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Characterization and complete genome sequences of *L. rhamnosus* DSM 14870 and *L. gasseri* DSM 14869 contained in the EcoVag® probiotic vaginal capsules

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A B S T R A C T

Lactobacillus rhamnosus DSM 14870 and *Lactobacillus gasseri* DSM 14869 were previously isolated from the vaginal epithelial cells (VEC) of healthy women and selected for the development of the vaginal EcoVag® probiotic capsules. EcoVag® was subsequently shown to provide long-term cure and reduce relapse of bacterial vaginosis (BV) as an adjunct to antibiotic therapy. To identify genes potentially involved in probiotic activity, we performed genome sequencing and characterization of the two strains. The complete genome analysis of both strains revealed the presence of genes encoding functions related to adhesion, exopolysaccharide (EPS) biosynthesis, antimicrobial activity, and CRISPR adaptive immunity but absence of antibiotic resistance genes. Interesting features of *L. rhamnosus* DSM 14870 genome include the presence of the *spaCBA-srtC* gene encoding spaCBA pili and interruption of the gene cluster encoding long galactose-rich EPS by integrases. Unique to *L. gasseri* DSM 14869 genome was the presence of a gene encoding a putative (1456 amino acid) new adhesin containing two rib/alpha-like repeats. *L. rhamnosus* DSM 14870 and *L. gasseri* DSM 14869 showed acidification of the culture medium (to pH 3.8) and a strong adhesion capability to the Caco-2 cell line and VEC. *L. gasseri* DSM 14869 could produce a thick (40 nm) EPS layer and hydrogen peroxide. *L. rhamnosus* DSM 14870 was shown to produce SpaCBA pili and a 20 nm EPS layer, and could inhibit the growth of *Gardnerella vaginalis,* a bacterium commonly associated with BV. The genome sequences provide a basis for further elucidation of the molecular basis for their probiotic functions.

1. Introduction

Bacterial vaginosis (BV) is a condition, characterized by a shift in vaginal microbiota from a *Lactobacillus*-dominant microbiota to a mixed microbiota dominated by *Gardnerella vaginalis* and anaerobic bacteria (Dols et al., 2011; Srinivasan et al., 2012). BV affects millions of women annually and is associated with various serious gynaecological and obstetrical complications, including adverse pregnancy outcomes such as premature rupture of membranes, post-delivery endometriosis and preterm birth, pelvic inflammatory diseases and increased risk of acquisition of HIV-1, human papillomavirus and herpes simplex virus 2 (Cohen et al., 2012; Donati et al., 2010; Shin and Kaul, 2008).

Antibiotic therapy with metronidazole or clindamycin, oral or vaginal, is the currently recommended treatment for BV and has been shown to eliminate BV-associated bacteria and the treatment efficacy is believed to be high. However, meta-analysis shows that the expected cure rate after one month is 70–80% for metronidazole (Kane and Pierce, 2001) and 82% for clindamycin (Joesoef and Schmid, 2005). Moreover, the recurrence rate is high, as shown by a large number of treated cases experiencing relapses (Bradshaw et al., 2006). Since antimicrobial treatment of urogenital infections is not always effective, and problems remain due to recurrent infections, alternative remedies are needed.

Lactobacilli are since long recognised to be a major part of the

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normal vaginal microbiota. In the individual woman, the microbiota is usually dominated by one to three *Lactobacillus* species and recent studies using genotyping methods have shown that the most frequently isolated species are *L. crispatus, L. jensenii, L. gasseri*, *L. iners* or *L. vaginalis* (Pendharkar et al., 2013; Ravel et al., 2011; Vasquez et al., 2002). Lactobacilli adhere to vaginal epithelial cells (VEC) and are involved in maintaining the normal vaginal microbiota by preventing overgrowth of pathogenic and opportunistic organisms (Ronnqvist et al., 2006).

Since bacterial vaginosis is caused by an imbalance of the normal vaginal microbiota, a rational therapeutic strategy would be to supply selected lactic acid bacteria in order to restore a healthy microbiota (Bolton et al., 2008; Reid et al., 2004; Reid et al., 2003). To meet this challenge, the probiotic preparation EcoVag® was developed. EcoVag® vaginal capsules consist of *L. gasseri* DSM 14869 and *L. rhamnosus* DSM 14870 (1×10^8 CFU of each strain), prebiotic carbohydrates lactitol monohydrate and anhydrous glucose as well as other excipients. The formulation was developed as a single unit capsule dosage form to be administered easily once a day just before bedtime. A previous pilot clinical study showed that daily vaginal administration of EcoVag® capsules for seven consecutive days in patients with BV was adequate to eliminate the symptoms within two to eight days in 19 out of 21 of women (90%) diagnosed with BV (Stray-Pedersen et al., 2003, unpublished data). We have also shown that adjunct treatment with vaginal EcoVag® capsules can improve the antibiotic treatment of BV (Larsson et al., 2011; Larsson et al., 2008, Pendharkar et al., 2015).

In this study, we performed complete genome sequencing and further characterization of *L. rhamnosus* DSM 14870 and *L. gasseri* DSM 14869 in an attempt to identify possible mechanisms of action of their probiotic activity.

