BACTERIAL AND FUNGAL PATHOGENICITY

Genetic modification of a vaginal strain of *Lactobacillus fermentum* and its maintenance within the reproductive tract after intravaginal administration

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Summary. Many micro-organisms cause important diseases of the female genital tract. Because systematic vaccination does not usually provide a good immune response at mucosal sites, commensal lactobacilli from the female genital tract were developed as vehicles to deliver continued doses of foreign antigen directly to the genital mucosal surface with the aim of stimulating strong local mucosal immune responses. Lactobacilli were shown to be common inhabitants of the genital tract of the animal model studied, the guinea-pig. One species, *Lactobacillus fermentum*, was found in all guinea-pigs studied and was chosen for genetic manipulation. Improved methods of electroporation were developed to enable the routine transformation of *L. fermentum* BR11 strain with the broad host range plasmid pNZ17. This recombinantly modified *Lactobacillus* strain was shown to possess good segregational stability over 120 generations in the absence of antibiotic selection. When this recombinant *L. fermentum* strain was administered to the vaginal tract of three guinea-pigs it persisted for only 5 days. Despite the relatively short period of persistence in these initial experiments, this novel vaccine approach could provide an effective means of stimulating mucosal immunity in the female genital tract.

Introduction

The female reproductive tract is susceptible to a wide range of infections by bacteria and viruses including *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *Treponema pallidum*, herpes simplex virus II, human papilloma virus and human immunodeficiency virus. Currently no vaccines against any of these are available commercially. The infections are usually sexually transmitted and many are restricted to the superficial layers of the vaginal or cervical epithelium. The reproductive tract mucosa forms part of the common mucosal immune system, which also includes the gastrointestinal, respiratory and urinary tracts and the conjunctivae. Local secretion of antigen-specific IgA at all mucosal sites is thought to be initiated locally in the mucosa-associated lymphoid tissue, such as the Peyer's patches of the small intestine and the lymphoid aggregates within the respiratory tract.^{1–3} Current approaches to vaccination against mucosal pathogens have utilised the knowledge that antigenic stimulation at one mucosal site

may lead to an immune response and protection at distant mucosal sites, *via* the dissemination of activated lymphocytes.^{1,4} The most common approach to date has been oral administration of antigen to activate the gut-associated lymphoid tissue and produce an immune response at distant mucosal sites, such as the reproductive tract.^{1,4} However, several studies have shown that while gut responses may be adequate, the responses at more distant sites are often weak, and that local antigen presentation may produce better immune responses.⁵ Menge *et al.*⁵ showed that a vigorous local immune response to keyhole limpet haemocyanin conjugated to cholera toxin B subunit was induced after direct immunisation of the female reproductive tract. Other investigators^{1,2} have also concluded that the level of specific antibody is influenced by the route of immunisation, with local administration of antigen being the most potent stimulator of specific IgA secretion.

Of the many factors that influence the level of mucosal immunity produced, the frequency and dose of the antigen are known to be very important.⁶ Repeated antigen doses often elicit stronger mucosal immune responses.⁶ Live vaccines with bacteria or

viruses as vehicles may be the best method to vaccinate against mucosal pathogens by providing a sustained antigenic stimulus over many days. Our approach is to try to use non-pathogenic indigenous bacteria, such as members of the genus *Lactobacillus*, as vehicles for delivering antigen to the vaginal mucosa. Lactobacilli are the dominant bacterial species of the healthy human vagina⁷ and are also routinely found as normal flora in a wide variety of animals.⁸⁻¹¹ Moreover, they are harmless commensals, devoid of pathogenic potential.

To be useful as vaccine vehicles, lactobacilli need to carry and express foreign antigens. The genetic modification of lactobacilli only became a practical proposition in 1988 when electroporation was first used to transform *L. casei* strains¹² and, thereafter, was successful with several other *Lactobacillus* sp.¹³⁻¹⁷ Genetic modification of lactobacilli has usually been achieved by the introduction of plasmids, either derived from other naturally occurring lactobacillus plasmids^{15, 17} or by utilising broad host range plasmid replicons from other gram-positive bacteria.^{13, 14, 16}

The aims of the present study were: to determine if lactobacilli could be isolated as common inhabitants from the vagina of the chosen animal model, the guinea-pig; to isolate plasmids from these vaginal lactobacilli; to transform a vaginal *Lactobacillus* strain with a plasmid (pNZ17) containing the broad host range replicon from plasmid pSH71; to assess the segregational stability of plasmid pNZ17 in the vaginal *Lactobacillus* strain and; and to administer the transformed strain intravaginally and assess its maintenance within the vagina.

