that modulate the way the harmful molecule acts in the body. Such drugs can block cell receptors, lower blood levels, or even alter the chemical composition of the harmful substance to make it more tolerable by the body. While both of these treatments have had success in treating patients, the patient is required to receive the treatment continuously for life. Both treatments are therefore expensive endeavors that many are certainly unable to afford.

Gene therapy provides a more permanent solution to the problem, in that it directly attempts to repair an individual's altered gene(s) to allow them to produce the necessary metabolic pathway constituents on their own. Gene therapy involves the removal of a subject's body cells to be subsequently cultured and genetically modified by a number of different means before being returned to the body. While gene therapy would provide a more permanent solution, several risk factors, including potential autoimmune response against the cells and potential viral infection of the genome, make this treatment highly controversial and hazardous.

It can therefore be concluded that for a metabolic disorder treatment to have the best effect, it needs to be semi-permanent, meaning only rarely requiring repetitious instances, physiologically safe, and have the potential to be terminated if the body reacts adversely to it. Several metabolic disorders may be curable when the concept of MME is applied as proposed in this paper. Microbes can be transformed to produce any number of

enzymes and factors which could potentially fill the metabolic void left by genetic mutation of the host.

Urea Cycle Disorders and Ammonia Oxidation

Ammonia is a byproduct of catabolism of nitrogenous organic molecules in several eukaryotic life forms. In mammalian metabolism, ammonia waste is produced from the deamination of amino acids. Deamination in the liver results in the production of ammonia and organic acid. The organic acid is used in intracellular mitochondria as an energy source for aerobic respiration, whereas the ammonia cannot be used for energy and must be converted to urea, a less toxic nitrogenous waste, and excreted from the body. Many enzymes are involved in this pathway, and malfunction in some of them can be disastrous (*13*).

The urea cycle is a highly complex metabolic pathway in the mammalian body involving enzymes present in the hepatic cell cytoplasm and within the hepatic cell mitochondria. After amino acid deamination, ammonia, existing as ammonium in the blood due to pH, travels to the liver where the hepatic cell mitochondria combine it with bicarbonate using the CPSI enzyme to produce carbamoyl phosphate. A series of enzymes then convert the substrate into many subsequent metabolites before finally converting it into either fumarate or urea. Urea is subsequently transported by the blood to the kidney, where it is filtered into urine and eventually excreted (*14*).

Deleterious mutations are the destructive factor in urea cycle diseases and are present in the individual at birth. Due to early exposure to compounds that could not be metabolized in such individuals, several harmful secondary symptoms can result from

urea cycle-related mutations. Among these are mental retardation due to ammonia build up in the cerebrum (*15*).

Ammonia oxidation, also called nitrification, was once thought to be a very well-known process in the nitrogen cycle. The process involves taking the toxic, nitrogenous waste ammonia and converting it to a less toxic form, nitrite, which is subsequently converted to nitrate. This process is carried out by various bacteria species in soil and marine environments and allows the nitrogen in ammonia to be reused in biological processes in higher organisms or to be rereleased into the atmosphere as nitrogen gas (*16*).

Another group of organisms has been shown to have an equally important role in ammonia oxidation in low oxygen conditions. These microorganisms belong to the domain Archaea, a group of prokaryotes completely separate from Bacteria. Archaea superficially resemble bacteria, but their genetic code and metabolic pathways actually better resemble members of Eukarya. Ammonia oxidizing archaea (AOA) use a yet to be understood metabolic pathway to aerobically convert ammonia to nitrate, similar to the bacteria mentioned previously. The key difference between AOA and ammonia oxidizing bacteria (AOB) is that AOA are largely more efficient in their metabolism of ammonia, being able to thrive even at nanomolar concentrations of ammonia. This results from the high affinity these organisms have for ammonia. AOA have been identified in both soil environments and marine environments, competing with both anammox and classical AOB (*16, 17*).

Transformation of *E. coli* with ammonia oxidation genes could provide a means by which ammonia in the body of a urea cycle disorder patient could be detoxified in an

effective manner. The *amoA* gene from AOA species could be a possible candidate by which an ammonia oxidative *E. coli* strain could be generated. An interesting experiment would be to construct two *E. coli* mutants, one with AOB ammonia oxidation genes and one with AOA ammonia oxidation genes. When colonized within a mouse model with high levels of ammonia, the strain that colonizes best would be the strain to use for urea cycle disorder treatment. The competing strains would also need to be analyzed for total ammonia oxidative activity.

