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REVIEW ARTICLE

Modification of an effector strain for replacement therapy of dental caries to enable clinical safety trials

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Abstract

Aims: To construct a genetically modified strain of *Streptococcus mutans* for dental caries prevention. The strain has significantly reduced cariogenicity owing to a deletion of the entire open reading frame for lactate dehydrogenase, and has excellent colonization potential through the production of a natural antibiotic called mutacin 1140. For use in human clinical trials, additional mutations were introduced to enable rapid elimination of the strain in case of adverse side effects and to increase genetic stability.

Methods: Deletion mutations were introduced into the *dal* gene for D-alanine biosynthesis and the *comE* gene for genetic transformation. The resulting strain, A2JM, was tested for dependence on exogenous D-alanine and its ability to be eradicated from colonized rats. The strain was also tested for its ability to exchange DNA with another strain of *S. mutans* in *in vitro* and *in vivo* models.

Conclusions: A2JM was completely dependent on exogenous D-alanine, but could colonize the oral cavity of rats in low numbers in the absence of dietary D-alanine. Results indicated that A2JM can scavenge D-alanine from other plaque bacteria. Lowering of the total oral bacterial load through daily application of chlorhexidine enabled virtually complete eradication of A2JM. The introduction of the *comE* gene did not significantly decrease the transformability of A2JM in *in vitro* or *in vivo* models. The addition of a deletion in the *comE* gene does, nonetheless, provide additional safety as it has a very low reversion frequency.

Significance and Impact of the Study: Based on the safety and efficacy profiles established *in vitro* and in animal models, A2JM appears suitable for safe use in human clinical trials.

Introduction

Streptococcus mutans effector strain BCS3-L1 was created using recombinant DNA technology for use in the prevention of dental caries (Hillman *et al.* 2000). Dental caries remain a major health problem afflicting a majority of the population worldwide. Those who suffer the worst oral health are found among the poor of all ages, with poor children and poor older people particularly vulnerable (Oral Health in America 2000). The caries incidence in Western Europe is comparable with that in the United States, while Canada and Japan report a caries rate almost double that in the United States. Many developing countries are facing an explosion in caries rates as diets and other socio-economic factors change rapidly. Clearly, additional caries prevention approaches that can augment existing ones (e.g., fluoride, sealants, brushing, flossing) are highly desirable. One such potential approach depends on the observation that the indigenous microflora can benefit its host by inhibiting the colonization or proliferation of many potential pathogens. In recent times, this observation has developed into a therapeutic approach called ‘replacement therapy’. A strong theoretical basis exists for the replacement therapy approach in the prevention of bacterial infections, which has been recently reviewed (Hillman 2002).

Strain JH1140 (Hillman *et al.* 1998) is a derivative of an *S. mutans* strain isolated from the saliva of a human subject. It naturally produces an antibiotic called mutacin 1140 that is capable of killing all other strains of *S. mutans* tested to date, which gives it a selective advantage in colonization as demonstrated in both animal models (Hillman *et al.* 1998) and in humans (Hillman *et al.* 1987). Recombinant DNA methods were used to delete essentially the entire open reading frame (ORF) for lactic acid dehydrogenase (LDH). This mutation created a metabolic blockade that was lethal when exchanged for the wild-type allele, but it was found that replacing the ORF for LDH with the ORF for alcohol dehydrogenase B from *Zymomonas mobilis* overcame this blockade to yield a viable strain called BCS3-L1 that produced wild-type levels of mutacin 1140 and no lactic acid (Chen *et al.* 1994; Hillman *et al.* 2000).

The ability of BCS3-L1 to serve as an effector strain in the replacement therapy of dental caries was extensively tested in the laboratory and in animal models (Hillman *et al.* 2000). The strain proved to have significantly reduced pathogenic potential; it persistently and pre-emptively colonized the niche on the tooth surface normally occupied by wild-type strains of *S. mutans*; it was genetically stable; and it showed no ill effects in acute or chronic toxicity studies. If BCS3-L1 could be shown to have similar properties in humans, it would serve as an idealized effector strain with the following advantages: (i) a single treatment regimen could, ideally, provide lifelong protection against tooth decay; (ii) the possibility of deleterious side effects are negligible as the effector strain is essentially identical to a micro-organism, which is found universally in humans; (iii) minimal patient education and compliance is required; and (iv) the therapeutic method would be cost effective and suitable for use in the population at large, and would be particularly well suited for use in developing countries.

For continued development of replacement therapy, trial of BCS3-L1 in human subjects is the necessary next step. To this point, the use of a persistently implanted, genetically modified bacterium has not been attempted for any purpose. As a consequence, due caution suggested that additional genetic modifications of BCS3-L1 were warranted in order to enable its rapid elimination from test subjects should an unexpected adverse event manifest itself. Also genetic modifications of BCS3-L1 were warranted to provide additional assurance that the strain has the maximum possible genetic stability. In this paper, we describe the construction of strain A2JM from BCS3-L1 by the addition of two mutations: first, a ‘recall’ mutation in the gene (*dal*) for alanine racemase, which make the strain dependent on exogenous D-alanine. As D-alanine is typically found in only trace amounts in most people’s diet (Nagata *et al.* 2006), the persistence of A2JM logically would depend on providing a D-alanine supplement to subjects participating in a safety trial. Second, BCS3-L1 has a naturally occurring mutation in *comC* (Li *et al.* 2001), which encodes a competence stimulating peptide (CSP), which could, in theory, be complemented by CSP in the environment. Thus, we also added a mutation in a gene (*comE*) important in the uptake of environmental DNA, which renders the strain less prone to transformation and which cannot be complemented. A2JM was extensively tested to assure its safe use in phase 1 clinical trials.

