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Full Length Research Paper

A recombinant lactobacillus strain expressing genes coding for restriction enzymes cleaving the HIV genomes for use as a live microbicide strategy against heterosexual transmission of HIV

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Using genetically engineered endogenous lactobacillus strains colonizing the vagina mucosa to express heterogenous proteins has of late joined the novel strategies aimed at developing a microbicides against HIV. Using the lactobacillus metabolic genome pathway, we found that these bacteria do not naturally produce restriction enzymes, but rather, have a number of putative alien genes of the type. In view of the antiviral defence role of restriction modification systems (RMS), we searched for enzymes that cleave HIV-1, 2 and other SIV genomes using theoretical computational methods. With over 200 such enzymes identified, we present herein a plasmid vector mediated strategy for modifying lactobacillus strains to express RMS islands as an approach to developing a live HIV microbicide. This model is transferable to other viral infections that find their way into humans through mucosal orifices.

Key words: HIV, recombinant Live Microbicides, Genetically modified Commensal bacteria (GMCBs), Bacteriovirogenomics, xRELAB.

INTRODUCTION

In the wake of increased virologic failure, resistance and toxicity to the currently approved Highly Active Antiretroviral Therapy (HAART) combinations (Mansky et al., 1995; DHHS, 2006; Hammer et al., 2006; Bartlett et al., 2006; Bartlett et al., 2001; Mayer et al., 2006), newer HIV inhibitory products are being explored (Gulick et al., 2006; Sansone et al., 2006; Pugach et al., 2006; Anastasopoulou et al., 2006; Norris et al., 2005; Norris et al., 2006; Davison et al., 2006; Delmedico et al., 2006; Markowitz et al., 2006). Many gene based models are currently being researched, ranging from the use of U1snRNAs to inhibit gene expression, RNA interference pathway to stop early and late HIV replication through post transcriptional gene slicing, and autologous T cell anti-HIV anti-sense Ribonucleic acid (RNA) delivered by the VRX496 lentiviral vector to ribozyme enzymes cleaving HIV RNA (Sajic et al., 2006; Barichievy et al., 2006; Meshcheryakova et al., 2006; Trebello et a., 2006; Liu et al., 2006). Recently, the use of recombinant lactobacillus developed by genetically modifying native vaginocervical (VC) commensal strains for use as live microbicides has became plausible strategy for preventing sexual transmission of HIV in women.

Base on the natural bacteria "restriction modification system (RMS) immune model (Murray, 2000; Nelson, 1972; Roberts and Macelis, 1991; Janulaitis et al 1992; Kessler and Manta, 1990; Nelson and McClelland, 1991; Radasci and Bickle, 1996; Barcus and Murray, 1995; Misaki, 2005) that renders bacteria cells immune to viral tropism through the production of restriction enzymes that recognize 4-8 bps nucleotide sequences in the invading DNA and cleave it, we hypothesized that, by modifying human cells susceptible to HIV tropism to accord the RMS properties, we could develop human gene therapies against HIV. Prospective *in vivo* viral-vector mediated delivery strategies for modifying human cells susceptible to HIV in this way were, however, overshadowed by the foresight of immunologic rejection and unpredictable safety (Web cutter Version 2.0. http://rna.lundberg.gu.se/cutter2/; Perelson et al., 1996; Perelson et al., 1997; Miller et al., 1993; Jolly, 1994; Weissman et al., 1995). We have hypothesized that through *ex-vivo* deli-

very; say by genetically modifying a native VC lactobacillus strain to accord it restriction and modification properties of RMS with potent activity against HIV genomes, if any, we could develop a novel live microbicide against HIV (Coombs et al., 2003; Bardeguez et al., 1997; Givan et al., 1997). We set of to analyse the pre-existing lactobacillus restriction and modification abilities, and search for other bacteria restriction enzymes that can cleave HIV 1, 2 and SIV genomes for use in this quest, and present here results of our findings.

METHODS

Design

Computer simulated assays, lactobacillus pathway genome data base [PGDB] search, and recombinant plasmid-vector-mediated genetic engineering modelling.