A major weakness of this approach is the lack of understanding of what effect oxidized ammonia products such as nitrate and nitrite could have on the total health of the gastrointestinal tract. As ammonia oxidation has never been carried out in the intestine, a thorough investigation of the long term affects the reaction could have on the host must be undertaken before even considered as a treatment option. While nitrate and nitrite are less toxic than ammonia, they may have long-term health risks if produced in large amounts in the colon.

Additionally, it must be determined if the location of ammonia oxidation in the intestine would even provide sufficient detoxification of ammonia to prevent urea cycle disorder symptoms from manifesting. The intestine may not be the optimal location for ammonia detoxification to occur. The transformed strains might not even be provided sufficient access to blood ammonia to be able to detoxify it from the colon. If a significant amount of ammonia is left in the blood even with ammonia oxidative *E. coli* in the colon, a different site of colonization should be considered.

Diabetes and Insulin Production

Diabetes is a disorder in which an individual's body is unable to regulate the glucose content of the blood due to a deficiency of a necessary signaling factor. Often, it is a mutation in the human INS gene that results in the disorder. The INS gene encodes for the signal peptide insulin. Insulin regulates blood glucose levels by causing body cells to internalize more glucose, thus removing the molecule from the blood. When a mutation exists in the INS gene, the body cannot produce its own fully functional insulin. When the cause of the disease is a deleterious mutation of the INS gene, the disorder is known as type II diabetes. Patients with this disease are forced to receive insulin usually in the form of an injection directly to the bloodstream in order to maintain blood glucose levels. Currently, insulin used by patients is produced by mutant *E. coli* strains transformed with the human INS gene (*18*).

Before the use of *E. coli,* patients had to receive insulin harvested from horses or other livestock. Using insulin produced by different animals was generally considered safe at the time. However, due to the slight variations in protein structure, patients would often have allergic reactions to the treatment, or the treatment would not be sufficiently effective to control blood sugar levels (*8*). Insulin production by *E. coli,* and afterward *Sacromyces cerviciae,* resulted in the availability of virtually endless supplies of the protein in human form. The body of patients therefore do not react adversely to the treatment.

While insulin has been produced by *E. coli* for years, the insulin producing bacteria have only been grown in *in vitro* conditions. The insulin is harvested from the cells in large bioreactors. Recombinant cells are grown and subsequently broken open to

release the human protein. Refinement of the cell lysate allows for the isolation of human insulin for packaging and shipping. The process kills all the cells and requires re-inoculation of the bioreactor with a fresh *E. coli* inoculum. Large sums of money are spent on media in which to grow the cells in and requires a great deal of coordination of advanced techniques to isolate the insulin itself from the cells (*19*).

Due to the native presence of E. *coli* in the intestine, it is feasible that recombinant cells expressing the human INS gene could colonize in the colon and produce insulin indefinitely within the host organism. The result would be a pool of insulin ever present in the colon, minimizing the need for further treatment. Only occasional emergency re-colonization of the recombinant strain would be required should the gut environment become hostile to the recombinant strain.

Several challenges would have to be overcome for such a treatment to be successful. First, no insulin producing *E. coli* mutant has thus far been designed to secrete the insulin itself. For a recombinant population of *E. coli* to thrive in the gut, it is imperative that they continue to live and propagate over many generations. Current methods of insulin harvest from *E. coli* involve the breaking open of the cells, which obviously kills them in the process. A mechanism of secretion must be established for a colonizing *E. coli* INS strain to be successful in treating diabetes. Secretion of insulin may be attainable via translocon insertion into the *E. coli* genome. The solution involves the insertion of a gene for a eukaryotic translocon protein that would allow for the active transport of the insulin peptide out of the bacterial cell.

The second foreseeable challenge would be the control of insulin production in the recombinant population. The production of the peptide would need to be only allowed

after high glucose intake. Too much insulin would not allow any glucose to be in the blood, which would be disastrous for the entire body. Putting the INS gene under operon regulation may present a possible solution. If the INS gene were incorporated into a lac operon, the gene would only be transcribed in the presence of lactose or a lactose analog. Having to take an enteric coated lactose pill to receive diabetes treatment would be a great deal less expensive than relying on insulin injections for life.