Materials and methods

Bacterial strains and media

Streptococcus mutans strains JH1140, BCS3-L1, JH1140 (*orfY*::Tn917) (Gutierrez *et al.* 1996) and NG8 (Lee *et al.* 1989) have been previously described. These strains were routinely grown in Todd Hewitt medium supplemented with 0·5% yeast extract (THYex) or semi-defined medium (SDM) (Hillman *et al.* 2000). Solid plates used the same medium with 7·5% agar added. Screening/Selection (S/S) medium for the selection of *S. mutans* contains per litre: 10-gm bacto tryptone, 5-gm yeast extract, 50-mg 2,3,5-triphenyl tetrazolium chloride (Sigma-Aldrich, St. Louis, MO, USA), 200-gm sucrose and 15-gm agar. Lactic acid producing colonies of *S. mutans* appear white on this medium and mutants defective in lactic acid production appear bright red. *Escherichia coli* strain DH5α used in the construction of A2JM was grown Luria Bertani (LB) medium. Tetracycline and/or erythromycin, when needed, were incorporated into media at 15 and 10 $\mu g\ ml^{-1}$, respectively. D-Alanine was added to a final concentration of 1 $mmol\ l^{-1}$.

Construction of an alanine racemase deficient mutant

The *S. mutans* genome database (http://www.genome.ou.edu/smutans.html) contains an ORF with high homology to alanine racemases from a variety of gram-positive and negative bacteria. This ORF plus 500 base pairs (bp) of 5′ and 3′ flanking DNA were amplified by PCR using primers (JA07: 5′-AGCTGGGGTCACTACTTTTC and JA08: 5′-TCTCAAAGGTAG-GCGTCAAG), designed from the database sequence. JH1140 genomic DNA served as template. The amplicon was gel purified and ligated into pCRBlunt (Invitrogen, Carlsbad, CA, USA) according to standard methods (Ausubel *et al.* 1997). An *EcoRV* fragment containing the cloned DNA was ligated into the *S. mutans* suicide plasmid, p95 (tet) (Hillman *et al.* 2000). Calcium treated cells of *E. coli* strain DH5α (Clonetech, Palo Alto, CA, USA) were transformed and spread on LB plates containing 15-μg ml^{-1} tetracycline. Clones that arose were purified on the selection medium and crack preps of 20 clones were examined by agarose gel electrophoresis to identify the ones that had a cloned insert of proper size. Plasmid DNA from one such isolate was purified using a Wizard miniprep kit (Promega, Madison, WI, USA) according to the manufacturer's instructions, and the insert sequenced in both directions using p95 primers to confirm the presence and fidelity (relative to the database sequence) of the cloned alanine racemase DNA sequence. Circle PCR mutagenesis (Hillman *et al.* 2000) using vent polymerase was used to delete the entire alanine racemase (*dal*) ORF except for five codons at the 5′ end and the five codons just prior to the translation stop codon at the 3′ end. In this fashion, recircularization of the PCR fragment resulted in the stably mutated allele but avoided any possible polar effects on downstream genes. The PCR product was gel purified, blunt end self-ligated, and transformed into DH5α using standard methods (Ausubel *et al.* 1997). Crack preps and sequencing of miniprep DNA were performed to identify a clone containing the proper in-frame deletion within the *dal* ORF. Purified p95 plasmid DNA containing the cloned *dal* gene with engineered deletion was used to transform BCS3-Ll using previously reported methods (Hillman *et al.* 2000) with selection on medium containing tetracycline. One clone which arose was analysed by Southern analysis to confirm that the genetic construct inserted by homologous (Campbell) recombination into the chromosome at the *dal* locus to yield a heterodiploid containing both wild-type and mutant *dal* alleles. Alanine racemase-deficient mutants were obtained by spontaneous resolution of the heterodiploid as follows: the heterodiploid was grown for 20 generations in SDM that contained 1-mmol l^{-1} D-alanine and no tetracycline. The culture was diluted and spread on solid medium of the same composition. Colonies that arose were replica patched onto D-alanine containing medium with and without tetracycline. Tetracycline sensitive colonies were purified and tested for growth on medium with and without D-alanine. Tetracycline sensitive, D-alanine dependent clones were analysed by Southern blot to verify that they contained no p95 DNA and only the mutated copy of the *dal* gene. One such clone, BCS3-Ll (*dal*), was chosen for further study. PCR primers were chosen that flanked the region of the deletion, and used to generate an amplicon that was sequenced to directly confirm the presence of the in-frame deletion.

Characterization of the *dal* mutation

The growth requirements of BCS3-Ll (*dal*) and A2JM containing both the *dal* and *comE* mutations were tested on a variety of rich and minimal media under aerobic and anaerobic conditions. Mid-exponential phase cells of BCS3-L1 (*dal*) and A2JM were centrifuged and washed with phosphate-buffered saline (PBS) and then resuspended at time zero in THYex medium with and without added D-alanine. The cultures were incubated at 37°C standing and the optical density and viable cell count measured at regular intervals. The samples were also examined by oil immersion microscopy following gram staining.

Spontaneous reversion of the *dal* mutation was tested by spreading 10^9 washed colony forming units of BCS3-L1 (*dal*) or A2JM grown in THYex broth with 1-mmol l^{-1} D-alanine on 100 THYex plates containing no D-alanine. The plates were incubated in candle jars at 37°C and examined for colonies daily for 1 week.

