Review Article

Rational Design of Improved Pharmabiotics

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Herein we review the most recent advances in probiotic research and applications with particular emphasis on the novel concept of patho-biotechnology: the application of pathogen-derived (ex vivo and in vivo) stress survival strategies for the design of more technologically robust and effective probiotic cultures with improved biotechnological and clinical applications.

1. Introduction

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit to the host" [1]. In acute infections, probiotics may boost the protection afforded by commensal microbiota through competitive interactions and direct antagonism of pathogens due in part to the production of antimicrobial factors [2]. In other clinical conditions, such as chronic infections and immuno-suppression, microbe-host signalling is probably more relevant to effective probiotic action. Gut homeostasis, the maintenance of a "balanced" and beneficial microbiota, requires continual signalling from bacteria within the gut lumen, maintaining the mucosal barrier while at the same time priming the gut for responses to injury [3]. Given these health-promoting benefits, improving probiotic stress tolerance and ability to grow and survive in foods prior to ingestion and subsequently within the animal host is an important clinical goal. This is particularly relevant given that many potentially beneficial probiotics often prove to be physiologically fragile; a significant limitation in clinical applications [4].

The patho-biotechnology concept [5–7] seeks to attain this goal, ultimately leading to the development of improved probiotic strains. A primary focus of this approach involves equipping probiotic bacteria with the genetic elements necessary to overcome the many stresses encountered during the probiotic life cycle (both external and internal to the host) as well as enabling probiotics to better deal with invading pathogens [8, 9]. This strategy can be divided into three distinct approaches (Figure 1). The first tackles the issue of probiotic storage and delivery by cloning and expression of pathogen specific stress survival mechanisms (facilitating improved survival at extremes of temperature and water availability), thus countering reductions in probiotic numbers which can occur during manufacture and storage of delivery matrices (such as foods and tablet formulations). The second approach aims to improve host persistence by expression of host specific survival strategies (or virulence associated factors—such as the ability to cope with bile; an important component of the bodies physicochemical defence system) thereby positively affecting the therapeutic efficacy of the probiotic. The final approach involves the development of the so-called "designer probiotics;" strains which specifically target invading pathogens by blocking crucial ligand-receptor interactions between the pathogen and host cell [10].

2. Improving Probiotic Storage and Delivery

The most common stresses encountered during the production of probiotic delivery matrices (food and/or tablet formulations) are temperature and water availability (a_w) [11]. The ability to cope with such stresses is a particularly

desirable trait in the selection of commercially viable probiotic strains. Common strategies employed by a variety of microbes to deal with both low a_w and temperature stress include the synthesis of chaperone proteins [12] as well as the accumulation of protective compounds, termed compatible solutes, including betaine, carnitine, and proline. These protective mechanisms help to stabilise protein structure and function, thus helping to maintain optimal metabolic output under a variety of stressful conditions [13].

Improving a strain's ability to synthesize chaperone proteins and/or accumulate compatible solutes is thus an obvious first step in the development of more robust probiotic strains. Bacteria have evolved sophisticated mechanisms for compatible solute accumulation, including both uptake and synthesis systems [13]. Indeed, the foodborne pathogen *Listeria monocytogenes* (an extensively well studied pathogen in terms of compatible solute accumulation [14]) possesses three distinct uptake systems (BetL, Gbu, and OpuC) and at least one compatible solute synthesis system (ProBA). By placing the *betL* gene (encoding the betaine uptake system BetL [15]) under the transcriptional control of the nisin inducible promoter P*nisA*, it was possible to assess the role of BetL (and thus betaine accumulation) in contributing to probiotic growth and survival under a variety of stresses likely encountered during food and/or tablet manufacture [8]. Our probiotic of choice, *Lactobacillus salivarius* UCC118, exhibits significantly lower betaine accumulation levels than *L. monocytogenes* and is correspondingly less physiologically robust than the pathogen. As expected, the *L. salivarius* betL$^+$ strain showed a significant increase in betaine accumulation compared to the wild type. Indeed, sufficient BetL was produced to confer increased salt tolerance [8], with growth of the transformed strain occurring at significantly higher salt concentrations than the parent. Furthermore, the presence of BetL resulted in a significant improvement in barotolerance. This is particularly significant given that high pressure processing is gaining increasing popularity as a novel nonthermal mechanism of food processing and preservation [16, 17]. Focusing on chaperone proteins, as opposed to compatible solutes as a means of improving cell viability under stress, Corcoran et al. [18] engineered the probiotic strain *Lactobacillus paracasei* to overproduce GroESL. As with our probiotics engineered to accumulate compatible solutes Corcoran's GroESL overproducing strain exhibited significantly improved technological robustness; resulted in a ~10-fold and 2-fold better survival following spray—and freeze—drying, respectively, when compared to the wild type unmodified strain.