Materials and methods

Experimental animals

Sexually mature (i.e., older than 6 weeks) female guinea-pigs were used.

Culture and enumeration of vaginal bacteria

The ano-genital area of each guinea-pig was cleansed with cotton-tipped swabs (Disposable Products Pty Ltd, South Australia) moistened with phosphate-buffered saline (PBS). If the vaginal closure membrane occluded the vaginal opening, the membrane was punctured with a second swab. A third swab was inserted into the vagina to a depth of 2.5 cm and rotated. The swab was placed in 1 ml of sterile PBS, 3-mm sterile glass beads were added, and the liquid and swab were thoroughly vortex mixed. Log₁₀ dilutions of the suspension were prepared in PBS, and 0.1 ml of each dilution was plated on de Mann, Rogosa, Sharpe agar (MRS; Unipath) and horse blood agar (HBA) to determine total *Lactobacillus* and aerobic counts, respectively. Plates were incubated at 37°C in air with CO₂ 5% for 48 h. For total anaerobic counts, dilutions

were plated on HBA and the plates were incubated for 48 h at 37°C in an anaerobic chamber (CO₂ 9.7%, H₂ 10.2%, N₂ 80.1%; Anaerobe Systems). MRS agar plates containing chloramphenicol 10 µg/ml were used for the isolation of chloramphenicol-resistant lactobacilli. Colony types were differentiated by morphological criteria, followed by Gram's stain. Representative isolates were identified to the genus level according to *Bergey's Manual of systematic bacteriology*.¹⁸ *Lactobacillus* spp. were identified by the use of the Microbact 24AN identification system (Disposable Products) and by reference to the VPI anaerobic manual¹⁹ and *Bergey's Manual of systematic bacteriology*.¹⁸

Lactobacillus strains and plasmids studied

The bacterial strains and plasmids used are shown in table I. *L. fermentum* BR11 was a guinea-pig vaginal isolate (obtained in this study) used for transformation, segregational and structural stability and colonisation studies. Other lactobacilli were isolated in this study or were obtained from the American Type Culture Collection as indicated in table I. The plasmid pNZ717 (kindly donated by G. Simons, NIZO, The Netherlands) is 5.7 kb in size and contains the broad host range replicon of the *Lactococcus lactis* subsp. *lactis* plasmid pSH71 and the chloramphenicol acetyltransferases (CAT) gene of pC194; *L. fermentum* CR1 refers to the *L. fermentum* BR11 isolate containing pNZ17.

Plasmid isolation from lactobacillus strains

Plasmids were isolated from vaginal and ATCC lactobacilli by a modification of the method of Anderson and McKay,²⁰ in which treatment with lysozyme 10 mg/ml for 60 min replaced the original step. Briefly, strains to be profiled were inoculated into MRS broth and grown overnight at 37°C in air with CO₂ 5%. For plasmid screening, the broth culture was diluted 1 in 50 and this was grown until the OD_{600nm} was c. 0.5. Plasmid DNA was isolated, resuspended in 0.02 ml of distilled water and treated with RNAaseA 100 µg/ml for 60 min. Routinely, 0.01 ml of the DNA solution was electrophoresed at 4.0 V/cm through agarose 0.8% in tris-borate-EDTA buffer containing ethidium bromide 0.5 µg/ml incorporated into the electrophoresis buffer. These electrophoresis conditions gave the optimal resolution of plasmid profiles.

Transformation of vaginal *L. fermentum* BR11

L. fermentum BR11 was rendered resistant to chloramphenicol and kanamycin by the introduction of plasmid pNZ17. Bacterial cells were cultivated in MRS broth overnight at 37°C in air with CO₂ 5% and then a 1 in 50 dilution in 10 ml of MRS broth was incubated until the OD_{600nm} reached 0.3. At this point, penicillin G (Sigma) was added to give a final con-

Table I. *Lactobacillus* strains and plasmids studied.