Construction of a *comE* mutant

The *com* region of the *S. mutans* chromosomal genomic DNA sequence was identified by homology to genes from closely related micro-organisms (e.g. *Bacillus subtilis*; http://pedant.-gsf.de/cgi-bin~wwwfly.pl?Set=Bacillus~subtilisl68&Page=index). A 4-kb region that included the *comC*, *D* and *E* genes was amplified using JH1140 DNA as template and the primers CJEJ02 (5′-ATTTTGGTATTTCAGTAGAGGTCAG) and CJEJ03 (5′GCTTCATTCATTTTGCTCTCC). The amplicon was ligated into pCR blunt and sequenced to confirm that the proper segment had been successfully cloned. Circle PCR mutagenesis was performed as described earlier. PCR primers were chosen such that religation of the amplicon resulted in virtually complete elimination of the *comE* ORF, but did not alter the reading frame of the altered gene, thereby obviating the possibility of polar effects. The modified fragment of DNA was excised using appropriate restriction enzymes and cloned into the *S. mutans* suicide vector pNM6E, a modified version of pVA891 (*erm*) (Macrina *et al.* 1980), in which the endogenous erythromycin resistance gene was deleted and replaced by the tetracycline resistance gene from pVA981. BCS3-Ll (*dal*) was transformed with the pNM6E construct with selection on THYex medium containing tetracycline (15 μg ml^{-1}). Heterodiploids were confirmed using Southern blot analysis, and spontaneous recombinants possessing only the mutant allele were identified as described earlier. One was chosen for further study. A PCR amplicon of the *comE* gene was sequenced to directly confirm the presence of the in-frame deletion. Identical *comE* mutations were constructed using heterodiploid intermediates in JH1140, BCS3-L1 and A2JM (*orjY*::Tn917) to serve as controls in the various studies.

In vitro and *in vivo* characterization of the *comE* mutation

A2JM (*orfY*::Tn917) was analysed for its ability to be converted to *dal*$^+$ by the wild-type strain NG8 *in vitro*. The frequency of NG8 conversion to *erm*^{r} by A2JM (*orfY*::Tn917) was also measured. This work was performed using a biofilm model as described previously (Li *et al.* 2001). Briefly, mixed culture biofilms of A2JM (*orfY*::Tn917) and NG8 were grown in 4X diluted THYex medium supplemented with D-alanine and heat-inactivated horse serum on polystyrene microtitre plates. Twenty microlitres of 1 : 1 ratio of A2JM (*orfY*::Tn917) and NG8 cell suspensions were mixed together and used as inoculum. The cultures were incubated at 37°C with 5% carbon dioxide for 16 h to allow biofilm formation. The liquid was gently removed from individual wells and mixed with 2 ml of prewarmed THYex containing D-alanine and 5% horse serum. The cultures were incubated at 37°C in 5% carbon dioxide for 3 h. The liquid was then removed and the biofilms cells were disrupted by gentle sonication in 2 ml of fresh medium. The cell suspensions were centrifuged, resuspended in 200 μl of medium and plated on duplicate THYex plates supplemented with 10 μg ml^{-1} erythromycin and no D-alanine. This medium would be permissive only for transformants of A2JM (*orfY*::Tn917) to D-alanine independence and NG8 to erythromycin resistance, and would be nonpermissive for the parent strains. All of the plates were incubated for 48 h, and the colonies which arose were purified on the selection medium. The purified colonies were tested on S/S medium with and without erythromycin to identify red-coloured colonies, which were *dal*$^+$ transformants of A2JM (*orfY*::Tn917), and white colonies which were *erm*^{r} transformants of NG8. The identity of transformants obtained using this method was confirmed using colony PCR amplification of the *dal* and *erm* genes and comparison of the amplicons to controls electrophoresed on agarose gels.

The effect of the *comE* mutation on transformability was determined *in vivo* using a cross-over design rodent model study. Approval for this and all other animal studies described later was obtained from the University of Florida's Institutional Animal Care and Use Committee. A total of 18 weanling rats were provided diet TD80406 (5% sucrose) and water containing 10-mmol^{-1} D-alanine *ad libitum*. Oral swab samples streaked on S/S medium confirmed that the animals did not harbour an indigenous *S. mutans* strain. Nine animals in group 1 were treated at 24 days of age with A2JM (*orfY*::Tn917) and nine animals in group 2 were orally implanted with NG8 using 10^9 CFU suspended in 100 μl of medium. The inoculation regimen was repeated on two consecutive days to insure rapid and efficient colonization of the test animals. Oral swab samples were analysed at weekly intervals to determine the proportion of the infecting strain to total cultivable flora. When the proportions stabilized at 6 weeks postinfection, animals in group 1 were treated by inoculating their teeth with 10^9 CFU of NG8. Similarly, animals in group 2 were treated with 10^9 CFU of A2JM (*orfY*::Tn917). At days 1, 3 and 7 following the superinfection step, three animals from each group were euthanized and their molar teeth extracted. The teeth were sonicated in 3 ml of PBS and the samples from both groups were spread on S/S medium containing erythromycin and no D-alanine to isolate transformants of A2JM (*orfY*::Tn9 17) to D-alanine independence and NG8 transformants to erythromycin resistance. The plates were incubated for 2 days at 37°C in candle jars, and the colonies that arose were counted. The transformation frequencies of A2JM (*orfY*::Tn917) to *dal*$^+$ and NG8 to *erm*^{r} were calculated based on the number of potential recipient cells found on day 1.