3. Host-Specific Adherence and Persistence

As well as the stresses encountered during processing and storage, probiotic bacteria must also overcome the physiochemical defences of the host in order to reach the gastrointestinal tract in sufficient numbers to exert a beneficial effect.

Recent work in our laboratory revealed that BetL significantly improved the tolerance of the probiotic strain

1. Delivery: engineering technological robustness

2. Survival: improved competitiveness in the GI tract

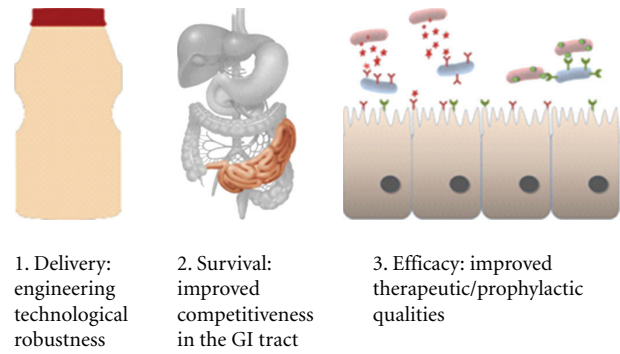3. Efficacy: improved therapeutic/prophylactic qualities

FIGURE 1: The patho-biotechnology concept involves three approaches to generating improved probiotic cultures, adapted from Sleator and Hill [19].

Bifidobacterium breve UCC2003 to gastric juice [9]. Interestingly, in support of this observation, Termont et al. [21] also reported similar results for an *L. lactis* strain expressing the *E. coli* trehalose synthesis genes, thus suggesting a novel protective role for compatible solutes in the gastric environment. Furthermore, inline with our previous observations with *L. salivarius* UCC118 [8], a significant osmoprotective effect was observed following the introduction of *betL* into *B. breve*, allowing significantly improved growth of the probiotic in conditions similar to those encountered in vivo (1.5% NaCl; equivalent to the osmolarity of the gut). In addition, *B. breve* strains expressing BetL were recovered at significantly higher levels than the wild type in the faeces, intestines, and caecum of inoculated animals. Finally, in addition to improved gastric transit and intestinal persistence (Figure 2(a)), the addition of BetL improved the clinical efficacy of the probiotic culture; mice fed *B. breve* UCC2003 (betL$^+$) exhibited significantly lower levels of systemic infection compared to the control strain following oral inoculation with *L. monocytogenes* (Figure 2(b)). Furthermore, in vitro bile tolerance of *B. breve* was significantly enhanced by heterologous expression of the *L. monocytogenes* bile resistance mechanism BilE (Figure 3(a)), a phenotype which most likely explains why the bilE$^+$ strain was recovered at significantly higher levels than the control strain from the faeces and intestines of mice, following oral inoculation (Figure 3(b)). In addition, the bilE$^+$ strain demonstrated increased clinical efficacy; by causing a reduction in *L. monocytogenes* recovered after oral inoculation (Figure 4).