Strains and plasmid	Relevant characteristics
<i>L. fermentum</i> BR 11	Guinea-pig vaginal isolate, this study
<i>L. acidophilus</i> BR 9	Guinea-pig vaginal isolate, this study
<i>L. sp.</i> BR 10	Guinea-pig vaginal isolate, this study
<i>L. sp.</i> BR 3	Guinea-pig vaginal isolate, this study
<i>L. sp.</i> WC 2	Guinea-pig vaginal isolate, this study
<i>L. casei</i> ATCC 393	ATCC isolate
<i>L. plantarum</i> ATCC 14917	ATCC isolate
<i>L. fermentum</i> CR1	<i>L. fermentum</i> BR11 containing pNZ17
Plasmid pNZ17	<i>Lactococcus lactis</i> /E. coli shuttle vector, 5.7 kb; pSH71 replicon; chloramphenicol acetyl transferase gene from pC194

centration of 10 µg/ml and growth was continued until the OD_{600nm} reached 0.6–0.7. Cells from the 10-ml culture were pelleted and washed three times in 0.5 M sucrose before being resuspended in a final volume of 0.5 ml of ice-cold 0.5 M sucrose. Cells were either used immediately or stored at –80°C for later use. Electrotransformation of lactobacilli was done with the BioRad Gene Pulser apparatus and chilled cuvettes with a 0.2-cm inter-electrode gap. pNZ17 plasmid DNA (0.5 µg) was added to 0.1 ml of a chilled suspension of *L. fermentum* cells and the mixture was held on ice for at least 5 min. Electrotransformation parameters included a field strength of 12.5 kV/cm with a parallel 200 ohm resistance and a capacitance of 25 µF. After the pulse, the cell-DNA suspension was returned to ice for 10 min and subsequently diluted 10-fold in MRS broth supplemented with 25 mM sucrose. Cell suspensions were incubated at 37°C in air with CO₂ 5% for 2 h to allow the cells to recover. Chloramphenicol was then added to the suspension to a final concentration of 0.1 µg/ml to induce expression of the CAT gene. Transformants were selected by plating 0.1 ml of the suspension on MRS agar plates containing chloramphenicol 10 µg/ml. Plates were incubated at 37°C for 48 h and transformants were confirmed by plasmid isolation.

Segregational stability of pNZ17 in *L. fermentum* CR1

The segregational stability of pNZ17 in the vaginal isolate *L. fermentum* CR1 was determined by repeated subculture in the absence of chloramphenicol; 0.1 ml of culture was diluted and plated on to MRS agar with and without chloramphenicol 10 µg/ml. After incubation for 24 h, the number of colonies in the presence and absence of chloramphenicol was counted. Cultures were passaged 22 times, which represents *c.* 120 generations for this strain.

Intravaginal administration of *L. fermentum* CR1

Lactobacilli were grown in the presence of chloram-

phenicol 10 µg/ml until the cells had reached late logarithmic phase (OD_{600nm} = 3.0). Bacterial cells were harvested by centrifugation at 4000 *g* for 5 min at 4°C, washed and resuspended in PBS to a concentration of 10¹¹ cfu/ml. The ano-genital area of each guinea-pig was cleansed and 0.2 ml of the *L. fermentum* strain CR1 suspension was flushed into the vagina with a smoothed glass pipette with a rubber suction bulb attached. In initial experiments, vaginal swabs were taken from four guinea pigs 1, 2, 3, 4 and 5 days after inoculation, and cultured to enumerate chloramphenicol-resistant bacteria. In subsequent experiments, the inoculation procedure was modified: 0.2 ml of the suspension were inoculated daily into the vagina of guinea-pigs for 5 days. After the final intravaginal inoculation, animals were swabbed daily for 7 days to assess whether vaginal colonisation by *L. fermentum* strain CR1 had occurred.