Enhanced Cellulose Catabolism

Cellulose is the most abundant carbohydrate on the planet, being produced in some capacity by all plants and even algae. The carbohydrate serves as a structural component in the cell wall of plant cells that give them their characteristic rigidity. Synthesis of cellulose is carried out by a series of cellulose synthase enzyme complexes near the plasma membrane of the plant cell. The complexes bind individual glucose ring molecules together via condensation reaction at carbons one and four. The particular type of bond formed between two glucose rings in cellulose chains is known as a beta glycosidic bond. Few organisms possess the proper enzymatic equipment to break this bond, thus releasing glucose for metabolic energy acquisition (*20*).

A cellulase is an enzyme involved with the degradation of cellulose. The term cellulase is somewhat generic, as there is not one specific enzyme that carries out the function. Cellulases are thus spread across a wide variety of organisms, both prokaryotic and eukaryotic, and degrade the polysaccharide chain in various ways. In general, cellulase enzymes fall into three main categories: endoglucanases, exoglucanases, and beta-glucosidases. Endoglucanases function by separating individual cellulose chains from the stacked configuration they naturally take. Exoglucanases cleave disaccharide

cellobiose units from the ends of the cellulose chains. Beta-glucosidease break the cellobiose units into individual glucose monomers that can be used for respiration (*21*).

E. coli strains have been transformed to degrade vegetable matter by action of cellulase enzymes in the biofuel industry. Genes from various cellulolytic bacterial species have been used to produce mutant cellulolytic *E. coli*. A study by Rodrigues et al. made use of an endoglucanase gene from Firmicute *Bacillus pumilus* and a beta-glucosidease gene from the thermophilic species *Fervidobacterium* to produce an *E. coli* mutant producing both of these enzymes for enhanced bioethanol production (*22*). The result was a strain that could grow on cellulose media twice as fast as strains possessing only one or the other cellulase genes.

Mammalian ruminants have a plethora of cellulolytic bacterial species that colonize their specialized intestinal tracts, typically from the genus *Clostridium (23).* As with *Lactobacillus*, however, it would be impossible to get these species to colonize the gut when ecological niches have already been established. Therefore, using a cellulolytic *E. coli* strain similar to the one mentioned earlier would be necessary to have cellulolytic activity in the intestine. The principal potential benefit that such a colonization could bring about is the ability for the body to obtain fuel from sources normally thought of as indigestible. Humans typically eat a wide variety of plant-based foods, but most of the energy in the form of cellulose is never accessed due to the limits of the native intestinal microbiota. Colonization by cellulolytic strains could allow malnourished people in impoverished situations to access energy from virtually any form of plant matter.

One foreseeable issue is the need for the secretion of cellulase enzymes to generate glucose outside of the bacterial cell wall. Only glucose formed in the lumen of

the colon could have the potential to be absorbed by the bloodstream and thus be used by the body. Fortunately, many strains already produced for the biofuel industry that secrete cellulase enzymes have been created. Thus, the genetic material encoding for cellulolytic ability from these strains would be optimal for generating an *E. coli* strain to be used practically.

Applications Outside the Intestine

While the gastrointestinal tract is a hub of sorts for metabolic activity of native microbiota, there are several other sites in and on the body where microorganisms abound. Other microbes could thus be exploited for metabolic enhancement as with *E. coli*. A very wide range of disorders could be treated in this manner.

For instance, the human skin is covered with several species of *Staphylococcus*, characterized by coccus morphology and gram positive staining. *Staphylococcus* bacteria typically exist in a commensal relationship with the host, posing no immediate threat. However, if left unattended, an open wound in the skin could serve as the site of a nosocomial infection by at least some *Staphylococcus* species. Therefore, *Staphylococcus* bacteria have a reputation for being opportunistic pathogens (*24*, *25*).

Despite the pathogenic potential, *Staphylococcus* species have been studied for their potential benefit to the host by preventing harmful, purely pathogenic microbes from colonizing on the surface of the skin. Moreover, it can be said that the concept of the nutrient niche hypothesis may apply even on the skin. The protective role of *Staphylococcus* bacteria could be amplified via genetic transformation.