Colonization and eradication of A2JM from the mouths of rats

The eradication of A2JM from the mouths of rats by the elimination of D-alanine from their diets was tested. An overnight culture of A2JM in THYex broth was used to inoculate twelve 24-day-old rats with 10^9 CFU. The inoculation was repeated on two successive days, and the animals were maintained on diet TD80406 containing 5% sucrose (Harlan Teklad, Madison, WI, USA) and fluoridated (1 ppm) tap water containing 10-mmol l^{-1} D-alanine *ad libitum*. After 3 weeks, during which A2JM could establish itself in the dental plaque, nine rats, chosen at random, had D-alanine removed from their drinking water and were treated twice daily for 30 s with a swab saturated with 0·12% chlorhexidine gluconate (Prodentec, Batesville, AR, USA). This regimen was designed to mimic chlorhexidine usage by human subjects. Three rats continued on the pre-eradication regimen and constituted the control group in this study. At weekly intervals following the elimination of D-alanine from the drinking water and initiation of chlorhexidine treatment, saliva and faeces from the rats were tested for A2JM by plating serial dilutions on S/S medium.

Acute and chronic toxicity studies of A2JM

Acute oral toxicity of A2JM in its delivery vehicle (SDM/30% glycerol) was tested by MicroTest Labs (Agawam, MA, USA) according to OECD (Organization for Economic Co-operation and Development) Guideline (420). Twenty adult Sprague Dawley rats were used, including 10 test and 10 control animals. Males and females were equally represented. After overnight fasting, each animal received gastric lavage of 5 ml of the test article containing 5×10^{10} CFU of A2JM in SDM/glycerol (70 : 30) or vehicle control, and the animals were observed for symptoms of toxicity immediately after the injection of the test article or blank and at least two more times in the next 4 h, and daily, thereafter, for 14 days. A separate acute oral toxicity test was performed using a dose of 5 g of D-alanine in water per kg body weight.

Chronic toxicity studies were performed by inoculating eight 24-day-old Sprague– Dawley rats with A2JM (10^9 CFU) in SDM/glycerol (70 : 30) on three successive days. Four sham-treated animals received only the vehicle. The A2JM-treated group was fed diet TD80406 and tap water containing 10-mmol l^{-1} D-alanine *ad libitum*. Sham-treated animals received the same diet but without D-alanine in their drinking water. After 52 weeks, tissue and blood specimens were collected from each animal and analysed by TherImmune Research Corporation (Gaithersburg, MD, USA).

Results

Introduction of a *dal* mutation into BCS3-L1 to serve as a recall mutation

D-Alanine is a crucial element in peptidoglycan (cell wall) cross-linking. It is synthesized by the action of alanine racemase (EC 5.1.1. l), which catalyses the pyrodoxal-dependent isomerization of L-alanine produced by central intermediary metabolism. D-Alanine can also be scavenged from the environment. While gram-negative bacteria appear to have distinct anabolic and catabolic activities (Wasserman *et al.* 1983), the gram-positive bacteria which have been studied to date probably possess a single, constitutive anabolic alanine racemase activity (Inagaki *et al.* 1986; Heaton *et al.* 1988). Mutants of alanine racemase have been found for a variety of bacterial species. In most cases, the mutants were dependent on environmenta1 D-alanine for survival. Free D-alanine is not a normal component in the human diet and there is no known pathway for its synthesis or utilization in humans. Because of its negligible toxicity and carcinogenicity, oral rinsing with D-alanine should be entirely safe. We therefore used the deletion of the gene (*dal*) for alanine racemase as the first step in the construction of a modified version of BCS3-L1 with additional safety measures for use in phase 1 clinical trials of replacement therapy.

The construction of the deletion mutation of *dal* used our previously described methodology involving PCR amplification of the target gene plus flanking DNA, and the resulting fragment was cloned into *E. coli*. Circle PCR mutagenesis was performed using primers that were designed to eliminate the entire ORF except for a small number of codons at the 5′ and 3′ ends of the target gene. After religation, the construct was introduced into BCS3-L1 (and other strains for use as controls; see later) with antibiotic (tetracycline) selection for the insertion of the vector into the chromosome via a single crossover using the homology provided by 5′ or 3′ flanking DNA. In this and subsequent studies using the *comE* gene, the observed transformations were performed using the recipient strain grown in THB (Todd Hewitt broth) containing 5% heat-inactivated horse serum and 1 μg ml^{-1} of synthetic CSP. The addition of CSP has been shown to promote transformation in a number of strains of *S. mutans*, but is especially important in JH1140 and its derivatives which are naturally deficient in the production of this molecule owing to a spontaneous mutation in *comC*. On average, transformation frequencies were on the order of one transformant per 10^9 recipients. Six transformants were picked and purified. The presence of two copies of the *dal* region DNA flanking the vector sequence was verified by Southern blot analysis. One verified, the clone was then grown in THyex supplemented with 1-mmol l^{-1} D-alanine in the absence of tetracycline for 20 generations to permit spontaneous recombination events that led to the loss of the vector plus either of the wild type of mutant allele of *dal*. Samples of the culture were plated on THYex medium containing D-alanine, and colonies that arose were replica patched onto media with and without tetracycline. Thirteen clones were isolated that could not grow in the presence of tetracycline, and of these, three demonstrated an inability to grow on THYex or SDM media unless supplemented with D-alanine. Southern blot analysis of these clones confirmed the absence of vector DNA and the *dal* ORF indicating that a second, spontaneous recombination event had occurred, which eliminated the wild-type *dal* gene. Sequencing a PCR amplicon of the *dal* gene using primers upstream and downstream of the *dal* ORF confirmed the absence of the *dal* ORF except for five codons at its 5′ and 3′ ends.