In addition to using pathogens as a source of host specific stress adaptation systems, certain probiotic (or GRAS) strains may also represent a less hazardous reservoir of stress survival genes. Indeed, Deou et al. [22], using a combination of genomics and transcriptome analysis, identified a number of key genes affecting the long gut residence time of the probiotic strain *Lactobacillus johnsonii* NCC533 (which persists for up to 12 days in the gut as opposed to 5 days for the type strain ATCC 33200). When ATCC 33200 DNA was hybridized against a microarray of NCC533, 233 genes were identified that were specific
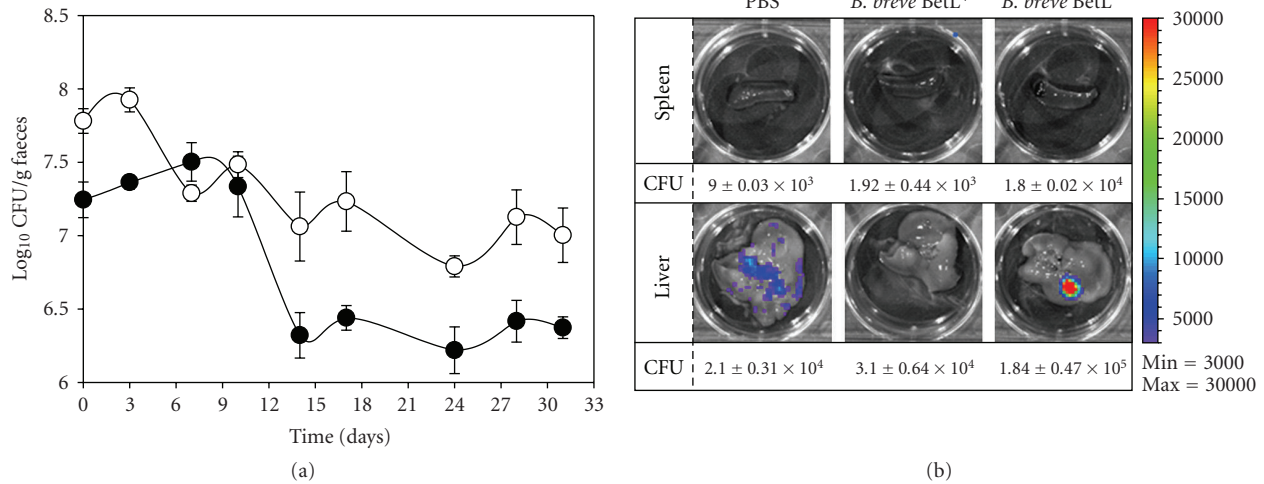
FIGURE 2: Recovery of *B. breve* BetL$^+$ (closed circles) and *B. breve* BetL$^-$ (open circles) from female BALB/c mice over 32 days of analysis. (a) Faeces for bacteriological analysis were obtained from five mice in each treatment group and viable counts of *B. breve* BetL$^-$ derivatives were determined, adapted from Sheehan et al. [9]. (b) Listerial infection in the livers and spleens of BALB/c mice. Animals were fed $\sim 10^9$ CFU mL^{-1} of either *B. breve* BetL$^+$ or the *betL* negative control strain *B. breve* BetL$^-$ for three consecutive days. A control group was fed PBS. On the fourth day, all animals were infected with $\sim 10^{11}$ CFU mL^{-1} luminescent *L. monocytogenes* EGD-e. Three days postlisterial infection the animals were sacrificed and the extent of listerial infection of the liver determined by measuring light intensity using an IVIS 100 system.
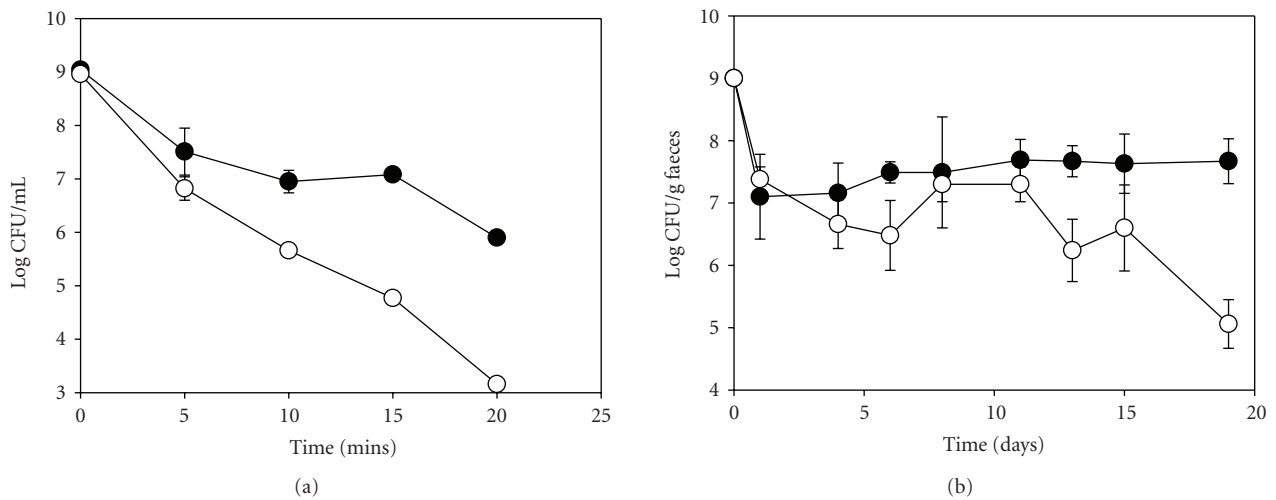

