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Short Communication

Identification and sequence analysis of two novel cryptic plasmids isolated from the vaginal mucosa of South African women

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ABSTRACT

The vaginal mucosa is dominated by Gram positive, rod shaped lactobacilli which serve as a natural barrier against infection. In both healthy- and bacterial vaginosis (BV)-infected women *Lactobacillus crispatus* and *Lactobacillus jensenii* have been found to be the predominant *Lactobacillus* species. Many studies have been conducted to assess factors influencing lactobacilli dominance in the vaginal microbiome. In the present study two plasmids, pLc4 and pLc17, isolated from vaginal *Lactobacillus* strains of both healthy and BV-infected women were characterized. The smaller plasmid, pLc4 (4224 bp), was detected in both *L. crispatus* and *L. jensenii* strains, while pLc17 was only detected in *L. crispatus*. Based on its nucleotide sequence pLc4 appears highly novel, with its replication protein having 44% identity to the replication initiation protein of pSMQ173b_03. Phylogenetic analysis with other Rolling Circle Replication plasmids confirmed that pLc4 shows a low degree of similarity to these plasmids. Plasmid pLc17 (16,663 bp) appears to carry both a RCR replicon and a theta replicon, which is rare in naturally occurring plasmids. pLc4 was maintained at a high copy number of 29, while pLc17 appears to be a medium copy number plasmid maintained at 11 copies per chromosome. While sequence analysis is a valuable tool to study cryptic plasmids, further function-based analysis will be required in order to fully elucidate the role of these plasmids within the vaginal milieu.

1. Introduction

Bacterial Vaginosis (BV) is the most common lower genital tract infection and impacts between 20 and 60% of women globally (Eade et al., 2012; Paria and Spear, 2014; Vallor et al., 2001). The microorganisms predominantly involved in BV are very diverse and include *Gardnerella vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticin*, *Peptostreptococcus*, and *Mobilancoccus* species, as well as anaerobic Gram negative rods such as *Prevotella* and *Bacteroides* species. BV is associated with Pelvic Inflammatory Disease, and it is hypothesized that it may increase susceptibility to Sexually Transmitted Infections (STI) and HIV (Paria and Spear, 2014; Sha et al., 2005; Vallor et al., 2001). The vaginal mucosa is dominated by Gram positive, rod-shaped *Lactobacillus* species which are thought to serve as a natural barrier against infection (Antonio et al., 1999; Damelin et al., 2010; Paria and Spear, 2014). Studies have shown that *Lactobacillus jensenii* (24%), *Lactobacillus crispatus* (22%), *Lactobacillus inners* (10%), *Lactobacillus gasserrii* (10%) and *Lactobacillus vaginalis* (9%) are the predominant culturable vaginal lactobacilli in South African woman (Damelin et al., 2011). Some, such as *L. crispatus,* have been found to have an inhibitory effect on *Escherichia coli*, the main causative agent of urinary tract infections (Ghartey et al., 2014). During BV infection pathogens disrupt normal healthy vaginal microflora, displacing the dominant lactobacilli (Vallor et al., 2001). The inhibitory characteristics exhibited by certain *L. crispatus* strains therefore make them good probiotic candidates for the prevention of BV and urinary tract infections (Ghartey et al., 2014; Ojalal et al., 2014). Researchers have previously proposed a HIV prevention strategy whereby anti-HIV molecules could be expressed within the cervico-vaginal milieu by endogenous vaginal *Lactobacillus* populations that have been engineered *in situ via* plasmid transduction (Damelin et al., 2010). The availability of diverse plasmids from predominant vaginal *Lactobacillus* species within the South African population could assist in the development of novel or improved engineering strategies.

Several studies have shown that plasmids found in probiotic lactobacilli play a vital role in their host survival. One such example is the *Lactobacilli brevis* KB 290, which carries nine plasmids that have been

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found to encode for proteins related to stress response and resistance (Fukao et al., 2013). Another example is *Lactobacilli paracasei* NFBC 338, a gastrointestinal tract (GIT) probiotic, which contains numerous plasmids possibly linked to biotin metabolism as well as adherence (Desmond et al., 2005). Probiotic strains of vaginal lactobacilli were analysed by Martin et al., 2008, where it was found that the majority of strains (90% of isolated probiotic strains) had plasmids (Martin et al., 2008). Given the prevalence of plasmids in beneficial bacteria it is probable that these plasmids play a role in enhancing vaginal colonization.

Despite evidence that supports the probiotic nature of *L. crispatus* within the vaginal milieu, very little is known about the plasmids it may harbour and the potential benefit (or costs) these may have on its host. This study presents the characterization of two novel plasmids, pLc4 and pLc17, extracted from *L. crispatus* and *L. jensenii* strains isolated from both healthy and BV infected individuals.

2. Methods and material

2.1. Bacterial strains and culture conditions

Vaginal *Lactobacillus* strains (*L. crispatus* ($n = 6$) and *L. jensenii* ($n = 2$)), previously isolated from the vaginal swabs of pre-menopausal South African volunteers with and without BV (Table 1), were routinely streaked on Man-Rogosa-Sharpe (MRS) agar (Oxoid) and grown anaerobically at 37 °C for 48 h or cultured in 50 ml MRS Broth (Oxoid) (in 50 ml polypropylene tubes) at 37 °C overnight without shaking. All strains were identified by 16S rRNA gene sequencing.