To establish a fortified skin microbiome, the pathogenicity of the *Staphylococcus* strain to be utilized must be removed completely. While several virulence factors exist,

removing genetic information for one or several master regulator transcription factors promoting virulent expression may be successful in preventing the transcription of virulence factor genes (*24, 25*). The non-pathogenic strain must then be transformed with one or several forms of antibiotic resistance, preventing its removal from the skin with antibiotic treatment. Subsequent antibiotic treatment would then kill transient pathogenic bacteria while allowing the resistant strain to flourish. When confronted with a skin environment where there is no available niche due to the overcolonization of the resistant strain, pathogens would be unable to compete and would thus die out. This method could provide much-needed extra defense for immunocompromised patients at highest risk for nosocomial infection.

Materials and Methods

Bacterial Strains

The *E. coli* strains used in this study (Table 1) were all derived from *E. coli* K12, a strain originally isolated from the colon of a diphtheria patient in Palo Alto, CA, in 1922. The MG1655 strain was derived from a K12 strain cured of a bacteriophage infection (*9*). MG1655 has since been widely used in laboratory applications. The MG1655 Str^r strain is a mutant spontaneously resistant to streptomycin (*11*).

MG1655 *ΔlacZ* is a knockout mutant for the *lacZ* gene (*2*). The gene encodes the enzyme beta-galactosidease and is normally under the regulation of an operon upstream of the promoter. The knockout mutant produces no beta-galactosidease as a direct result of the genotype.

MG1655 *lacZ** is a clone of the MG1655 *ΔlacZ* strain with the addition of a plasmid vector containing a kanamycin resistance cassette and a functional *lacZ* gene

under lac operon regulation. The strain thus has restored production of beta-galactosidase when under lactose induction. The vector was constructed from the amplified DNA of an *E. coli* strain from the ASKA collection (*12*).

Table 1. *Bacterial Strains Used and Supporting Information*

***E. coli* Strain**	**Resistance**	**Phenotype**	**Reference**
MG1655 Str^r	Streptomycin, Nalidixic Acid	Wild type MG1655 with spontaneous antibiotic resistance	(*11*)
MG1655 *ΔlacZ*	Streptomycin, Chloramphenicol	Deletion mutation of *lacZ*, resulting in β-galactosidease deficiency	(*2*)
MG1655 *lacZ**	Streptomycin, Kanamycin	MG1655 *ΔlacZ* with ASKA lac operon controlled *lacZ* vector	(*12*), this study

MG1655 *lacZ** Construction

Construction of the *lacZ* recovery strain MG1655 *lacZ** was carried out via electroporation of the MG1655 *ΔlacZ* mutant with isolated and amplified plasmid vector pCA24N from an ASKA collection strain overexpressing *lacZ*. Polymerase chain reaction was performed to amplify the pCA24N vector. The reaction was gel purified following separation on a 0.7% agarose gel. The MinElute Gel Extraction kit (#28604)

was used to isolate the amplified plasmid. Electroporation of the MG1655 *ΔlacZ* strain with the isolated plasmid was performed at 24.5 kV. The strain was subsequently incubated in SOC media for one hour at 37^{o}C prior to plating onto agar medium with kanamycin. Single colony frozen stocks of the generated MG1655 *lacZ** mutant were subsequently made (*12*).

Biolog Metabolic Analysis of Strains

The Biolog GEN III MicroPlate test panel was used to investigate the metabolic capabilities of the strains used before colonization in streptomycin-treated mice. Each strain was inoculated onto agar media plates with proper antibiotics from frozen stock. The plates were allowed to incubate for 18 hours at 37^{o}C. The cultures were then removed and inoculated into aliquots of IF-A solution to an optical density (OD) of 0.05. The cell suspension was then loaded into each well of the Biolog microplate (100µL per well). The plates were then allowed to incubate for 18 hours before data analysis was performed (*26*).