Results

Ecological studies of the guinea-pig vagina

The vaginal flora of all guinea-pigs examined consisted almost exclusively of gram-positive bacteria; gram-negative bacteria were isolated only occasionally (table II). The dominant aerobic genera isolated were *Corynebacterium* and *Enterococcus*; the dominant anaerobic genus isolated was *Propionibacterium*. *Bifidobacterium* spp. and *Bacteroides* spp. were also isolated from some animals. Lactobacilli were isolated consistently from all guinea-pigs and, although not numerically the most prevalent organisms, they always represented a significant component of the microbial flora (10²–10³ cfu/swab). *L. fermentum* was isolated from all guinea-pigs examined. Several other species of *Lactobacillus* were isolated routinely but could not be definitively identified to species level. The most common gram-negative organism isolated in this study was *Proteus* sp.—presumably faecal contaminants. No attempts were made to classify organisms other than lactobacilli to species level. One strain of *L. fermentum*,

Table II. Culture and enumeration of guinea-pig vaginal bacteria

Organism	cfu/swab
Aerobes and facultative anaerobes	
<i>Corynebacterium</i> sp. 1	10 ⁵
<i>Corynebacterium</i> sp. 2	10 ³
<i>Staphylococcus</i> sp.	10 ⁴
<i>Enterococcus</i> sp.	10 ⁵
<i>L. fermentum</i>	10 ²
<i>L. acidophilus</i>	10 ²
<i>Lactobacillus</i> sp.	10 ⁴
<i>Streptococcus</i> sp.	10 ⁵
Obligate anaerobes	
<i>Propionibacterium</i> sp.	10 ⁵
<i>Bifidobacterium</i> sp.	10 ⁴

Fig. 1. Agarose gel of plasmid preparations of vaginal and culture collection lactobacilli. Lane 1, mol. wt markers; 2, *L. fermentum* BR 11; 3, *L. fermentum* BR11 containing pNZ17 (= *L. fermentum* CR 1); 4, *L. casei* ATCC 393; 5, *L. plantarum* ATCC 14917; 6, *L. sp.* WC 2; 7, *Lactobacillus* sp. BR 10; 8, *E. coli* containing pNZ17. → indicates chromosomal DNA; — indicates plasmid DNA bands.

Fig. 2. Segregational stability of pNZ17 in *L. fermentum* strain CR1 after repeated subculture with (—) or without (---) chloramphenicol.

sensitive to chloramphenicol 5 µg/ml, was designated BR11 and chosen for further study.

Plasmid isolation from vaginal lactobacilli

Plasmid profiles of several vaginal and culture collection lactobacilli are shown in fig. 1. Extra-chromosomal DNA was detected in most of the lactobacilli examined (*L. fermentum* BR11, *L. casei* ATCC 393, *L. plantarum* ATCC 14917, *Lactobacillus* sp. BR 10). All preparations still contained some evi-

dence of chromosomal DNA contamination (→ in fig. 1) despite efforts to use optimal procedures for plasmid isolation. The high mol. wt plasmid migrating above the contaminating chromosomal DNA in most lanes possibly represents the lactose fermentation plasmid which is a common feature of many lactic acid bacteria. Furthermore, several strains contained plasmids of lower mol. wt (*L. fermentum* BR11, *L. plantarum* ATCC 14917). Plasmid preparations of *L. casei* ATCC 393 and *L. plantarum* ATCC 14917 were made to assess the reliability of the modified Anderson and

Fig. 3. The persistence of *L. fermentum* CR1 in the guinea-pig vagina after intravaginal inoculation; ----, number of Cm^r CR1 bacteria on day 0; —, number of Cm^r bacteria removed from the vagina following inoculation of 10^{11} CR1 bacteria on five successive administrations as indicated by (↑).

McKay method. *L. casei* ATCC 393 is known from previous reports to contain a large 27-kb Lac⁺ plasmid (as shown in fig. 1, lane 4), and *L. plantarum* ATCC 14917 to contain an 8-kb plasmid (as shown in fig. 1, lane 5). Similar plasmid profiles were obtained repeatedly in this work, confirming the suitability of the modified procedure.

Electrotransformation of vaginal strain *L. fermentum* BR11 with pNZ17

L. fermentum BR11 was regularly transformed with plasmid pNZ17 by the electroporation protocol described, with transformation efficiencies up to 2×10^3 transformants/ μ g of pNZ17. Culture collection lactobacilli gave higher transformation efficiencies of 10^4 transformants/ μ g or better (data not shown). Transformation of *L. fermentum* BR11 with pNZ17 was confirmed by agarose gel electrophoresis (fig. 1, lane 3). Restriction endonuclease digestion of this plasmid preparation with *Pst*I, which attacks a single site in pNZ17, gave a 5.7-kb linear plasmid band identical in size to *Pst*I-digested pNZ17 from *Escherichia coli* (data not shown).

