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Genetically modified *Streptococcus mutans* for the prevention of dental caries

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Abstract

There are many examples of positive and negative interactions between different species of bacteria inhabiting the same ecosystem. This observation provides the basis for a novel approach to preventing microbial diseases called replacement therapy. In this approach, a harmless effector strain is permanently implanted in the host's microflora. Once established, the presence of the effector strain prevents the colonization or outgrowth of a particular pathogen. In the case of dental caries, replacement therapy has involved construction of an effector strain called BCS3-L1, which was derived from a clinical *Streptococcus mutans* isolate. Recombinant DNA technology was used to delete the gene encoding lactate dehydrogenase in BCS3-L1 making it entirely deficient in lactic acid production. This effector strain was also designed to produce elevated amounts of a novel peptide antibiotic called mutacin 1140 that gives it a strong selective advantage over most other strains of *S. mutans.* In laboratory and rodent model studies, BCS3-L1 was found to be genetically stable and to produce no apparent deleterious side effects during prolonged colonization. BCS3-L1 was significantly less cariogenic than wild-type *S. mutans* in gnotobiotic rats, and it did not contribute at all to the cariogenic potential of the indigenous flora of conventional Sprague-Dawley rats. And, its strong colonization properties indicated that a single application of the BCS3-L1 effector strain to human subjects should result in its permanent implantation and displacement over time of indigenous, disease-causing *S. mutans* strains. Thus, BCS3-L1 replacement therapy for the prevention of dental caries is an example of biofilm engineering that offers the potential for a highly efficient, cost effective augmentation of conventional prevention strategies. It is hoped that the eventual success of replacement therapy for the prevention of dental caries will stimulate the use of this approach in the prevention of other bacterial diseases.

Introduction

As reviewed by Florey (1946), the use of beneficial bacteria to fight harmful bacteria was first attempted over a century ago when Cantani employed a harmless organism referred to as 'Bacto. Termo' to treat tuberculosis. Since then, there have been dozens of reports describing both positive and negative bacterial interactions in which the presence of a particular indigenous microorganism promotes or deters the presence of a pathogen. The reason for the abiding interest in this area of microbiology is the prospect of preventing an infection by an approach traditionally called 'replacement therapy', or, more recently, 'probiotics'. In this approach, a naturally occurring or laboratory-derived effector strain is used to intentionally colonize the niche in susceptible host tissues that is normally colonized by the pathogen. By being better adapted than the pathogen, a well-designed effector strain will prevent colonization or outgrowth of the pathogen by blocking attachment sites, competing for essential nutrients, or other mechanisms. In this fashion, the host is protected for as long as the effector strain persists as a member of the indigenous flora, which, ideally, is for the lifetime of the host.

Application of replacement therapy to various diseases

As a rule, instances of naturally occurring bacterial interactions are detected during *in vitro* cultivation, and their occurrence *in vivo* is inferred from correlations provided by cultivation studies. For example, the pre-

dominant microorganisms in the pharynx of healthy neonates are one or more species of alpha-hemolytic streptococci, and the absence of these species was shown to correlate with a significantly increased risk of infections including sepsis, meningitis, pneumonia, and cystitis (reviewed by Sprunt & Leidy 1988). Strain 215 is a naturally occurring alpha-hemolytic Streptococcus that was chosen to serve as an effector strain based largely on its *in vitro* ability to inhibit a variety of common pathogens that initially colonize the pharynx. When given to a group of infants lacking an indigenous alpha-hemolytic Streptococcus in their throat flora, strain 215 usually became the predominant microorganism in pharyngeal cultures, and the numbers of potential pathogens declined to low or undetectable levels. These infants suffered a significantly lower incidence of infections than uninoculated controls (Sprunt et al. 1980). Also, in older individuals, natural or antibiotic-induced low levels of alpha-hemolytic streptococci in the pharynx have been shown to correlate with increased susceptibility to *Streptococcus pyogenes* infections (Sanders 1969; Crowe & Sanders 1973; Sanders et al. 1976; Fujimori et al. 1997). This suggests the potential for replacement therapy in the prevention of streptococcal pharyngitis in susceptible subjects.