FIGURE 3: (a) Survival of stationary phase *B. breve* in 1% porcine bile. *B. breve* bilE$^+$ (closed circles) and *B. breve* bilE$^-$ (open circles). Overnight cultures were inoculated (3%) into GM17 and MRS broth containing 1% porcine bile. Viable cell counts were performed by serial dilution in one-quarter strength Ringer's solution followed by plating onto GM17Cm5 or RCMCm4, respectively. Standard deviations of triplicate results are represented by error bars. (b) Effect of bilE on the gastrointestinal persistence of *B. breve* bilE$^+$ (closed circles) and *B. breve* bilE$^-$ (open circles) were used for peroral inoculation of female BALB/c mice ($n = 5$). *B. breve* counts were determined in stools at 48-hour intervals, adapted from Watson et al. [20].

for the long-gut-persistence isolate. Furthermore, whole-genome transcription analysis of NCC533 identified 174 genes consistently expressed in the jejunum of mice mono-colonized with this strain. Fusion of the two microarray data sets identified three loci that were both expressed in vivo and specific to the long-gut-persistence isolate. The identified genes included two glycosyltransferase genes in the exopolysaccharide synthesis operon; genes encoding a sugar phosphotransferase system (PTS) transporter annotated as mannose PTS; and a gene whose product shares 30% amino acid identity with immunoglobulin A proteases from pathogenic bacteria. Knockout mutants of all three genetic loci were tested in vivo revealing an important role for the PTS and immunoglobulin A like protease in increasing gut residence time. These genes thus may represent important stress adaptation systems for the generation of pharmabiotics with improved gastrointestinal persistence. Collectively, the data indicates that rational genetic manipulation of selected
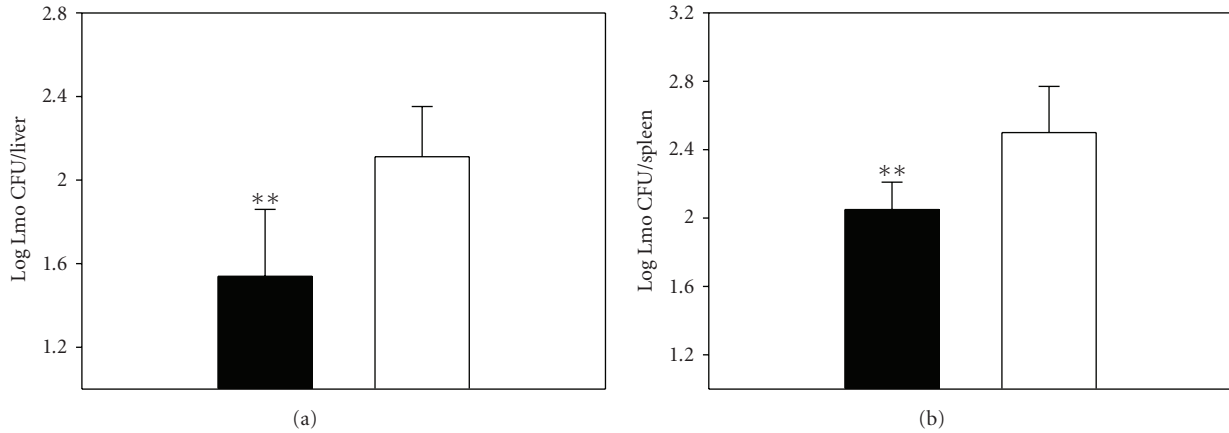
(a)