Segregational stability of plasmid pNZ17 in *L. fermentum* strain CR1

Even in the absence of the selective agent chloramphenicol, plasmid pNZ17 was maintained relatively stably in *L. fermentum* strain CR1 for at least 120

generations (fig. 2). During the first 50 generations there was virtually no loss of plasmid; there was a 30% loss of plasmid during the next 70 generations.

Intravaginal administration of *L. fermentum* strain CR1

After intravaginal inoculation of *L. fermentum* strain CR1 into three guinea-pigs, its maintenance in the vagina was assessed by the ability to recover chloramphenicol-resistant organisms (fig. 3). In initial experiments, *L. fermentum* strain CR1 cells were given once only on day 0. In all experimental animals, the number of chloramphenicol-resistant bacteria (Cm^r) recovered from the vaginal tract decreased daily from 10^4 on day 1, to < 10 by day 7. The modified procedure of inoculation daily for 5 days did not significantly increase the persistence of Cm^r bacteria: 3×10^5 organisms were recovered by vaginal swabbing 1 day after the fifth inoculation and the numbers had fallen to 3×10^2 organisms by day 7. Thereafter, Cm^r bacteria were below the detection level of 10^1 .

Discussion

Systemic immunisation has generally proved to be ineffective against mucosal pathogens, primarily because, although it induces strong systemic antibody responses, it usually fails to induce a significant response at the appropriate mucosal surface.² In

contrast, direct mucosal immunisation often stimulates a more effective mucosal response.¹⁻³ However, most of these studies have focused on either the gastrointestinal or respiratory tracts as the mucosal sites of administration. Very few studies have focused on the female reproductive tract, despite the importance of local infections at this site.⁵ However, limited studies have shown that administration of antigens intravaginally does have the potential for the induction of a strong local immune response.⁵ In a mouse model, the presentation of intraluminal antigen to either dendritic or follicular cells within the vaginal epithelium or stroma, has been shown to result in the migration of these antigen-presenting cells to local lymph nodes, either inguinal or those ventral to the vagina.²¹ These activated cells presumably then go on to secrete specific antibody back into the vaginal mucosa.

One of the requirements for effective mucosal immunity, typified by the production of sIgA (and perhaps IgG originating from the circulation), is that the antigen provides a sustained stimulus.^{6,22} Live micro-organisms can provide this sustained stimulus and thereby induce stronger mucosal responses. Therefore, our approach was to utilise non-pathogenic bacteria that are normal residents of the female reproductive tract as vehicles for the delivery of antigens locally and hence the induction of a local immune response. Lactobacilli are an excellent choice as the delivery vehicle for several reasons; lactobacilli are generally non-pathogenic organisms; they are present in large numbers at several mucosal surfaces;⁷ and they have a natural ability to stimulate the immune system.

While the present results indicate that lactobacilli are not the dominant bacterial genera of the chosen animal model, the guinea-pig, as they are in man, they did constitute a constant and significant part of the vaginal microflora in all guinea-pigs examined. Other gram-positive species, including corynebacteria and enterococci were found to be the dominant aerobic genera and perhaps these micro-organisms also could be used as vaccine delivery vehicles. These two genera also are found as a substantial component of the indigenous microflora of mice, rats, monkeys and other mammals.⁸⁻¹⁰ However, the fact that lactobacilli are not the most prevalent micro-organisms is not of great concern, because the main requirement is for the vaccine organism to be able to colonise the vagina and to be able to compete with the established and more numerous resident micro-organisms. The fact that lactobacilli were present in significant numbers in all guinea-pigs examined, indicates that they are able to satisfy this requirement.