Mouse Co-colonization with MG1655 WT and Mutant Strains

Initial streptomycin sulfate treatment (5 g/L in drinking water) was performed on weaned BALB/c mice (Charles River, Kingston, NY) 48 hours before strain inoculation to clear their colons of resident Enterobacteria. Food and streptomycin water were removed 24 hours before inoculation to encourage mouse ingestion of strain inoculum. Single-colony inoculations of broth cultures for competing strains were made after removal of food and water. After an 18-hour incubation, 5 µL were taken from each culture and added to 990 µL of 20% sucrose solution. This was repeated for each mouse. The bacterial suspensions were then serially diluted to obtain viable count data. The

sucrose suspension was fed to each mouse and food and streptomycin water were returned. The mice were given 15% lactose in solution with the drinking water to simulate a high lactose diet. Fecal collections were performed 5 hours after inoculation. One gram of feces was collected per mouse and diluted in 10 mL of tryptone each. Ten-fold serial dilutions of the fecal suspensions were performed. Dilutions were then plated onto MacConkey agar media containing streptomycin and either nalidixic acid, chloramphenicol, or kanamycin, depending on the strain to be isolated. CFU counts were then enumerated for each dilution plate. Subsequent collections occurred every day for 15 days total (*2*).

Results

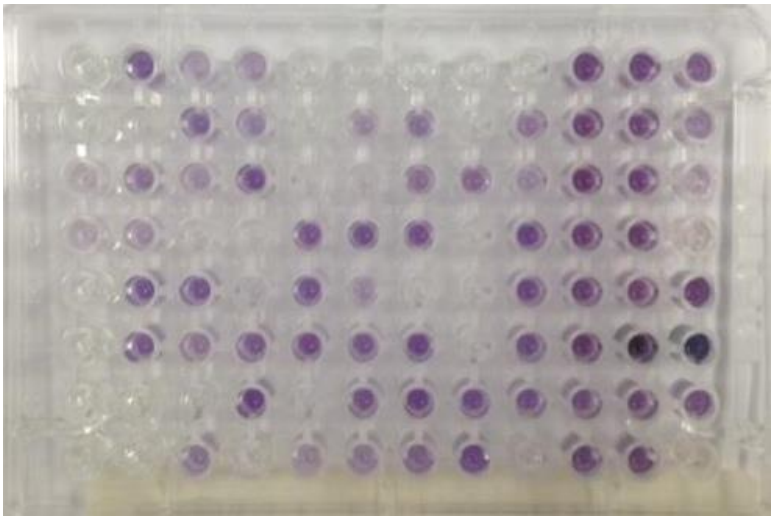
Metabolic Analysis of MG1655 *ΔlacZ* Confirms Strain Metabolic Deficiency

The Biolog test panel measured the ability of *E. coli* MG1655 *ΔlacZ* to grow on 95 different carbon sources (Figure 1). Through this assay, it was confirmed that MG1655 *ΔlacZ* does not grow on lactose. It is expected that the wild type strain will retain the ability to grow on lactose. Further metabolic analysis will be performed on restored the MG1655 *lacZ** strain to ensure the strain is capable of growing on lactose. Spectrophotometric analysis of the test panel media will provide an empirical measure of the carbon catabolic activity of each strain. Special attention will be paid to lactose.

It is worth noting that the MG1655 *ΔlacZ* Biolog plate revealed that the strain is capable of growing on cellobiose, the disaccharide product of cellulose carbohydrate chain breakdown. It is also worth noting that the strain was able to grow on several antibiotic containing wells, including those with nalidixic acid and vancomycin. This is

surprising, as resistance to these antibiotics has not been previously described for this strain.

A)



B)

GEN III MicroPlate ™

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butyric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

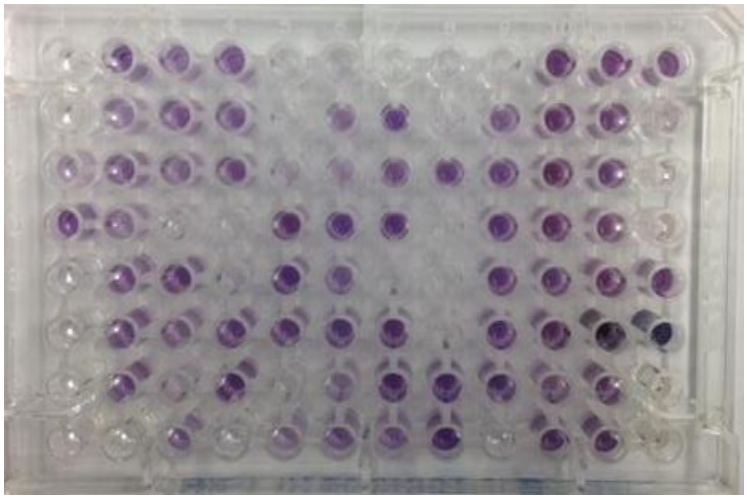
Figure 1. Biolog GEN III MicroPlate test panel of MG1655 ***ΔlacZ.*** When the assay plate (A) is compared with the test panel grid (B), the catabolic activity of the strain can be determined. Assay plate and test grid provided by Biolog, Inc.