Materials

291 restriction enzymes from Promega database (http://www.promega.com/default.asp), the curated metabolic pathway genome of *Lactobacillus plantinuum-LacplantCyc* (http://www.lacplantcyc.nl/): the 9037 base pair HIV-1/SIVcpz (A1.BY.97.97BL006_AF193275), 9713 base pair HIV-2/SIVssm (A.GM.x.MCN13_AY509259) and 9719 base pair other SIV (B.FR.83.HXB2_LAI_IIIB_BRU_K03455) genomes obtained from the HIV gene sequence database (www.hiv.lanl.gov/content/hiv-db/ALIGN_CURRENT/ALIGN-INDEX.html, webcutter version2 (http://rna.lundberg.gu.se/cutter2/), pOSEL144 (Web cutter Version 2.0. http://rna.lundberg.gu.se/cutter2/) and pubMed literature on the pOSEL144 plasmid vector model (http://www.ncbi.nlm.nih.gov/).

Interventions

We searched for restriction enzymes cleaving the HIV genomes by feeding the above HIV subtype 1, 2 and other SIV whole genome in webcutter version 2.0 preset to recognize 6 palindromic nucleoside base pair sequences. In addition, we prompted the LacplantCyc pathway of *L. plantinuum* to identify lactobacilli restriction enzyme producing abilities. The biotechnology strategies for modifying the plasmid pOSEL144 to express heterogenous protens was reviewed on PubMED using a search dated 17/03/2007 prompted by the key words “Plasmid vector pOSEL144”

RESULTS

Webcutter V2.0 preset to identify enzymes

Of the 291 enzymes analyzed, 222 (76.3%) cleaved the HIV-1/SIVcpz genome as compared to 201 (69.1%) and 207 (71.1%) for HIV-2/SIVssm and other SIV respectively. 17 (5.8%) enzymes had 10 or > cuts in the HIV-1/SIVcpz genome as compared to 25 (8.9%) and 18 (6.2%) for HIV-2/SIVssm and other SIV, respectively. There were 6 of the enzymes demonstrating a mutual potent activity of 10 or > cuts against all three HIV-1/HIV-2/SIV genomes: Eco-130, EcoT141, ApoI, AcsI, BssTII, and StyI (Tables 1 - 4).

LacplantCyc pathway genome database

Of the 129 pathways and 704 predicted reactions involving some 670 chemical species and 710 enzymes, none originally involved the production of a restriction enzyme by lactobacillus. However, among putative alien genes acquired by horizontal gene transfer, there was one RMS genotype within the Lactobacillus PGDB, of *Staphylococcus aureus* origin: hsdR (putative type I restriction enzyme) and hsdM protein (methyltranferase). This implies that the native strains of lactobacillus do not naturally produce restriction enzymes (Coombs et al., 2003; Bardeguez et al., 1997; Givan et al., 1997; Patterson et al., 1998; Hladik et al., 1999; Anderson et al., 1998; Belec et al., 1995; Canchaya et al., 2006; Clerici, 1999; McLean and Rosenstein, 2000; Klebanoff, 1991; Theresa et al., 2003; Liu., et al 2006) (Table 5)

Plasmid vector pOSEL144

Searching PubMED, 22755 papers were found on plasmid vector pOSEL144. A summary of the latest literature revealed that plasmid vector pOSEL144 is readily amenable to modifications for use in engineering strains of lactobacilli expressing heterogeneous proteins. Chang et al. (2003) recently demonstrated inhibition of HIV infectivity by a natural human isolate of *Lactobacillus jensenii* engineered to express functional two-domain CD4 using a similar technology (Theresa et al., 2003). More recently, Liu et al. (2006) demonstrated Engineered Vaginal Lactobacillus Strain for Mucosal Delivery of the Human Immunodeficiency Virus Inhibitor Cyanovirin-N (Liu et al., 2006). Using pOSEL144 modified to carry restriction enzymes cleaving the HIV genome, it is therefore possible to genetically engineer lactobacilli that can express these enzymes.

