JMBR: A Peer-review Journal of Biomedical Sciences
December 2014, Vol. 13 No.2 pp 27-33

MOLECULAR CHARACTERIZATION OF FIBRONECTIN-BINDING PROTEIN OF LACTOBACILLUS PENTOSUS AND LACTOBACILLUS PLANTARUM STRAINS

*KINGSLEY C ANUKAM, AND ABIODUN M EMOKPAE

ABSTRACT

The ability of lactobacilli to adhere to vaginal epithelial surfaces and intestinal tracts is believed to be important to allow colonization and host interaction for persistence. Bacterial adhesins molecules are proteins of which the human host fibronectin serves as a substrate for the attachment of bacteria and play a significant role in cellular processes. Lactobacilli, especially L. pentosus and L. plantarum strains are closely related in their fermentative characteristics and are versatile in colonizing both the human, dairy and plant micro-ecology. Bioinformatic tools were used to characterize the secondary and putative three-dimensional structures of fibronectin-binding proteins (FnBP) predicted in the genome of L. pentosus and L. plantarum strains. The FnBP gene tree and in silico PCR clearly shows that L. pentosus KCA1 fibronectin-binding protein can be distinguished from other pentosus and plantarum strains, thus suggesting a potential marker for differentiation. In addition, FnBP of L. pentosus KCA1 contains 56 leucine, and 36 valine residues, which is higher when compared with other selected strains. Also the number of carbon C (2862) and hydrogen H atoms (4551) are slightly higher than other strains, thus suggesting the uniqueness of the KCA1 FnBP. The 3-D model generated a QMEAN Z-score of -2.33, -2.74, -2.67, and -2.77 for L. pentosus KCA1, L. pentosus IG1, L. pentosus MP-10 and L. plantarum WCFS1 respectively. In vitro fibronectin-binding capability of L. pentosus KCA1 using a fibronectin-binding assay showed that L. pentosus KCA1 can effectively bind to anti-human fibronectin antibody.

INTRODUCTION

Lactobacilli have long been known as the quintessence of a constituent healthy vaginal and gut micro-ecology. Several studies have indicated that colonization of the host intestinal epithelium by lactobacilli requires adhesive properties necessary for persistence of a particular strain in the digestive track [1]. In general, bacterial organisms that occupy a particular niche in the host must bind with some form of appendages that enable them not to be eroded. The microbiota, including pathogens may have evolved with these adhesive mechanisms for colonization and survival [2]. Lactobacilli colonizing the vaginal mucosa are faced with the turbulent activities usually associated with menstrual cycle and other activities that disrupt the vaginal microbiome. Lactobacilli protect the urogenital tract from pathogen invasion and prevent urogenital and sexually transmitted diseases from manifesting [3]. Several mechanisms have been postulated including but not limited to (a) production of lactic acid, hydrogen peroxide (H_2O_2), bacteriocins, and biosurfactants that directly kill or inhibit

KEYWORDS: *Fibronectin-binding protein, Lactobacillus pentosus, vaginal colonization.*

Kingsley C Anukam, and Abiodun M Emokpae
Department of Medical Laboratory Science,
School of Basic Medical Sciences, University of Benin.

Correspondence

TWAS Genomics Research Unit,
Department of Medical Laboratory Science,
University of Benin
kanukam@gmail.com

bacterial and viral pathogens [4] (b) stimulation of host defense mechanisms against pathogens [5]; and (c) formation of microcolonies that adhere to the epithelial cell receptors and form a physical barrier to pathogen adhesion [6].

Most high-affinity bacterial adhesins molecules are proteins, and a major target for them is the host fibronectin [7]. Fibronectin is a large dimeric glycoprotein found in body fluids, present in soluble forms and on the surfaces of cells as fibrillar form and in the extracellular matrix (ECM). It functions to connect the intracellular cytoskeleton to the exterior ECM on which cells exist [8]. The ability to bind to the human protein fibronectin is a characteristic that has been reported for many pathogens [9], as well as key mediator for persistence of lactobacilli colonizing the human vaginal mucosa [10]. For example Staphylococcus aureus and Streptococcus pyogenes, encoded with Fibronectin-binding proteins (FnBPs) with related sequence organization have been reported to mediate bacterial adhesion to and invasion of host cells [11]. The binding of pathogenic Streptococcus pyogenes and Staphylococcus aureus to epithelial cells via fibronectin facilitates their internalization and systemic spread within the host [12]. Several authors have focused on FnBPs of pathogens for which recent structural data have significantly provided insight on the interactions in invasion and the role as virulence factors in infections.