Replacement therapy using strain 502A, a naturally occurring *Staphylococcus aureus* strain of low virulence, was very successful in curtailing various diseases caused by this species (Shinefield et al. 1971; Perl & Golub 1998). After gaining relatively widespread acceptance in the 1960s and 1970s, 502A has been little used partly because of lack of need and partly because of infections that have been reported following its use (Drutz et al. 1966; Blair & Tull 1969; Houck et al. 1972). Although the benefits of using strain 502A far outweigh the hazards (Light et al. 1967; Houck et al. 1972), this work emphasized the potential difficulty of using naturally occurring effector strains that may have residual pathogenic potential.

Application of replacement therapy to dental caries

A large body of literature implicates *Streptococcus mutans* as being the principle etiologic agent of dental caries (reviewed by Anderson 1992). *S. mutans* strain BCS3-L1 is a genetically modified effector strain designed for use in replacement therapy of dental caries (Hillman et al. 2000). Construction of BCS3-L1 took into account the following logical prerequisites:

1. It must have a significantly reduced pathogenic potential;
2. It must persistently and preemptively colonize the *S. mutans* niche, thereby preventing colonization by wild-type (disease-causing) strains whenever the host comes into contact with them;
3. Ideally, it should be able to aggressively displace indigenous strains of *S. mutans*, thereby allowing even previously infected subjects to be treated with replacement therapy;
4. It must be generally safe and not predispose the host to other disease conditions.

Pathogenicity

Several different approaches have been examined to satisfy the first of these prerequisites for effector strain construction. In accord with the acidogenic theory of dental caries, lactic acid production by *S. mutans* has long been considered to be the main pathogenic mechanism for production of caries lesions. Consequently, lactate dehydrogenase (LDH) deficiency was chosen as the approach for reducing acidogenicity in construction of BCS3-L1. Earlier work (Johnson et al. 1980) with a closely related *S. rattus* strain had provided convincing evidence for the effectiveness of this approach. LDH-deficient mutants were virtually non-cariogenic in gnotobiotic rats and did not contribute significantly to the cariogenic potential of the indigenous flora in conventional pathogen-free rats. Attempts to transfer these findings directly to *S. mutans* proved to be difficult. LDH-deficient mutants of various strains of *S. mutans* were not found using the same screening methods used to isolate mutants of *S. rattus.* Cloning the structural gene encoding the *S. mutans* LDH (Hillman et al. 1990) provided the basis for solving this puzzle. Standard insertional mutagenesis methods failed to yield LDH-deficient clones (Duncan et al. 1991; Hillman et al. 1994), suggesting that LDH-deficiency was a lethal mutation in most *S. mutans* strains. This hypothesis was definitively proven by creation of a temperature sensitive LDH mutant (Chen et al. 1994). This isolate grew well at 30 °C but did not grow at 42 °C under a variety of cultivation conditions. Chemostat studies indicated that some aspect of glucose metabolism was toxic during growth under the non-permissive condition (Hillman et al. 1996). The toxic effect could be

overcome by limiting the amount of environmental glucose. This and other data accorded with studies of *S. mutans* central intermediary metabolism indicating that this organism has enzymatic activities, including pyruvate formate-lyase (Abbe et al. 1982; Takahashi et al. 1982) and pyruvate dehydrogenase (Carlsson et al. 1985; Hillman et al. 1987a), for pyruvate dissimilation. However, at high sugar concentrations, the levels of activity of these enzymes are apparently insufficient to compensate for the absence of LDH. It was found (Hillman et al. 1996) that a supplemental alcohol dehydrogenase (ADH) activity, when expressed in the temperature sensitive LDH mutant, could complement LDH deficiency.

With this background of information, BCS3-L1 construction started with the *ldh* gene cloned into an appropriate suicide vector for *S. mutans.* Essentially the entire gene except for transcription and translation signal sequences was deleted and replaced with the *Zymomonas mobilis* open reading frame for alcohol dehydrogenase (ADH) II. Transformation of the recombinant molecule into the *S. mutans* starting strain, JH1140, and allelic exchange resulted in the isogenic mutant, BCS3-L1. This effector strain had no measurable LDH activity and ca. 10-fold elevated levels of ADH activity relative to its parent. Fermentation end-product analysis showed that BCS3-L1 made no detectable lactic acid. As predicted from earlier work with *S. rattus*, much of the metabolized carbon was converted to the neutral end-products, ethanol and acetoin. Under various cultivation conditions, including growth on a variety of sugars and polyols, such as sucrose, fructose, lactose, mannitol, and sorbitol, BCS3-L1 yielded final pH values that were 0.4 to 1.2 pH units higher than those of its parent, JH1140. The reduced acidogenic potential of BCS3-L1 was reflected in its dramatically decreased cariogenic potential as shown in several animal models (Hillman et al. 2000). The results of these studies provided strong evidence that an LDH-deficient *S. mutans* strain such as BCS3-L1 has significantly reduced pathogenic potential, and thus satisfies the first prerequisite for use as an effector strain in replacement therapy for dental caries.