DISCUSSION

Our results indicate that the HIV genome is still highly susceptible to the activity of various restriction enzymes, and many enzymes have the ability to cleave the genome in more than one place, with 17 (5.8%), 25 (8.9%) and 18 (6.2%) cleaving HIV-1/SIVcpz, HIV-2/SIVssm and Other SIV in 10. or more areas respectively. The size of genome has no influence on enzyme activity, but rather the presence of specificity palindromes. Taking the smaller HIV-1/SIVcpz genome A1.BY.97.97BL006_AF-193275 with 9037 base pairs for instance, we observed that 222 (76.3%) cleaved this gene compared to 201 (69.1) for the HIV-2/SIVssm A.GM.x.MCN13_AY509259 genome with 9713 base pairs and 207 (71.1) in the Other SIV genome B.FR.83.HXB2_LAI_IIIB_BRU_K03455 with 9719 base pairs.

Generally, there are four classes of restriction modification systems (RMS), with each class possessing differing biochemical structure and function. Within the same class of RMS however, genes from one bacteria species are

Table 1. Summary of Enzyme activity cutting 10 or > times.

Summary of Enzyme activity cutting 10 or > times		
Of the 291 enzymes analyzed, 222 (76.3%) cleaved the HIV-1/SIVcpz genome as compared to 201 (69.1%) and 207 (71.1%) for HIV-2/SIVssm and Other SIV respectively. 17(5.8%) enzymes exhibited 10 or > cuts [x 10=2 (BseRI, DraI), x 11=3 (BanII, Eco24I ,FriOI), x 13=3 (Eam1104I, EarI, Ksp632I), x 16=5 (BssT1I, Eco130I, EcoT14I, ErhI, StyI), x 17=2 (BstSFI, SfcI), and x 24=2 (AcsI, ApoI)] in the HIV-1/SIVcpz genome as compared to 25(8.9%) for HIV-2/SIVssm[x10=9(AccB1I, BanI, BshNI, DraII ,Eam1104I, EarI, Eco64I, EcoO109I, Ksp632I), x11=9 (BssT1I, BstSFI, CfrI, EaeI, Eco130I, EcoT14I, ErhI,SfcI, StyI), x 12=5 (BstYI, MflI, MspA1I, NspBII, XhoII), x16=2 (AcsI, ApoI) and 18 (6.2%). For Other SIV [x10=3(MslI, MspA1I, NspBII), x11=2 (DraI, Eco57I), x12=5 (BssT1I, Eco130I, EcoT14I, ErhI, StyI), x13=2 (BstSFI, SfcI), x17=6 (AcsI, ApoI, BstX2I, BstYI, MflI, XhoII). Details of enzyme activity on each HIV genomes are shown in Tables 1, 2 and 3.		
HIV subtype/(genome)/bps/N(%) enzymes	**No. cuts**	**Enzymes: Number (Name)**
HIV-1/SIVcpz/ (A1.BY.97.97BL006_AF193275) /9037 base pairs/17(5.8%)	10	2 (BseRI, DraI),
	11	3 (BanII, Eco24I, FriOI)
	13	3 (Eam1104I, EarI, Ksp632I)
	16	5 (BssT1I, Eco130I, EcoT14I, ErhI, StyI)
	17	2 (BstSFI, SfcI)
	24	2 (AcsI, ApoI)
HIV-2/SIVssm/ (A.GM.x.MCN13_AY509259)/ 9713 base pair/25(8.9%)	10	9 (AccB1I, BanI, BshNI, DraII, Eam1104I, EarI, Eco64I, EcoO109I, Ksp632I)
	11	9 (BssT1I, BstSFI, CfrI, EaeI, Eco130I, EcoT14I, ErhI, SfcI, StyI)
	12	5 BstYI, MflI, MspA1I, NspBII, XhoII)
	16	2 (AcsI, ApoI)

Table 2. Putative enzyme activity on HIV-1/SIVcpz genome A1.BY.97.97BL006_ AF193275 (9037 base pairs).

Frequency of cuts	**No. of enzyme (%)**	**Enzymes cleaving 10 or > times**
1	72 (24.7)	
2	46 (15.8)	
3	16 (5.5)	
4	13 (4.5)	x 10 (BseRI, DraI),
5	20 (6.9)	x 11 (BanII, Eco24I ,FriOI),
6	19 (6.5)	x 13 (Eam1104I, EarI, Ksp632I),
7	14 (4.8)	x 16 (BssT1I, Eco130I, EcoT14I, ErhI, StyI),
8	4 (1.4)	x 17 (BstSFI, SfcI), and
9	1 (0.0)	x 24 (AcsI, ApoI)
10	2 (0.7)	
11	3 (1.0)	
13	3 (1.0)	
16	5 (1.7)	
17	2 (0.7)	
24	2 (0.7)	
Totals	222 (76.3)	17(7.7 % of 222; 5.8% of N=291)