Lactobacilli, especially L. pentosus and L. plantarum strains are closely related in their fermentative characteristics and phylogenetically related in terms of 16S rRNA nucleotide base sequence. As these two Lactobacillus species are very versatile in colonizing both the human, dairy and plant micro-ecology, it would seem logical to examine their genetic fingerprint that may be involved in adhesion. FnBP appear to be encoded in the genome of sequenced lactobacilli as the proteins can be extracted from the cell surface from both human and plant origin but the exact role is not yet understood. The purpose of this study was to characterize the secondary and putative three-dimensional structures of fibronectin-binding proteins predicted in the genome of L. pentosus and L. plantarum strains. In addition the FnBP capability of Lactobacillus pentosus KCA1, a newly sequenced strain isolated from the vagina of a healthy woman [13] with genetic open reading frames (ORF) known to encode FnBP was investigated in vitro.

MATERIALS AND METHODS

Bacterial organisms and Fibronectin-binding protein sequence alignments

Lactobacillus pentosus KCA1 was generously donated by TWAS Genomics research Unit of the Department of Medical Laboratory Science, University of Benin. Complete genome sequence and gene annotations of L. pentosus KCA1 have been deposited in the NCBI bioproject database (http://www.ncbi.nlm.nih.gov/bioproject/169225)

Open reading frame (ORF) coding for fibronectin-binding protein (FnBP) of Lactobacillus pentosus KCA1 was predicted with GeneMark software [14] and is available in the EMBL gene bank database (http://www.uniprot.org/taxonomy/1136177) .

Lactobacillus pentosus strains, such as L. pentosus IG1, and L. pentosus MP-10 are closely related to the reference L. pentosus KCA1, based on the gene trees of the three housekeeping genes (recA, dnaK, and pheS) [13]. The genome sequence and annotations of these strains are available in the gene bank database. Due to the 16S rRNA sequence

similarity between pentosus and plantarum strains, we selected eight L. plantarum strains based on product annotation hit (Fibronectin-binding protein), gene name, matrix score and E-value of zero and having 92% amino acid identity to Fibronectin-binding protein of L. pentosus KCA1 for comparisons. Basic Local Alignment Search Tool (BLAST) algorithm was utilized in the uniprot database (http://www.uniprot.org/BLASTp) for amino acid identity alignments. Multiple-sequence alignments and neighborjoining (NJ) trees were generated using the ClustalX program [15].

Fibronectin-binding gene cassette organizations

The genome sequence was deposited in the RAST (Rapid Annotation using Subsystem Technology) annotation pipeline with Taxonomy ID 1136177 (http://rast.nmpdr.org/) [16]. We searched through the features of L. pentosus KCA1, both graphically and through a table. Both allow quick navigation and filtering for features of interest. We searched for features in the RAST subsystem having a role in fibronectin-binding protein.

In silico PCR for distinguishing fibronectin-binding proteins of L. pentosus and L. plantarum strains.

Genomic information was retrieved from the EMBL database and NCBI microbial genome database. The nucleotide base sequences coding for the selected L. pentosus and L. plantarum strains fibronectin binding proteins were used for primer design. Both the forward and reverse primers including the internal oligonucleotide probes were selected with the primer3 software tool (http://primer3plus.com). The selected primers were imported into in-silico PCR online software (http://insilico.ehu.es/PCR/) that simulates against all sequenced genomes of Lactobacillus species available in the non-redundant database of the NCBI.

Protein Parameters

We used ProtParam, a bioinformatic tool which allows the computation of various physical and chemical parameters for a given protein stored in Swiss-prot or TrEMBL or for a user entered sequence. The computed parameters include the molecular weight, theoretical pI, (isoelectric point), amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (http://web.expasy.org/protparam/)[17].