Phenotypic testing of BCS3-L1 (*dal*) showed that the mutant is entirely dependent on exogenous D-alanine. When the cells were transferred during logarithmic growth from medium containing D-alanine and placed in medium without D-alanine, there was a short initial period of continued growth, during which, presumably, internal stores of D-alanine were depleted. The growth of the mutant then stopped and the optical density and viable cell counts declined over time (Fig. 1a). The decline in the optical density observed in the absence of

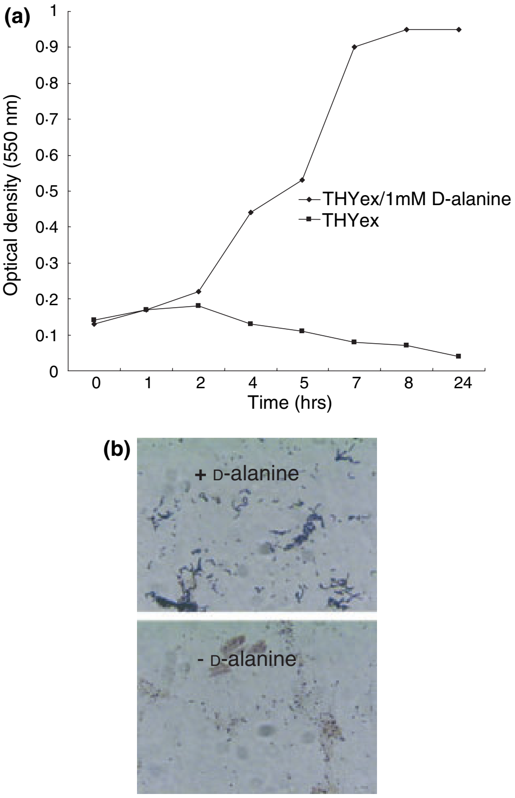

Figure 1 (a) Effect of D-alanine starvation on the growth of BCS3-L1 (*dal*). Mid-exponential phase cells were washed by centrifugation with phosphate-buffered saline (PBS) and resuspended in prewarmed media with or without added 1-mmol l^{-1} D-alanine. The optical density of the cultures were taken at the indicated times. (b) Effect of D-alanine starvation on cells of BCS3-L1 (*dal*). Two hours after transferring BCS3-L1 cells into media with or without D-alanine, samples were gram stained and examined by light microscopy.

D-alanine was attributed to cell lysis, as evidenced by the appearance of cell debris and red stained cells when gram-stained specimens were examined microscopically (Fig. 1b). A variety of other media and growth conditions were also tested, and BCS3-L1 (*dal*) was uniformly unable to grow in the absence of added D-alanine.

Spontaneous reversion of BCS3-L1 (*dal*) to growth in the absence of exogenous D-alanine was tested by spreading a total of 10^{11} CFU on media lacking D-alanine. After 1 week of incubation, no *S. mutans* colonies were observed, indicating that the reversion of *dal* or second site mutations that enabled endogenous D-alanine synthesis did not occur.

Introduction of a *comE* mutation into BCS3-L1 (*dal*) to create A2JM

In addition to the *comC* mutation, which is naturally present in JH1140 and its derivatives, we introduced a mutation in *comE*, which cannot be complemented by other strains of *S. mutans* or other species growing in the same habitat. The construction of the *comE* mutation followed the same format as described for the *dal* mutation. It was installed on the BCS3-L1 (*dal*) chromosome using pNM6E, and heterodiploid intermediates were confirmed by Southern blots. The resolution of the heterodiploids by a spontaneous recombination event provided six erythromycin-sensitive clones. As a mutation in *comE* does not provide an easily measured phenotype, all six of the clones were screened by colony PCR, which identified one clone, called A2JM, that lacked the ORF for *comE* and which did not have any vector DNA.

The effect of the *comE* mutation on transformability in biofilms was determined. In this model, we constructed a variation of A2JM in which the transposon, Tn917, was inserted in the gene cluster responsible for mutacin 1140 biosynthesis. This was done in order to prevent mutacin production by A2JM from killing the donor strain, NG8, before it had a chance to exchange genetic material. Using a biofilm model (Li *et al.* 2001), only limited genetic exchange occurred. The average total transformation frequencies, including the transformation of A2JM (*orfY*::Tn917) to dal^{+} and NG8 to erm^{r}, was $1{\cdot}40 \times 10^{-9}$. A second model to determine the transformation frequencies using planktonic cells gave similar results (data not shown). The frequency of NG8 transformation to erm^{r} was approximately twice that of A2JM (*orfY*::Tn917) transformation to dal^{+}. The transformants of NG8 to erythromycin resistance were tested by PCR to confirm that they had acquired the *erm* gene present in Tn917. Additional PCR results indicated that the entire lan gene cluster was present in only about 50% of these transformants. The mechanism for the acquisition of the erythromycin-resistance gene independent of flanking lan gene DNA by the wild-type strains of *S. mutans* is likely to involve a process analogous to zygotic induction, wherein Tn917, present on a fragment of A2JM transforming DNA, is induced to transpose onto the host chromosome. However, we did not experimentally confirm this hypothesis.

Genetic exchange between A2JM (*orfY*::Tn917) and NG8 was also examined in an animal model in which one strain was first implanted and then the animals were challenged with the second strain. Isolation of A2JM (*orfY*::Tn917) clones that were transformed to D-alanine independence and NG8 clones that were transformed to erythromycin resistance were selected using the methods described earlier. Under the conditions used, no transformants of either type were found in samples taken at any of the time points. The number of potential recipients and donors were determined from the total number of A2JM (*orfY*::Tn917) and NG8 present on the dentition, and allowed us to determine that the *in vivo* frequency of transformation was $<10^{-7}$.