(b)

FIGURE 4: *Improved clinical efficacy*. Probiotic dosing of BALB/c mice with *B. breve bilE$^+$* (black) significantly (∗∗, $P < .001$) reduces the level of subsequent *L. monocytogenes* infection when compared to the wild-type *B. breve bilE$^-$* strain. Bacterial growth was followed in (a) the liver and (b) the spleen 3 days postinfection, adapted from Watson et al. [20].

probiotic strains can significantly improve delivery to and persistance within the GI tract [22].

However, despite the potential benefits of such rationally designed pharmabiotics, there are also some significant limitations which still need to be addressed, not least of which are the safety concerns surrounding the development of genetically modified cultures. Natural acquisition of horizontally encoded virulence factors, for example, phage-encoded toxins or acquisition of antibiotic resistance markers, in combination with the modified ability of the microorganism to survive the innate immune defences of the gastrointestinal tract (low pH, elevated osmolarity, and bile salts) could conceivably result in the emergence of a potentially deadly strain rather than the expected beneficial one. It is hoped that some of these concerns may be allayed by proper adherence to rigid biological containment and safety procedures as discussed later.

4. Expanding the Patho-Biotechnology Concept by Directed Evolution

In addition to an already existing array of useful pathogen derived stress survival systems [4], it may be possible to artificially engineer improved systems using a directed evolution approach. One such approach involves the use of the *E. coli* mutator strain XL1-Red; deficient in three of the primary DNA repair pathways (*mutS, mutD*, and *mutT*), plasmid replication in this strain results in a mutation rate ~5000-fold higher than that of the wild type. Thus, with each successive generation, random point mutations (some of which are beneficial) are introduced into the gene of interest, creating a bank of mutant genes from which the most effective can be selected based on an improved phenotype (e.g., increased osmotolerance, etc.).

We employed this technique to engineer proline hyperproducing strains of *E. coli* with a significantly increased ability to tolerate elevated osmolarities (Figure 5). Bacterial proline synthesis from glutamate occurs via three enzymatic
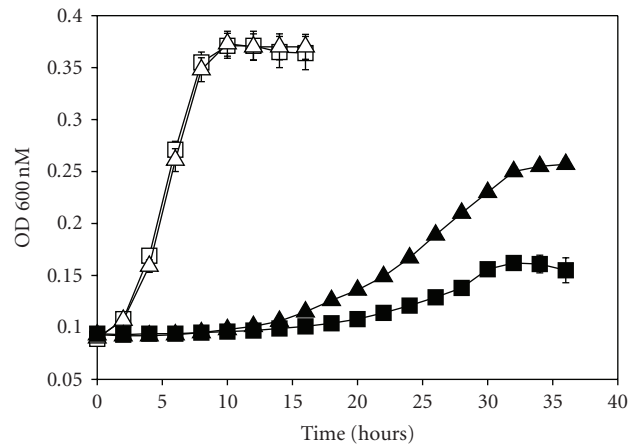