Metabolic Analysis of MG1655 Str^{r} Confirms Lactose Catabolism

The Biolog test panel was also used to measure the catabolic activity of the MG1655 Str^{r} strain (Figure 2). The Biolog assay confirmed that the strain does indeed catabolize lactose, affirming its potential as a competitor against the mutant *ΔlacZ* strain in a streptomycin-treated mouse colonization.

The strain also grew in the presence of naldixic acid and vancomycin, similar to the *ΔlacZ* strain. Resistance to these antibiotics is not imbued to these bacteria via gene vector, suggesting the strains obtained resistance to the antibiotics through a different means.

A)



B)

GEN III MicroPlate ™

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butyric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Figure 2. Biolog GEN III MicroPlate test panel of MG1655 Str^{r}. When the assay plate (A) is compared with the test panel grid (B), the catabolic activity of the strain can be determined. Assay plate and test grid provided by Biolog, Inc.

Due to outside circumstances, the mouse colonization portion of the experiment was unable to be performed before time of this publication.

Discussion

The lack of lactose catabolism in the MG1655 *ΔlacZ* strain allows it to act as a negative control in the experiment. It can be expected in the lactose-fed mouse colonization that the colony forming unit (CFU) count of each strain will be indicative of which strain is the better colonizer in a high-lactose environment. Whether the knockout strain is outcompeted or not by the wild type will provide an indication of the importance of lactose to colon colonizing microbiota.

The mouse colonization with MG1655 Str^r and MG1655 *ΔlacZ* is expected to reveal aspects of the importance of lactose catabolism in the mouse intestine. With the high influx of lactose sugar into the colon due to the treatment in the study, it can be expected that this carbon source will play a role in strain selection, resulting in the out-competition of one strain over the other. It is likely that the MG1655 Str^r will outcompete the MG1655 *ΔlacZ* due to the ability the former strain has to utilize lactose sugar as a carbon source. The nutrient niche hypothesis states that the more nutrient sources a bacterium has access to in the colon, the better it competes in the gut ecosystem (*2*). The fact that the mutant strain cannot utilize lactose, which will be prevalent in the colon with the treatment, points to the likelihood that it will be outcompeted. However, if the strain is not outcompeted, then it will be shown that lactose is not an important sugar to the enterobacterial community in regard to niche competition.

The cellobiose catabolism could be important in regard to the potential transformation of *E. coli* to cellulolytic bacterium, a subject discussed in length earlier in this paper. If the mutant is capable of digesting cellobiose, then it is possible that the wild type strain is capable of doing the same. If the wild type is capable of digesting

cellobiose, then transforming the strain to digest cellulose polysaccharide fully would be an easier feat to accomplish. A transformation with a gene to break down cellulose into disaccharide cellobiose units would be the only necessary procedure to make *E. coli* completely cellulolytic.

Conclusions

While still very much only an idea, there are fragments of the MME hypothesis scattered throughout current microbe-host interaction studies. Microorganisms and host animals may rely on one another more than previously thought, especially in the sense of the latter on the former. In the modern era of antibiotic treatments and sanitization, humans have effectively lowered the risk of infection, but they have simultaneously downplayed the importance of native microbiota to health. By strategically enhancing the microbes already present in and on the human body, sufferers of disorders and diseases thought previously to be incurable may have a chance to live a much more normal life.