Colonization

Transmission of mutans streptococci within the human population has been extensively studied. Most studies support the idea that this organism is usually transmitted from mother (primary caretaker) to child within a several year period following the onset of tooth eruption (Davey & Rogers 1984; Berkowitz & Jones 1985; Caufield & Walker 1989; Alaluusua 1991; Genco & Loos 1991). Numerous studies (Krasse et al. 1967; Jordan et al. 1972; Ruangsri & Orstavik 1977; Svanberg & Krasse 1981) have documented the difficulty of persistently introducing laboratory strains of mutans streptococci into the mouths of humans, particularly if they already harbored an indigenous strain of this organism.

From the standpoint of replacement therapy of caries, these results suggest that implantation of an effector strain would best be accomplished in children immediately after the onset of tooth eruption and before their acquisition of a wild-type strain. In order to prevent supercolonization by wild-type strains when the host comes in contact with them, an effector strain should have some significant selective advantage to colonization. This would also enable subjects who have already been infected with wild-type *S. mutans* to be treated by replacement therapy. The ability of an effector strain to preemptively colonize the human oral cavity and aggressively displace indigenous wild-type strains was initially thought to be a complex phenomenon dependent on a large number of phenotypic properties. However, it was discovered that a single phenotypic property could provide the necessary selective advantage. A naturally occurring strain of *S. mutans* was isolated from a human subject that produces a bacteriocin called mutacin 1140 that is capable of killing virtually all other strains of mutans streptococci against which it was tested (Hillman et al. 1984). Mutants were isolated that produced no detectable mutacin 1140 or that produced approximately three-fold elevated amounts. The mutants were used in a rat model to correlate mutacin production to preemptive colonization and aggressive displacement.

A correlation was also made between mutacin 1140 production and the ability of *S. mutans* to persistently colonize the oral cavities of human subjects and aggressively displace indigenous mutans streptococci (Hillman et al. 1985, 1987b). Three years following a single, 3 min infection regimen involving brushing and flossing of a concentrated cell suspension onto and between the teeth, all of the subjects remained colonized by the mutant strain producing 3-fold elevated amounts of mutacin 1140 (Hillman et al. 1989). No other strains of mutans streptococci were observed in saliva and plaque samples of these colonized volunteers. The same results were found recently, 14

years after colonization, for at least two of three subjects who are still available for testing. These results indicate that this strain of *S. mutans* succeeded in satisfying the second and third prerequisites for use as an effector strain in replacement therapy: It persistently and preemptively colonized the *S. mutans* niche in the human oral cavity and it aggressively displaced indigenous strains of this organism. Consequently, *S. mutans* strain JH1140, which has a spontaneous mutation resulting in ca. 3-fold elevated production of mutacin 1140, served as the starting strain for construction of BCS3-L1 described above. Introduction of the *ldh* mutant allele into JH1140 had no measurable effects on phenotypic properties known to be important in the natural history of infection by *S. mutans.* This includes intracellular polysaccharide storage and aciduricity. Interestingly, production of extracellular polysaccharide (glucan), which serves as an important holdfast during colonization, was somewhat increased during *in vitro* cultivation in the presence of sucrose, probably because of the pH dependence of the glucosyl transferase activities. It is not known if this effect is also manifested during *in vivo* growth.

Safety and stability

To serve as an effector strain in replacement therapy of dental caries, BCS3-L1 must be safe in several important regards. First, it must be genetically stable. In the case of BCS3-L1, reacquisition of an acidogenic phenotype by spontaneous reversion is extremely unlikely because construction of BCS3-L1 involved deletion of essentially the entire *ldh* open reading frame. Although horizontal transmission of an *ldh* gene is possible, repeated attempts have failed to demonstrate transduction of *S. mutans* by bacteriophage (A. Delisle, personal communication). In addition, transformability is known to be very strain dependent (Perry & Kuramitsu 1981; Westergran & Emilson 1983), and although it has been shown (Li et al. 2001) that BCS3-L1 has a low transformation frequency due to a natural mutation in its gene for competence stimulating peptide, allelic exchange has been used to delete the *comE* gene to provide added assurance that exogenous DNA will not transform this strain.