FIGURE 5: Growth of a proline hyperproducing strain of *E. coli* expressing a mutated version of the listerial proB gene relative to a wild-type *E. coli* in M9 minimal medium of elevated osmolarity. Growth (as determined by turbidity using a Spectra max 340 spectrophotometer, Molecular Devices), was measured both in the presence (closed symbols) and absence (open symbols) of 4% NaCl. (□, ■) CSH26C control strain, (△, ▲) CSH26proB^{mut}. Each point represents the mean value of three independent experiments.

reactions, catalyzed by γ-glutamyl kinase (GK) (*proB* product), γ-glutamyl phosphate reductase (GPR) (*proA* product), and [1]Δ-pyrroline-5-carboxylate reductase (P5C) (*proC* product). For both prokaryotic and eukaryotic systems proline synthesis from glutamate is regulated by feedback inhibition of the first enzyme in the pathway (GK). Thus, it is possible to isolate proline hyperproducing strains by screening for isolates exhibiting reduced proline-mediated feedback inhibition of GK activity (as a consequence of single-base-pair substitutions in the *proB* gene). This was achieved by passaging the listerial *proBA* operon through *E. coli* XL1-Red thus creating a bank of randomly mutated *proBA* operons. The resulting gene bank was then transformed into *E. coli* CSH26 (a proline auxotroph) and successful transformants were

screened for proline hyperproduction. Three independent proline overproducing mutants were obtained (each carrying point mutations at a different location within the *proB* gene). These strains, heterologously expressing the mutated listerial *proBA* operon, were shown to be considerably more osmotolerant than strains expressing the wild-type listerial *proBA* [23]. Thus, while complementation with wild-type listerial *proBA* offers a significant degree of osmoprotection, the bioengineered *proBA* operon is far more effective, proving that the directed evolution approach provides a new dimension to the patho-biotechnology concept. It is of course entirely likely that this directed evolution approach may well dispense with the need for pathogens altogether as a source of stress survival systems in favour of selectively enhancing the probiotic's own gene complement.

5. Designer Probiotics

Faced with an emerging pandemic of antibiotic resistance, clinicians and scientists alike are now struggling to find viable therapeutic alternatives to our failing antibiotic wonder drugs. Many disease-causing bacteria exploit oligosaccharides displayed on the surface of host cells as receptors for toxins and/or adhesins, enabling adherence to the host and entry of the pathogen or secreted toxins into the host cell. Blocking this adherence prevents infection, while toxin neutralization ameliorates symptoms until the pathogen is eventually overcome by the host's immune system. "Designer probiotics" have been engineered to express receptor-mimic structures on their surface which fool the pathogen into thinking that the administered probiotic is in fact their target host cell [10, 24–26]. When administered orally, these engineered probiotics bind to and neutralize toxins in the gut lumen and interfere with pathogen adherence to the intestinal epithelium—thus essentially "mopping up" the infection. One such construct consists of an *E. coli* strain expressing a chimeric lipopolysaccharide (LPS) terminating in a shiga toxin (Stx) receptor. One milligram dry weight of this recombinant strain has been shown to neutralize >100 μ g of Stx1 and Stx2 [24]. Paton et al. [25, 26] have also constructed probiotics with receptor blocking potential against Enterotoxigenic *E. coli* (ETEC) toxin LT and cholera toxin (Ctx). Designer probiotics have also been developed to combat HIV, in addition to treating infections. Rao et al. [27] recently described the construction of a probiotic strain of *E. coli*, engineered to secrete HIV-gp41-haemolysin A hybrid peptides which block HIV fusion and entry into host cells. When administered orally or as a rectal suppository, this "live microbicide" adheres the gut mucosa and secretes the peptide in situ, thereby providing protection in advance of HIV exposure for up to a month. Other anti-HIV probiotics currently in development include a genetically engineered *Streptococcus gordonii* which produces cyanovirin-N, a potent HIV-inactivating protein originally isolated from cyanobacterium, and a natural human vaginal isolate of *Lactobacillus jensenii* modified to secrete two-domain CD4 which inhibits HIV entry into target cells [28]. Notwithstanding in vitro and in vivo efficacy in