Plasmids are commonly used for the introduction of foreign DNA into bacterial cells. Plasmids, cryptic or coding for phenotypically defined characteristics, such as carbohydrate fermentation, have been shown to be ubiquitous in naturally occurring *Lactobacillus* strains. Another important characteristic of many of the

lactobacillus plasmids investigated previously has been the broad host range of their replication control elements. The use of resident plasmid replicons as the basis for the construction of cloning vectors is quite common.^{14,15,17} In the present study, all vaginal lactobacilli showed evidence of plasmid DNA, and, therefore, the construction of cloning vectors based on their replicons would be feasible. However, we have not pursued this approach at this stage. Instead, we chose to base our cloning vehicles on plasmids with a broad host range among gram-positive bacteria. Plasmids belonging to this family of small plasmids replicate by rolling circle replication and are dispersed throughout the gram-positive genera. The plasmid chosen contained the replicon of pSH71, a cryptic plasmid of *Lactococcus lactis* subsp. *lactis*, which has been shown to function in several industrial lactobacilli.^{14,15} In the present study, plasmid pNZ17 readily transformed the vaginal *L. fermentum* BR11 isolate at efficiencies of 2×10^3 . Penicillin G added to culture media of logarithmically replicating cells has been shown by several groups, including our own (data not shown) to increase the transformability of gram-positive bacteria, as have other cell-wall modifiers, such as glycine and lysozyme.¹³ Although the transformation efficiency of our vaginal *L. fermentum* strain was lower than that of the highly transformable *L. casei* ATCC 393, the transformation protocol was found to be simple and reliable.

If a recombinant vector is to be used to deliver foreign antigen to the reproductive tract, it is desirable that it is stably maintained in the host strain under non-selective conditions. Bates *et al.*¹⁶ reported variable stabilities of recombinant plasmids in different *Lactobacillus* strains, perhaps due to incompatibility of the introduced plasmid with the resident lactobacillus plasmid. Posno *et al.*¹⁵ showed that the plasmid pGK12, which contains the replicon of the *Lactococcus lactis* subsp. *cremoris* pWV01, a plasmid very closely related to the pSH71 replicon used in the present work, was stable in a *L. plantarum* strain, but was highly unstable in *L. pentosus*. The presence of a resident plasmid in their host strain was not mentioned. The high-level stability of pNZ17 in the vaginal isolate *L. fermentum* CR1 in the present study may be explained by compatibility with the resident plasmids of the host *L. fermentum* BR11 strain. The large plasmids naturally occurring in this strain may not be of the ssDNA plasmid family and, therefore, plasmid incompatibility was not a significant problem. The segregational and structural stability of vaccine plasmids may alter when foreign genes are inserted, and this needs to be investigated further.

Under the conditions used in this work, the transformed vaginal *L. fermentum* CR1 strain did not persist for long periods in the vagina after intravaginal administration. Previous studies^{23,24} have indicated that, to achieve effective colonisation of the female reproductive tract by lactobacilli, it is important to control a range of factors including the growth phase

of the inoculated cells, the nature and pH of the cell suspension media and the cell concentration of the inoculum. Although these factors were considered in the design of the present experiments, the intravaginal administration of *L. fermentum* strain CR1 failed to establish it within the vagina of the guinea-pigs. Recolonisation of the vaginal ecosystem by a recombinantly modified bacterium is obviously complex, and other factors that may have contributed to the short-term persistence include: in-vivo segregational instability; change in the adhesive properties of the vaginal isolate due to in-vitro manipulation; colonisation resistance of the native vaginal microflora; and removal of the recombinant lactobacilli by the daily swabbing routine before they had a chance to become established. Factors which promote efficient colonisation by the modified micro-organism will need to be investigated further.

This study demonstrated that naturally occurring isolates of vaginal lactobacilli are amenable to genetic modification. Previous studies on genetic manipulation and colonisation with lactobacilli have focused

on culture collection or dairy strains. *Lactobacillus* strains often demonstrate host specificities and thus the use of a naturally occurring vaginal isolate to deliver antigen within the genital tract is desirable. However, further experiments are required to find optimal conditions for the delivery of these strains to the female reproductive tract. While it is not yet fully proven that colonisation of the reproductive tract with recombinantly modified lactobacilli will lead to production of sufficient titre of antibodies to be protective against invasion by the pathogen, this approach to vaccination should result in antibodies of the IgA class, at the correct tissue location, the vaginal mucosa. Therefore, we believe that the use of genetically modified commensal micro-organisms, such as lactobacilli, has great potential in the development of vaccines against a range of diseases of the female reproductive tract.

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