References

1. J. Li, W. Zhang, C. Wang, Q. Yu, R. Dai, X. Pei, Lactococcus lactis expressing food-grade beta-galactosidase alleviates lactose intolerance symptoms in post-weaning Balb/c mice. *Applied microbiology and biotechnology* **96**, 1499-1506 (2012); published online EpubDec (10.1007/s00253-012-3977-4).
2. A. J. Fabich, S. A. Jones, F. Z. Chowdhury, A. Cernosek, A. Anderson, D. Smalley, J. W. McHargue, G. A. Hightower, J. T. Smith, S. M. Autieri, M. P. Leatham, J. J. Lins, R. L. Allen, D. C. Laux, P. S. Cohen, T. Conway, Comparison of carbon nutrition for pathogenic and commensal Escherichia coli strains in the mouse intestine. *Infection and immunity* **76**, 1143-1152 (2008); published online EpubMar (10.1128/IAI.01386-07).
3. S. A. Jones, F. Z. Chowdhury, A. J. Fabich, A. Anderson, D. M. Schreiner, A. L. House, S. M. Autieri, M. P. Leatham, J. J. Lins, M. Jorgensen, P. S. Cohen, T. Conway, Respiration of Escherichia coli in the mouse intestine. *Infection and immunity* **75**, 4891-4899 (2007); published online EpubOct (10.1128/IAI.00484-07).
4. T. A. Snider, A. J. Fabich, T. Conway, K. D. Clinkenbeard, E. coli O157:H7 catabolism of intestinal mucin-derived carbohydrates and colonization. *Veterinary microbiology* **136**, 150-154 (2009); published online EpubApr 14 (10.1016/j.vetmic.2008.10.033).
5. T. Shi, K. Nishiyama, K. Nakamata, N. P. Aryantini, D. Mikumo, Y. Oda, Y. Yamamoto, T. Mukai, I. N. Sujaya, T. Urashima, K. Fukuda, Isolation of potential probiotic Lactobacillus rhamnosus strains from traditional fermented mare milk produced in Sumbawa Island of Indonesia. *Bioscience, biotechnology, and biochemistry* **76**, 1897-1903 (2012)10.1271/bbb.120385).
6. Y. E. Hoy-Schulz, K. Jannat, T. Roberts, S. H. Zaidi, L. Unicomb, S. Luby, J. Parsonnet, Safety and acceptability of Lactobacillus reuteri DSM 17938 and Bifidobacterium longum subspecies infantis 35624 in Bangladeshi infants: a phase I randomized clinical trial. *BMC complementary and alternative medicine* **16**, 44 (2016)10.1186/s12906-016-1016-1).
7. F. Armougom, M. Henry, B. Vialettes, D. Raccah, D. Raoult, Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. *PloS one* **4**, e7125 (2009)10.1371/journal.pone.0007125).
8. J. Szewczak, A. Bierczynska-Krzysik, M. Piejko, P. Mak, D. Stadnik, Isolation and Characterization of Acetylated Derivative of Recombinant Insulin Lispro Produced in Escherichia coli. *Pharmaceutical research* **32**, 2450-2457 (2015); published online EpubJul (10.1007/s11095-015-1637-y).
9. M. P. Leatham-Jensen, J. Frimodt-Moller, J. Adediran, M. E. Mokszycki, M. E. Banner, J. E. Caughron, K. A. Krogfelt, T. Conway, P. S. Cohen, The streptomycin-treated mouse intestine selects Escherichia coli envZ missense mutants that interact with dense and diverse intestinal microbiota. *Infection and immunity* **80**, 1716-1727 (2012); published online EpubMay (10.1128/IAI.06193-11).