animal models, further refinements to the receptor-mimic probiotics might be necessary before initiating Phase I clinical trials. Patho-biotechnology, the introduction of genes to improve resistance to stomach acid, or otherwise promote adherence and survival in the gut, for example, would enable dose regimes to be substantially lowered thus providing greater efficacy and further cost benefits.

In addition to infection control probiotics (and other nonpathogenic bacteria) are also being engineered to function as novel vaccine delivery vehicles which can stimulate both innate and acquired immunity but lack the possibility of reversion to virulence which exists with more conventional pathogenic platforms. Guimarães et al. [29] recently described the construction of an *L. lactis* strain expressing *inlA*, encoding internalin A, a surface protein related to invasion in *L. monocytogenes*. In this instance, the otherwise noninvasive *L. lactis* strain is now capable of invading the small intestine and delivering molecules (DNA or protein) into mammalian epithelial cells, making it a safer and more attractive alternative to attenuated *L. monocytogenes* as an antigen delivery vehicle. However, while undoubtedly safer than using attenuated pathogens, equipping probiotic strains with the ability to traverse the epithelium introduces a unique set of safety concerns which need to be addressed.

Probiotic vaccine carriers administered by the mucosal route mimic the immune response elicited by natural infection and can lead to long lasting protective mucosal and systemic responses [30]. Mucosal vaccine delivery (those administered orally, anally, or by nasal spray) also offers significant technological and commercial advantages over traditional formulations including reduced pain and the possibility of cross-contamination associated with intramuscular injection as well as the lack of a requirement for medically trained personnel to administer the vaccine [31].

6. Biological Containment and Safety

Despite their obvious clinical potential, the use of genetically modified organisms in food and medicine raises legitimate concerns about their propagation in the environment and about the dissemination of antibiotic markers or other genetic modifications to other microorganisms. At least some of these concerns might be allayed by the implementation of stringent biocontainment measures. Recently, Steidler et al. [32] identified the thymidylate synthase (*thyA*) gene as a target gene that combines the advantages of passive and active containment systems. Thymine auxotrophy involves activation of the SOS repair system and DNA fragmentation, thereby constituting an indigenous suicide system. Thymine and thymidine growth dependence differs from most other auxotrophys in that absence of the essential component is bactericidal in the former and bacteriostatic in the latter. Thus, *thyA*-deficient bacteria cannot accumulate in the environment. This approach addresses biosafety concerns on a number of levels. Firstly, no resistance marker is required to guarantee stable inheritance of the transgene(s), thus overcoming any potential problems associated with dissemination of antibiotic resistance. Second, accumulation

of the genetically modified organism in the environment is highly unlikely given that rapid death occurs upon thymidine starvation. Finally, should an intact *thyA* be acquired from closely related bacteria by means of homologous recombination then the transgene(s) would be lost?

7. Conclusion and Future Prospects

Engineered probiotics thus have the potential to alleviate the symptoms of chronic gastrointestinal disorders and associated sequelae, to fight infection, modulate the immune system, and act as delivery vehicles for bioactive molecules [31]. Notwithstanding these impressive health benefits, probiotic research has really only begun to achieve scientific credibility over the last decade [19], this despite the fact that Yakult launched the first probiotic fermented food drink in Japan in 1935; long before the appearance of the first commercially available antibiotics.

However, the increasing emergence of antibiotic resistance, coupled with a significant decline in production of new antibacterials, means that probiotics are finally coming of age, representing a real alternative to traditional drug based therapies [33, 34].

Acknowledgments

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