2.2. Plasmid extraction from Lactobacillus strains

Plasmid DNA was extracted from *L. crispatus* and *L. jensenii* (Table 1) strains using the Zyppy™ Plasmid Miniprep Kit (Zymo Research; Irvine CA U.S.A) with minor modifications to the lysis step. Briefly, overnight cultures were grown in 50 ml MRS broth as described above until an $OD_{600nm} > 0.8$. The cells were harvested by centrifugation at 6000 x *g* for 5 min and the pellets were resuspended in 600 µl sucrose-lysozyme solution as previously described (O'Sullivan and Klaenhammer, 1993). The suspensions were incubated at 37 °C for 1 h. Plasmids were then extracted from the treated suspensions using the kit as per the manufacturer's instructions.

2.3. Plasmid DNA sequencing, analysis and confirmation

Extracted plasmid DNA was purified using the Qiaex II Gel Extraction kit (Qiagen). Sequencing libraries were prepared with the Illumina Nextera XT library preparation kit. Libraries were individually indexed, pooled in equimolar concentrations and sequenced with a MiSeq reagent V3 kit (2 × 300 bp) (Illumina). A 10% PhiX V3 spike for low diversity libraries was included as per the manufacturer's instructions (Illumina Nextera XT guide). The fastq files generated were analysed using CLC Genomics Workbench version 6.5. Read pairs were merged and sequential reference assemblies to phiX174, *L. crispatus* (FN692037.1) and *L. jensenii* (FN557015.1) genomes were performed to remove contaminating DNA sequences. A *de novo* assembly was performed using all remaining unmapped reads. The *de novo* assembly was performed using similarity and length fractions of 0.9 and 0.95, respectively, with scaffolding turned off. Annotated plasmid sequences are available on GenBank under accession numbers KR052811 and KP984530 for pLc17 and pLc4, respectively. Softberry (www.softberry.com) and CLC Genomics Workbench (Qiagen) were used for ORF prediction. BLAST analysis to identify related ORFs was performed using megaBLAST against the NCBInr database, or in the case of replication proteins against the ACLAME database (http://aclame.ulb.ac.be/). Circoletto (http://tinyurl.com/he9cqhm) and Easyfig (Sullivan et al., 2011; http://mjsull.github.io/Easyfig/) were used for plasmid comparisons. For phylogenetic tree construction, the full-length amino acid sequences of selected replication proteins were aligned using MEGA6 (Tamura et al., 2013), and the neighbour-joining tree was constructed using the built-in program (Felsenstein, 1985). Secondary DNA structures were predicted using the Fold server (RNAstructure webserver https://rna.urmc.rochester.edu/). Plasmid specific primers were designed to confirm the plasmid assemblies (Table 2). PCR reactions contained 1 × Dream *Taq* Buffer, 2 µM dNTPs, 1 µl of DNA template and 1.25 U of Dream *Taq* DNA Polymerase. Before amplification, plasmid DNA was denatured at 95 °C for 3 min. The amplification consisted of 34 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s for pLc4 and 56 °C for 30 s for pLc17, and extension at 72 °C for 1.25 min.

Table 1
Lactobacilli DNA samples isolated from healthy and infected women.

Sample	Organism	Patient condition	Plasmid name
L1	*L. crispatus*	Healthy patient	pLc17
L2	*L. crispatus*	BV infected patient	pLc17
L3	*L. jensenii*	Healthy patient	pLc4
L4	*L. jensenii*	BV infected patient	pLc4
L5	*L. crispatus*	Healthy patient	pLc17
L6	*L. crispatus*	BV infected patient	pLc17
L7	*L. crispatus*	Healthy patient	pLc17
L8	*L. crispatus*	BV infected patient	pLc4

2.4. Plasmid copy number determination

Plasmid copy numbers were determined by quantitative PCR. Total DNA was extracted from vaginal swabs using the QuickDNA™ Fungal/Bacterial DNA Kit (Zymo Research) [11 swabs from healthy individuals and 11 from BV-infected individuals]. Prior to extraction the swabs were submerged in 1 ml of sterile 0.8% (wt/vol) saline (prepared with RNase free H_2O, pH 7.0), vigorously agitated to dislodge adhering bacterial cells and 600 µl of the cell suspension was subsequently used for the extraction which was performed according the manufacturer's instructions.

A primer set specific to each plasmid (pLc4 and pLc17) was designed (Table 2) using Primer3 software (Utergasser et al., 2012). The Human EGFR gene was used to normalise for residual human DNA contamination.

A 10-fold serial dilution series of the plasmid DNA ranging from 10 to 1,000,000 copies/µl was used to construct the standard curves. The concentration of the plasmid DNA was measured using the Qubit™ DNA BR assay kit and the corresponding copy number was calculated using the following equation.

$$\text{Number of copies} = \frac{(\text{amount} \times 6.022 \times 10^{23}) = (\text{ng} \times \text{number/mole})}{(\text{length} \times 1 \times 10^{9} \times 650)(\text{bp} \times \text{ng/g} \times \text{g/mole of bp})}$$

The qPCR amplification was performed using a Rotor-gene® Q (QIAGEN) with the Kapa SYBR® Fast qPCR Master mix (2×) kit (Kapa Biosystems). Human Genomic DNA (Roche Diagnostics) and plasmid DNA was added to each 10 µl reaction containing forward and reverse primers (10 µM), and Master mix (1×). The thermal cycling parameters are as described in Table 2. The melting curve was measured by cycling from an annealing temperature of 60 °C through to a melting temperature of 95 °C at a ramp rate of 0.5 °C every 2 s. The Cq value in each dilution was measured in triplicate and plotted against the logarithm of their initial template copy number. Each standard curve was generated by a linear regression of the plotted points. The slope of each standard curve was used to calculate the amplification efficiency. Plasmid copy number (PCN) was determined according to Skulj et al., 2008.