Eradication studies

In a preliminary study, it was observed that BCS3-L1 (*dal*) colonized the teeth of Sprague–Dawley rats with or without D-alanine supplementation in their water. The average number of BCS3-L1 (*dal*) was significantly higher [$t(12) = 5{\cdot}382$, $P < 0{\cdot}001$] in animals provided with D-alanine in their drinking water (0·223% of total cultivable flora ± 0·103, mean ± SD) than in animals not receiving the supplement (0·097% ± 0·078, mean ± SD) (weeks 1 through 14). After removing D-alanine from the drinking water of group 1 animals (weeks 15–21), this difference disappeared [$t(17) = 0{\cdot}17$, $P = 0{\cdot}862$], indicating that D-alanine supplementation was necessary to maintain the higher level of colonization by BCS3-L1 (*dal*) in group 1 animals. After 4 months of infection, BCS3-L1 (*dal*) isolated from oral swab samples of animals not given D-alanine in their drinking water were still completely dependent on environmental D-alanine for growth *in vitro*. This result indicated that reversion to D-alanine independence was not the cause for the continued presence of the effector strain in animals not provided with dietary D-alanine. Incorporation of the rat diet TD80406 into microbiological cultivation medium to a concentration of 10% (w/v) indicated that the diet did not contain measurable amounts of D-alanine. It therefore seemed likely that BCS3-L1 (*dal*) was receiving D-alanine by cross-feeding from other plaque micro-organisms at a rate sufficient to enable its survival in the absence of dietary D-alanine. As rodents are coprophagic, it is also possible that fecal micro-organisms were responsible for cross-feeding BCS3-L1 (*dal*) with D-alanine. To test these possibilities, plaque and fecal samples were cross-streaked with BCS3-L1 (*dal*) on agar medium containing no D-alanine. Neither plaque nor faecal flora provided sufficient D-alanine to enable the growth of BCS3-L1 (*dal*) under these conditions of *in vitro* cultivation. However, autoclaved medium on which plaque bacteria had been grown was found to support the growth of BCS3-L1 (*dal*) without the addition of supplemental D-alanine. Apparently, killed plaque bacteria may liberate sufficient D-alanine to permit cross-feeding.

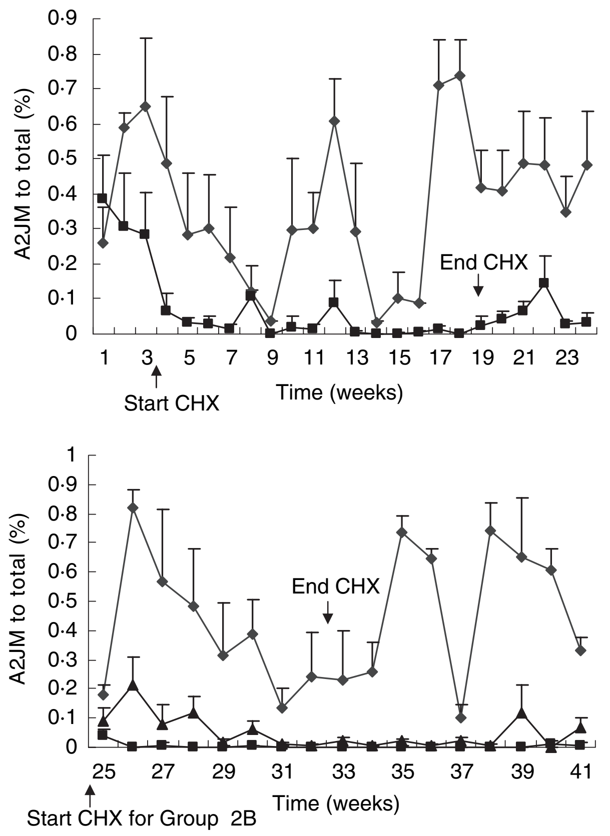

Figure 2 (a) Reduction of A2JM below detectable levels in the mouths of rats. Rats colonized with A2JM were treated by removing D-alanine from their drinking water and treatment with 0·12% chlorhexidine to eradicate the strain. The graph shows the proportion of A2JM to total bacteria recovered from plaque for the control (◆, group 1) and treated (■, group 2) animals for weeks 1–24. (b) Continued eradication of A2JM from the mouths of rats. The graph shows the proportion of A2JM to total bacteria recovered from plaque of control rats, rats that showed eradication below detectable levels without recurrence after the first treatment with chlorhexidine (group 2A), and rats that showed recurrence after the first chlorhexidine treatment and were retreated (group 2B). (◆, Group 1; ■, group 2, ▲, group 3).

These results suggested that virtually complete eradication of a D-alanine-dependent effector strain, such as A2JM, planned for use in clinical trials, may occur if the total oral microbial load is reduced, thereby limiting the potential for cross-feeding. Twelve rats, including three controls and nine experimental animals, were colonized with A2JM. Control animals (group 1) remained colonized by A2JM throughout the study (Fig. 2a,b). The average level of colonization over the entire period of the experiment was calculated to be approximately 0·42% of the total cultivable oral flora and 0·13% of the total cultivable fecal flora. Three weeks following implantation, the D-alanine was removed from the drinking water of the experimental rats (Fig. 2a, group 2), and they were treated twice daily for 30 s using cotton-tipped swabs saturated with chlorhexidine. Treatment with chlorhexidine virtually eliminated the recovery of A2JM from oral swab samples of group 2 animals within 12 weeks. As reinfection through coprophagy was felt to be a likely confounding factor in the interpretation of the effects of chlorhexidine on the oral levels of A2JM in rats, treatment was continued an additional 4 weeks to reduce A2JM in faecal samples to virtually undetectable levels.