transferable to another species/strain. The class I 'RMS' to which *S. aureus*' putative alien genes found in the lactobacillus belong, is controlled mainly by 3 of 6 gene products: the specific restriction endonuclease (hsdR), Specificty host DNA stretch (hsdS) and a specific DNA methyltransferase (hsdM). All 3 proteins products of these genes recognize the same 4 - 8 base pair palindromic nucleotide sequence in the invading viral DNA with the former catalysing cleavage of foreign double standard DNA and the later functioning to protect the host genomic

Table 3. Table of enzyme activity on HIV-2/SIVssm Genome A.GM.x.MCN13_AY509259 9713 base pairs.

Frequency of cuts	No. of enzyme (%)	Enzymes cleaving 10 or > times
1	41 (14.1)	x10 (AccB1I, BanI, BshNI, DraII ,Eam1104I, EarI, Eco64I, EcoO109I, Ksp632I) x11 (BssT1I, BstSFI, CfrI, EaeI, Eco130I, EcoT14I, ErhI,SfcI, StyI) x12 (BstYI, MflI, MspA1I, NspBII, XhoII) x16 (AcsI, ApoI)
2	53 (18.2)	
3	29 (1.0)	
4	9 (3.1)	
5	16 (5.5)	
6	10 (3.4)	
7	5 (1.4)	
8	6 (2.1)	
9	7 (1.7)	
10	9 (3.1)	
11	9 (3.1)	
12	5 (1.7)	
16	2 (0.7)	
Totals	201 (69.1)	25 (12.4 % of 201; 8.9% of 291)

Table 4. Table of enzyme activity on Other SIV genome B.FR.83.HXB2_LAI_IIIB_BRU_K03455 (9719 base pairs).

Frequency of cuts	No. of enzyme (%)	Enzymes cleaving 10 or > times
1	63 (21.7)	x10 (MslI, MspA1I, NspBII), x11 (DraI, Eco57I), x12 (BssT1I, Eco130I, EcoT14I, ErhI, StyI), x13 (BstSFI, SfcI), x17 (AcsI, ApoI, BstX2I, BstYI, MflI, XhoII)
2	41 (14.1)	
3	11 (3.8)	
4	30 (10.3)	
5	14 (4.8)	
6	8 (2.8)	
7	11 (3.8)	
8	11 (3.8)	
10	3 (1.0)	
11	2 (0.7)	
12	5 (1.7)	
13	2 (0.7)	
17	6 (2.1)	
Totals	207 (71.1**)**	18 (8.7 % of 207; 6.2% of N=291)

Table 5. Putative alien genes resulting from horizontal gene transfer (based on homology BLAST analysis) of gram-positive bacterial origin. Adapted and Modified from http://www.lacplantcyc.nl.

Orf	Organism (best BLAST hit)	E-score	Identity >	Function	Remarks
Gram-positive origin					
lp_0275	*Staphylococcus aureus* (N315)	1.00E-123	52%	manganese transporter	Best hits:*S.aureus* (E-123), Listeria (E-103), Nostoc (E-100), Agrobacterium (E-92), Rhizobium (E-89)
lp_0918	*Staphylococcus aureus* (Mu50)	1.00E-110	29%	hypothetical protein	Only 1 good hit *S.aureus* (E-110, 29% identity over 1000 aa)
lp_0938	*Staphylococcus aureus* (Mu50)	0	42%	hsdR, putative type I restriction enzyme	also hits with gram-negatives
lp_0939	*Staphylococcus aureus* (N315)	0	60%	hsdM protein	Best hits with gram positives
lp_0941	*Listeria innocua*	1.00E-103	58%	integrase	Hits with Listeria are by far the best
lp_1299	*Staphylococcus aureus* (Mu50)	1.00E-103	41%	hypothetical protein	2e hit S.aureus, 3e hit *L. lactis* (6E-41, 24% identity)