Mutacin 1140 has been shown (Hillman et al. 1998) to be a member of a small class of antibiotics called lantibiotics. It is a small (2263 Da), very stable peptide containing modified amino acids, lanthionine, methyllanthione, didehydroalanine and didehydrobutyrine, characteristic of lantibiotics. Sufficient mutacin 1140 has not been purified to directly test its toxicity. However, the prototype lantibiotic, nisin, is known to have extremely low toxicity (Hurst 1981), and has been developed and used for decades as a food preservative that is generally recognized as safe. It was shown (Hillman et al. 2000) that after colonization for 6 months, the mean weights of conventional Sprague-Dawley rats colonized with BCS3-L1 did not differ significantly from animals colonized with JH1140 or *S. mutans*-free control animals. Histopathological examination revealed no treatment-related lesions in any of the major organs examined.

Mutacin production by BCS3-L1 and the change in fermentation products resulting from LDH deficiency could conceivably upset plaque ecology and lead to the bloom of another microorganism with pathogenic potential. Recent studies (reviewed by Costerton 1995) have provided an appreciation for the complicated structural architecture of biofilms, presumably including dental plaque. Following specific initial attachment to a surface, the growth of cells leads to the formation of a thick layer of differentiated mushroom- and pillar-like structures consisting of cells embedded in their extracellular polysaccharide matrix. Between these cellular structures are water-filled spaces that serve as channels for the introduction of nutrients and the elimination of waste products. In biofilms consisting of two or more species of bacteria, each cellular structure may be pure or mixtures of cells depending on the pressures imposed by positive and negative bacterial interactions. These interactions may also extend over a finite area to affect the general composition of plaque in a particular habitat. Clearly, however, there is a physical limit to this sphere of influence. The mutacin 1140 up-producing strain of *S. mutans* eliminated mutacin-sensitive indigenous strains of *S. mutans* but had no effect on indigenous *S. oralis* strains that were equally sensitive to mutacin killing *in vitro* (Hillman et al. 1987b). These results indicate that *S. mutans* has a physically distinct habitat that is separated from the *S. oralis* habitat by a distance sufficient for dilution to reduce the concentration of mutacin below its minimal inhibitory concentration. A similar explanation could account for the failure to observe qualitative or quantitative changes in the plaque of rats following long-term infection with an LDH deficient mutant, even though the mutant's metabolic end-products are certain to be different from those of the wild-type strain (Stashenko & Hillman 1989).

A final aspect of replacement therapy safety is the requirement for controlled spread of the effector strain within the population. Mutacin 1140 up-production clearly provides a selective advantage to BCS3-L1 colonization, but the minimal infectious dose has not been determined for this strain or any *S. mutans* strain in humans. As described above, horizontal transmission of natural strains appears to be a rare event, but mutacin up-production may promote its occurrence. Wives and children of the two subjects infected with the mutacin up-producing *S. mutans* strain were not colonized when tested 14 years after the initial infection regimen (J.D. Hillman, unpublished result). Clearly, additional studies with larger populations will have to be performed to properly measure the potential for horizontal transmission. It is expected that, like wild-type strains of *S. mutans*, vertical transmission of BCS3-L1 from mother to child will occur at a high frequency.

Concluding remarks

In general, replacement therapy using a carefully constructed effector strain provides a number of advantages over conventional prevention strategies. In the case of dental caries, a single colonization regimen that leads to persistent colonization by the effector strain should provide lifelong protection. In the event that the effector strain does not persist indefinitely in some subjects, reapplication could be performed as the need arises without added concern for safety or effectiveness.

One of the greatest advantages of replacement therapy is that there is no need for patient compliance. Conscientious use of conventional prevention methods (brushing, flossing, topical fluoride, controlled sugar consumption, etc.) is sufficient in most cases to maintain the *S. mutans* level below its minimal pathogenic dose. The fact that dental caries remains as one of the most common infectious diseases afflicting humans is a clear indication that a truly effective prevention strategy must avoid the need to rely on patient compliance.

The current resurgence of various infectious diseases indicates that traditional and antibiotic-based therapies alone will not suffice. The continued study of bacterial interactions as they occur *in vivo* will inevitably lead to the identification of naturally occurring effector strains for the replacement therapy of various infections. If ultimately successful, the use of genetic engineering to tailor an effector strain for replacement therapy of dental caries would encourage similar efforts to prevent other infectious diseases.

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