10. J. Miller, *Experiments in molecular genetics*. (1972).
11. E. J. Gauger, M. P. Leatham, R. Mercado-Lubo, D. C. Laux, T. Conway, P. S. Cohen, Role of motility and the flhDC Operon in Escherichia coli MG1655 colonization of the mouse intestine. *Infection and immunity* **75**, 3315-3324 (2007); published online EpubJul (10.1128/IAI.00052-07).
12. M. Kitagawa, T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, H. Mori, Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. *DNA research : an international journal for rapid publication of reports on genes and genomes* **12**, 291-299 (2005)10.1093/dnares/dsi012).
13. J. P. Colombo, Congenital disorders of the urea cycle and ammonia detoxication. *Monographs in paediatrics* **1**, 1-150 (1971).
14. M. Serrano, A. Ormazabal, M. A. Vilaseca, N. Lambruschini, R. Garcia-Romero, S. Meavilla, B. Perez-Duenas, M. Pineda, A. Garcia-Cazorla, J. Campistol, R. Artuch, Assessment of plasma ammonia and glutamine concentrations in urea cycle disorders. *Clinical biochemistry* **44**, 742-744 (2011); published online EpubJun (10.1016/j.clinbiochem.2011.03.136).
15. W. Smith, G. A. Diaz, U. Lichter-Konecki, S. A. Berry, C. O. Harding, S. E. McCandless, C. LeMons, J. Mauney, K. Dickinson, D. F. Coakley, T. Moors, M. Mokhtarani, B. F. Scharschmidt, B. Lee, Ammonia control in children ages 2 months through 5 years with urea cycle disorders: comparison of sodium phenylbutyrate and glycerol phenylbutyrate. *The Journal of pediatrics* **162**, 1228-1234, 1234 e1221 (2013); published online EpubJun (10.1016/j.jpeds.2012.11.084).
16. A. Meyer, A. Focks, V. Radl, G. Welzl, I. Schoning, M. Schloter, Influence of Land Use Intensity on the Diversity of Ammonia Oxidizing Bacteria and Archaea in Soils from Grassland Ecosystems. *Microb Ecol* **67**, 161-166 (2014); published online EpubJan (DOI 10.1007/s00248-013-0310-4).
17. M. Konneke, D. M. Schubert, P. C. Brown, M. Hugler, S. Standfest, T. Schwander, L. S. von Borzyskowski, T. J. Erb, D. A. Stahl, I. A. Berg, Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO2 fixation. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 8239-8244 (2014); published online EpubJun 3 (DOI 10.1073/pnas.1402028111).
18. C. Konidaris, P. G. Mitlianga, G. K. Papadopoulos, No specific reactivity to E. coli glutamic acid decarboxylase from sera of newly-diagnosed insulin dependent diabetic patients. *International journal of immunopathology and pharmacology* **16**, 129-138 (2003); published online EpubMay-Aug (
19. C. Chakraborty, Overexpression for commercial production of recombinant human insulin as A-chain and B-chain fusion protein in Escherichia coli through genetically engineered plasmids. *Indian journal of pathology & microbiology* **47**, 569-573 (2004); published online EpubOct (
20. J. S. Griffiths, K. Sola, R. Kushwaha, P. Lam, M. Tateno, R. Young, C. Voiniciuc, G. Dean, S. D. Mansfield, S. DeBolt, G. W. Haughn, Unidirectional movement of cellulose synthase complexes in Arabidopsis seed coat epidermal

cells deposit cellulose involved in mucilage extrusion, adherence, and ray formation. *Plant physiology* **168**, 502-520 (2015); published online EpubJun (10.1104/pp.15.00478).

21. W. K. Wang, K. Kruus, J. H. Wu, Cloning and expression of the Clostridium thermocellum celS gene in Escherichia coli. *Applied microbiology and biotechnology* **42**, 346-352 (1994); published online EpubNov (
22. A. L. Rodrigues, A. Cavalett, A. O. S. Lima, *Enhancement of Escherichia coli cellulolytic activity by co-production of beta-glucosidase and endoglucanase enzymes*. 2010 (2010).
23. E. Faure, C. Bagnara, A. Belaich, J. P. Belaich, Cloning and expression of two cellulase genes of Clostridium cellulolyticum in Escherichia coli. *Gene* **65**, 51-58 (1988); published online EpubMay 15 (
24. R. Arya, S. A. Princy, Exploration of Modulated Genetic Circuits Governing Virulence Determinants in Staphylococcus aureus. *Indian journal of microbiology* **56**, 19-27 (2016); published online EpubMar (10.1007/s12088-015-0555-3).
25. A. F. Haag, F. Bagnoli, The Role of Two-Component Signal Transduction Systems in Staphylococcus aureus Virulence Regulation. *Current topics in microbiology and immunology*, (2016); published online EpubJan 5 (10.1007/82_2015_5019).
26. A. J. Fabich, M. P. Leatham, J. E. Grissom, G. Wiley, H. Lai, F. Najar, B. A. Roe, P. S. Cohen, T. Conway, Genotype and phenotypes of an intestine-adapted Escherichia coli K-12 mutant selected by animal passage for superior colonization. *Infection and immunity* **79**, 2430-2439 (2011); published online EpubJun (10.1128/IAI.01199-10).