DNA from cleavage by the cognate restriction enzyme (Murray, 2000; Nelson, 1972; Roberts and Macelis, 1991; Janulaitis et al 1992; Kessler and Manta, 1990; Nelson and McClelland, 1991; Radasci and Bickle, 1996; Barcus and Murray, 1995). In nature, the genes for coding for restriction-modification systems are often clustered together in a region called an "immigration control region". The fact that in the same class of RMS, these genes, or their proteins, from differing species/strain origin can recombine to yield restriction enzymes with completely new properties, besides the diversity of enzymes shown here to cleave the HIV genomes offers avenues for fighting resistance. Although vector mediated *in vivo* use of restriction enzymes as a gene based therapy against HIV is possible, there are dangers to the human genome that contains several of the recognition palindromes (Misaki, 2005; Web cutter Version 2.0. http://rna.lundberg.gu.se/cutter2/; Perelson et al., 1996; Perelson et al., 1997; Miller et al., 1993; Jolly, 1994; Weissman et al., 1995). In addition, immunologic rejection is bound to occur towards the cell-lines transduced by these viral vectors. This leaves only an *ex vivo* strategy for the therapeutic application of these enzymes.

Lactobacillus bacteria form the predominant commensals of the vagino-cervical mucosa. Recent studies have revealed that these bacteria actually form exist in form of biofilm lining in the mucosa (Coombs et al., 2003; Bardeguez et al., 1997; Givan et al., 1997; Patterson et al., 1998; Hladik et al., 1999; Anderson et al., 1998; Belec et al., 1995; Canchaya et al., 2006; Clerici, 1999; McLean and Rosenstein, 2000; Klebanoff, 1991). Similarly, several recent strategies aimed at modifying lactobacillus to express anti-HIV heterogenous proteins as a live microbicide strategy against HIV transmission have emerged (Theresa et al**.,** 2003; Liu, et al 2006). Our findings that highlight the presence of putative alien restriction genes and proteins from *S. aureus* reveals that the lactobacillus metabolic genome pathway genome is innately readily amenable to genetic modification and thus artificial engineering to express restriction enzyme genes. Plasmid vectors, such as the pOSEL144, can readily be modified to carry these genes into lactobacillus (Liu, et al 2006). It is believed that modification of native commensal lactobacillus to accord them abilities to express genes coding for restriction enzymes that cleave HIV proviral DNA offers a novel "live-microbicide" strategy for preventing HIV infection in high-risk women. Recombinant strains of lactobacillus expressing other antiviral proteins such as Cynavarin N and CD4-Ligands have already been demonstrated elsewhere to last over 2 weeks after application in the VC mucosa, offering a user friendly convenience by eliminating the need for immediate-pre-coital application (Liu, et al 2006). The United Nations (UN) Millennium Development Goals (MDGs) Genomics working Group of the Task Force on Science, Technology, and innovation (Task force 10) recently ranked the 10 most vital biotechnologies for improving global heath (UNDP 2001; UNMP 2005; and 51). The developing world too, listed its own priority applications for these (Greenwood et al., 2006; Salamanca-Buentello, 2005). In all 8 MDGs, Regenerative Medicine, an emerging interdisciplinary field of research and clinical applications focused on the repair, replacement, or regeneration of cells, tissues, or organs to restore impaired function resulting from any cause, had its profound role. Through its approach of nanaotechnology (NT) based medicine (nanomedicine), defined as the study, design, application, and exploitation of novel properties and phenomena of functional materials, devices, systems at a nanometer scale (1 - 100 nanometers; 1 nanometer = 1 x 10^{-9} of a meter: that is, at atomic and molecular levels), regenerative medicine offers many solutions to the health problems of the developing world (Singer et al., 2005) such as the one discussed in this context. Among the top 3 in 10 listed, immune system enhancement by engineered immune cells and novel vaccination strategies ranked 3rd.

Conclusion

Our results indicate that a diversity of bacteria restriction enzymes are capable of cleaving the HIV genome, and their application in viral infections is feasible, since many human orifices where these viruses primary enter are colonised by bacteria species. Using this approach may provide a novel line of antiviral therapeutic strategies. Although HIV is discussed as the primary pathology here, this science is transferable to other mucosal acquired viral infections such as HPV, HBV, HCV, SARS, and influenza. In the next 5 -10 years, I speculate that using this approach will form a basis for immunisation, vaccination, and microbiocide interventions against several viral infectious pathogens

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