Six of the nine animals (Fig. 2b, group 2A) that had received chlorhexidine treatment did not experience a significant resurgence of A2JM following the cessation of chlorhexidine treatment. Both oral and faecal samples only sporadically yielded distinctive red, irregular, mucoid A2JM colonies on S/S medium, which has previously been shown to have a plating efficiency >95% relative to blood agar and other permissive media. Between the period of week 25 and 41, the average level of oral colonization by A2JM was approximately 0·005% of cultivable bacteria, which is 100-fold lower than that observed in control animals over the same time period. Three of the animals (Fig. 2b, group 2B) that had received chlorhexidine treatment did experience a small but significant resurgence of A2JM following the cessation of chlorhexidine treatment. The average level of A2JM was found to be 0·075% between weeks 20 and 24, as compared with 0·45% for control animals. No other differences between group 2A and 2B animals were noted that could account for the observed difference in response to the eradication method. It is possible that the variability inherent in the chlorhexidine application technique designed for rats in this study is the basis for the observed differences between group 2A and group 2B. Seven independent A2JM colonies isolated during the resurgence period (weeks 20–24) from each of the three animals in the control (group 1) and the nine animals in the chlorhexidine- treated (group 2) groups were compared with A2JM recovered from a frozen glycerol stab for their minimal inhibitory concentration (MIC) and minimal bacteriocidal concentration (MBC) of chlorhexidine. No resistance to chlorhexidine was observed under the conditions used for testing. The MIC and MBC were 0·039% (v/v) for all isolates and for the A2JM control.

Although demonstrating a significant reduction in the levels of A2JM, the three chlorhexidine-treated animals showing resurgence (group 2B) were retreated for another 12 weeks by a daily application of chlorhexidine twice. The levels of A2JM again fell to virtually unmeasurable levels in oral and faecal samples. The cessation of chlorhexidine treatment was followed by a small but significant resurgence of A2JM, with an average level of 0·05% as compared with 0·6% for the control during the period from 38 to 4 1 weeks.

Toxicity studies

Gastric lavage of 10 adult male and female Sprague–Dawley rats with a dose of A2JM equivalent to a dose intended for human application (5×10^{10} CFU in SDM/glycerol) produced no changes relative to control animals receiving the vehicle alone. The animals were observed for changes in the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous system function, motor activity and behaviour pattern. Particular attention was directed to the observation of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma over a 14-day period following treatment. Likewise, the administration of D-alanine at 5-g kg^{-1} body weight produced no measurable deleterious effects.

A2JM was shown to cause no adverse side effects in laboratory rats for a period of 1 year following treatment with the effector strain suspended in the described pharmaceutical carrier. Persistent colonization by A2JM was demonstrated over that period of time. Similarly, the ingestion of D-alanine in drinking water at a concentration of 10 mmol l^{-1} for 1 year did not produce any adverse side effects in the laboratory rats. Daily in-life observations made during the animal study yielded nothing unusual with regard to the general condition, behaviour, body weight, food and water consumption for the A2JM inoculated animals and D-alanine-treated animals. Serum clinical chemistry using the Vitros 250 chemistry analyzer (Ortho-Clinical Diagnostics) showed no differences between treated and control animals for levels of albumin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, calcium, cholesterol, chloride, creatine kinase, creatinine, enzymatic carbon dioxide, glucose, potassium, sodium, phosphorus, total bilirubin, total protein and triglycerides. Haematological examinations revealed no differences between treated and control groups with regard to white blood cells, red blood cells, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelets, mean platelet volume, neutrophils, lymphocytes, monocytes, eosinophils and basophils. Histopathological examination of paraffin embedded, sectioned and haematoxylin and eosin-stained tissues, were performed microscopically by a board-certified veterinary pathologist. No test-related pathology findings were observed for any of the 48 tissues examined, including oral and craniofacial tissues, such as salivary glands, tongue, mandibular lymph nodes, optic nerve, eyes, and lacrimal glands.

Discussion

Safety testing of a genetically modified bacterium for use in replacement therapy for the prevention of a disease carries an implicit need to provide assurance that the effector strain can be eradicated or brought below its minimum pathogenic dose quickly in the case of an unexpected adverse event. In the case of BCS3-L1, designed as an effector strain for the prevention of dental caries, the most obvious potential adverse event is the acquisition of a wild-type LDH gene that would restore the ability of the cell to produce lactic acid. Such an event would initially occur in one cell among the millions that are present in the mouth. If a lactic acid producing revertant had a selective advantage over the effector strain, the ultimate result would be the outgrowth of a lactic acid producing strain equivalent to the one already present in the mouths of humans. Co-infection studies in which mixtures of A2JM and JH1140 were introduced into the mouths of rats indicated that lactic acid production did not provide a selective advantage (data not shown). Mutacin 1140 does not logically add any additional risk as this molecule is naturally produced by certain wild-type strains of *S. mutans*, and, in one study, was produced by over 2% of *S. mutans* isolates recovered from a random population of 164 subjects (data not shown). Nonetheless, extreme caution is advised when working with a new therapeutic agent, particularly when it is the first of its kind. For this reason, additional modifications were made to BCS3-L1 to create A2JM whose sole purpose is performing safety testing in human trials. It is anticipated that once safety is established, further testing of efficacy will be performed using the idealized effector strain, BCS3-L1.