2. Materials and methods

2.1. Source of L. rhamnosus DSM 14870 and L. gasseri DSM 14869 strains

Lactobacillus strains were previously isolated from vaginal mucosal epithelial cells obtained from 26 Norwegian healthy and fertile women, with no vaginal infection or disease and who use no medication. The vaginal samples were collected during routine gynaecological examination by an experienced gynaecologist at the Department of Obstetrics and Gynecology, Levanger Hospital, Nord-Trøndelag county, Norway from 1995 to 1999. VEC were harvested using sterile cotton tips and transferred to sterile phosphate buffered saline (PBS, pH 7.2). The cells were washed twice with sterile saline, and the cell preparation with the adhered bacteria was seeded (using a streaking method) directly onto MRS agar plates acidified to pH 5.4 for selection of lactobacilli and incubated at 37 °C under anaerobic conditions for three days. Fifty-five *Lactobacillus* strains were isolated and tested with regard to decreasing the pH in growth culture medium and the 18 highest acid producer strains were further tested for growth in MRS broth, H_2O_2 production and inhibition of *G. vaginalis* (Fig. S1). Four strains fulfilled the condition of fast-growing in MRS broth and strong production (+ + +) of H_2O_2 (including *L. gasseri* DSM 14869) while a fifth one, *L. rhamnosus* DSM 14870, showed fast-growing capability in MRS and inhibition of *G. vaginalis* DSM 4944 *in vitro*. Among these five strains, *L. gasseri* DSM 14869 and *L. rhamnosus* DSM 14870 showed the best adherence to Caco-2 cells and vaginal epithelial cells. In this study, these two strains were further characterized at the genotypic and phenotypic level.

2.2. Genome sequencing

For DNA preparation, the two *Lactobacillus* strains were grown in MRS medium (OD_{600} 0.8). Two ml of culture was pelleted, washed twice with 50 mM Tris and resuspended in 50 mM Tris containing lysozyme (10 mg/ml) and mutanolysin (100 U/ml). After incubation for 1 h at 50 °C, the genomic DNA was extracted using the DNAeasy Blood and Tissue kit (Qiagen GmbH, Hilsen, Germany) according to the manufacturer's recommended protocol for Gram-positive bacteria.

Purified genomic DNA was sheared into smaller fragments to the desired size by sonication. Small (500 bp) and long insert (5–6 K) libraries were constructed according to the protocol provided by the manufacturer (Illumina) and paired-end sequencing was performed with the next-generation sequencing using Illumina HiSeq 2000 system (BGI, Shenzhen, China). The short reads were assembled into genome sequence using SOAPdenovo 1.05. Gaps were filled by direct sequencing of PCR products amplified with oligonucleotide primers designed to anneal to each end of neighboring contigs. The existence of plasmid (s) was predicted by GC depth analysis, aligment of the assembly results with the plasmid database by BLAST and the presence of circular sequences with a length corresponding to plasmids.

rRNA, tRNA and sRNA were predicted using rRNAmmer, tRNAscan and Rfam softwares respectively. Open reading frames (ORF) were predicted using the Glimmer 3.0 software. The corresponding functional annotation information were obtained using BLASTP alignment (identity $\geq 40\%$; E-value $\leq 10^{-5}$, match length ≥ 40 amino acids) between the gene sequences and the following databases: COG (clusters of orthologous groups of proteins), KEGG (Kyoto Encyclopedia of Genes and Genomes), SwissProt, TrEMBL, NCBI nr (National Center for Biotechnology Information, non redundant) database, and GO (gene ontology). Manual annotations were also determined by comparing all the predicted and translated ORFs with the public protein sequence database at NCBI using BLASTP with default parameter settings. Sequences of proteins of interest were also individually search against Pfam protein families database (Pfam 27.0, European Bioinformatics Institute (EMBL-EBI) and Conserved Domains Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) at NCBI. Genome visualization showing GC content, GC skew ((G-C)/(G + C)), rRNA, tRNA and the distribution of COG annotated gene was obtained by CGView.

Transposon sequences were predicted using RepeatMasker software (using Repbase database) and the RepeatProteinMasker software (using its database of transposable element protein) while tandem repeats were predicted by the Tandem Repeat Finder software. The presence of putative antibiotic resistance genes was determined by searching against the antibiotic resistance gene database (ARDB) at Center for Bioinformatics and Computational Biology, University of Maryland (http://ardb.cbcb.umd.edu/blast/genome.shtml). Possible genome islands were predicted using BLAT (standalone BLAT v. 34) by alignment between target genome and the genome island database originally extracted by the SIGI-HMM software. Prophages were predicted using BLAT comparison (standalone BLAT v. 34) between sequenced genome and a prophage database generated by Prophinder software and ACLAME database. The CRISPR Finder software was used to search for CRISPR direct repeats and spacers (Grissa et al., 2007). Synteny analysis between genome of sequenced and reference strains (*L. gasseri* ATCC 33323, *L. rhamnosus* GG and *L. rhamnosus* Lc705) was performed with the aid of MUMmer. Alignment at the amino acid level was analysed using BlastP m8 (identity $\geq 85\%$; e-value $\leq 10^{-5}$). Core Pan analysis was performed using real_CorePanGene.pl Version 2 at BGI. The complete genomes from the human intestinal isolate *L. rhamnosus* GG ATCC 53103 (FM179322), the dairy isolate *L. rhamnosus* Lc705 (NC_013199.1) and the human vaginal isolate *L. gasseri* ATCC 33323 (CP000413.1) were obtained from NCBI. At the time of preparation of materials for this publication, the only complete genome sequence of *L. gasseri* available was that for the type strain ATCC 33323.

Based on the results from a BLASTP search of the UniProtKB database, ORFs were assigned gene names according to the best protein homolog exceeding 40% amino acid identity and e-value less than 10^{-5}. The complete genomes of *L. gasseri* DSM 14869 and *L. rhamnosus* DSM 14870 were annotated using BLASTP search of the UniProtKB/Swiss-Prot and UniProtKB/TrEMBL databases and deposited in Genbank (National Center for Biotechnology Information).

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