A mutation in the *dal* gene, which encodes alanine racemase necessary for endogenous synthesis of D-alanine was introduced first into BCS3-L1 to make the strain dependent on environmental D-alanine. As the human diet typically contains very little of this compound, it was felt that the *dal* mutation would serve as a useful 'recall' mutation in the event of an unexpected adverse event. Next, a deletion mutation was introduced into the *comE* gene encoding a portion of the two component regulatory system involved in DNA uptake. This mutation was intended to augment the natural, spontaneous insertion mutation in *comC* present in JH1140 and its derivatives which is potentially revertable and may be complemented by CSP produced by other bacteria in the local environment. Construction of both mutants involved a strategy previously employed by us to delete essentially the entire ORF, thereby minimizing the potential for reversion. This hypothesis was confirmed by the selection for revertants on medium lacking D-alanine, which demonstrated that the reversion frequency is $<10^{-11}$. As the deletion of the *ldh* gene for LDH and the *comE* gene for genetic competence followed the same format as that used in deleting the *dal* gene, it is reasonable to presume that the reversion frequencies for these mutations are also very low. Although *dal*, *comE* and *ldh* appear to be cistrons or exist at the distal end of an operon, another aspect of the genetic construction took into account the potential for polar effects and other unknown epigenetic effects induced by deletion mutations. By leaving the signal sequences for these genes and five codons in frame at the beginning and end of each ORF, these phenomena are less likely to be of concern. Finally, the construction used a suicide vector containing the deleted gene plus a region of flanking DNA large enough to ensure homologous recombination. A single cross-over event created a heterodiploid intermediate that was confirmed by Southern blot analysis, and resolution of the heterodiploid by a spontaneous homologous recombination event provided a mixture of clones that contained either the wild-type of the mutant allele. PCR amplification of the gene in question allowed us to definitively prove that a deletion mutation had been created according to the proposed scheme. This method also resulted in the complete removal of the vector DNA and its antibiotic resistance markers from the A2JM chromosome. The result was a true isogenic mutant.

Deletion of the *dal* gene produced a mutant that was completely dependent on exogenous D-alanine. In this regard, *S. mutans* is similar to certain other gram-positive bacteria that have been studied, where only a single pathway exists for the conversion of L-alanine to the D isomer. It was shown *in vitro* that in the absence of D-alanine, the growth of the *dal* mutant ceased and cell lysis began, presumably owing to the weakening of the cell wall. Colonization of the rat oral cavity by the *dal* mutant could be achieved and maintained at wild-type levels by the addition of D-alanine to the drinking water. Removal of D-alanine from the drinking water caused a significant reduction in the levels of colonization but not complete eradication of the *dal* mutant. Testing of persistent isolates indicated that they retained their dependence on D-alanine, and indicated that the normal environment of the oral cavity provided a source of this compound. D-Alanine was not found in saliva and did not appear to be excreted by plaque bacteria under *in vitro* conditions of growth. Killed plaque bacteria did provide sufficient D-alanine for the *dal* mutant to grow in one *in vitro* model, suggesting that the normal turnover of cells in the oral cavity may permit a *dal* mutant to scavenge enough D-alanine to maintain a low level of colonization.

This hypothesis was tested by determining the levels of A2JM colonization in the oral cavities of rats with and without D-alanine supplementation in their drinking water. It was shown that reducing the overall bacterial load by daily administration (twice) of chlorhexidine resulted in prolonged reduction of A2JM below detectable limits in the mouths of six of the nine test animals. Retreatment of the three animals that showed outgrowth following the initial chlorhexidine therapy resulted in a sustained reduction of A2JM below detectable levels, although eradication below detectable levels in one of the animals was not achieved. It was shown that A2JM did not acquire measurable resistance to chlorhexidine throughout the course of this study.

The effect of introducing the *comE* mutation was measured in both *in vitro* and *in vivo* models. The transformation of JH1140 and its derivatives were difficult to achieve throughout the course of our work on them (Chen *et al.* 1994; Hillman *et al.* 1994, 1996, 2000). This observation is typical for most strains of *S. mutans*, but in the case of JH1140 and its derivatives, there is the additional feature of a naturally occurring mutation in *comC* (Li *et al.* 2001). The transformation frequency reported here for A2JM (*orfY*::Tn917) is not significantly different from the transformation frequency reported previously for JH1140. This suggests that the complementation of CSP does not occur under the conditions of testing that were used. Nonetheless, the *comE* mutation provides an additional level of assurance for genetic stability without, apparently, altering other important phenotypic properties of the effector strain. Transfer of the *erm* marker on Tn917 from A2JM (*orfY*::Tn917) to NG8 was observed, also at a low frequency. Whether this occurred via a homologous recombination event using DNA flanking, the transposon or a 'zygotic induction' event in which a fragment of A2JM (*orfY*::Tn917) DNA entering NG8 cells enabled the transposition of Tn917 was not determined. In the former case, Tn917 should be located on the same site in the NG8 chromosome and the A2JM chromosome. In the latter case, the location of Tn917 should vary from clone to clone among the NG8 transformants. As mentioned, mutacin 1140-producing strains of *S. mutans* are common in the human population. This finding suggests that the opportunity exists for natural transmission of mutacin 1140 in the human population regardless of the proposed application of A2JM as an effector strain in replacement therapy of dental caries. Interestingly, mutacin 1140 production by other oral species has not been found, which may indicate that there are species-specific constraints for the production of this molecule that we are unaware of. The lan gene cluster is very large (*c.*10 kb) and is required in its entirety for mature mutacin 1140 production. This, combined with the fact that A2JM aggressively displaces indigenous *S. mutans*, should significantly limit the opportunity for transformation of mutacin and the other pertinent genetic markers to occur.

Acute and chronic toxicity tests of A2JM and D-alanine in rats add support to the assertion that replacement therapy using a genetically modified strain of *S. mutans* will not have deleterious side effects. The ultimate effector strain for use in efficacy trials and which ultimately will be developed for the marketplace is not expected to employ the D-alanine dependence, which is intended strictly to enable safety trials. One of the main advantages of replacement therapy over conventional modes of decay prevention, such as brushing and flossing is that it does not require patient compliance. The fact that dental caries remains the single most common chronic infectious disease in humans is a clear indication that, even with good dental education, compliance is a major hurdle. It therefore makes no sense to rely on the patient to use a D-alanine mouthrinse daily in order to maintain the effector strain at the levels necessary for it to persistently colonize the teeth and prevent colonization by disease-causing strains whenever the host comes in contact with them.

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