Secondary and 3-D structure predictions

The secondary structures of the fibronectin-binding proteins were predicted with online PSIPRED [18] and Jpred3 [19] protein structure prediction servers. For the 3-D modeling, the amino acid sequences of the fibronectin-binding proteins were imported into Swiss-model server (http://swissmodel.expasy.org), which compares with non-redundant set of protein database (PDB) structures [20].

Fibronectin-binding assay

Binding of L. pentosus KCA1 to immobilized human fibronectin (Sigma) was performed in 96-well Polysorp plates (Nunc) with bacterial cells grown to late exponential phase (OD550 of 3.5-4; $1.2x10^9$ to $1.4x10^9$ cfu/ml) [12]. Plates were covered with 50 µg/ml of fibronectin in carbonate/bicarbonate buffer 50 mmol/L, pH 9.6 at 4°C overnight. Wells were washed three times with PBS and blocked for 1 h with PBS plus 1% Tween 20. One hundred µl of each strain were added to each well in PBS adjusted to an OD550nm of 1 ($7x10^8$ cfu/ml) and plates were incubated overnight at 4°C. After removing non-adhered cells by three washes with 200µl of PBS plus 0.05% Tween 20 (PBST), the plates were dried and adhered cells were detected by staining with crystal violet (1mg/ml for 45 min). After washing, the colorant was released with citrate buffer 50 mmol/L pH 4.0 (100µl per well) and the

absorbance at 600nm was determined in a Multiskan Ascent plate reader (Thermo-Labsystems,). Inhibition of binding by soluble fibronectin was assessed by adding different quantities of fibronectin (1 to 10 µg per well) to the binding assay described above. Blank wells without bound fibronectin were run as controls in all experiments and their absorbance values were subtracted from the values of wells covered with fibronectin. Experiments were carried out in triplicates with bacteria coming from independent cultures.

Results and Discussion

The important roles of Lactobacillus cell-surface proteins including fibronectin-binding proteins (FnBP) in governing interactions with the host environment, at the level of initial colonization, long-term persistence, and potentially the modulatory roles on both the innate and adaptive immune responses, and the rest of the microbiota by surface exclusion have been well established [21].

The translated amino acid query sequence of Fibronectin-Binding Protein (FnBP) of Lactobacillus pentosus KCA1 (KCA1_1548) gave a BLASTp hit with a score of 2927 in the uniprot database. The ORF for FnBP of L. pentosus KCA1 is 568 amino acids in length and the BLASTp hit produced ten similar organisms with the same number of amino acid sequences representing L. pentosus and L. plantarum strains as found in the non-redundant database of the NCBI. Interestingly, the amino acid sequence of L. pentosus KCA1 produced a hit with 99% identity to L. pentosus IG1 and MP-10 and 92% amino acid identity to the eight L. plantarum strains identified in the database (http://www.uniprot.org/BLASTp).

However, using the Clustal W algorithm (http://www.genome.jp/tools/clustalw/) the fibronectin-binding protein sequence alignment matrices (%) of the selected organisms differed slightly as shown in Table 1. Among the plantarum strains, with the exception of L. plantarum JDM1 and L. plantarum ZJ316, other strains have the same sequence identity. This can be visualized from the phylogenetic neighbour joining tree (NJT) demonstrating the relatedness of the L. plantarum strains (Figure 1) The gene tree clearly shows that L. pentosus KCA1 fibronectin binding protein can be distinguished from other pentosus strains as the relationships at the node is obviously resolved. This suggests that FnBP can be used as a distinguishing marker between L. pentosus KCA1 and other pentosus including L. plantarum strains. However, FnBP of L. pentosus KCA1 is closely related to the L. pentosus IG1 and MP-10 FnBP than to L. plantarum strains. This is similar to our previous findings where the gene trees of the three conserved (housekeeping) genes (recA, dnaK, pheS) suggests that L. pentosus KCA1 is closer to L. pentosus IG1 and L. pentosus MP- 10 with higher percentage identity than to L. plantarum WCFS1 housekeeping genes [13].

The primer sequences (Forward primer -F 5'-CCGAATTACGCCAAACCTTA-3' and Reverse primer –R 5'-TTGCAAATATTTCCGCATCA-3') for L. pentosus KCA1 FnBP amplification were different from L. pentosus IG1 and L. pentosus MP-10 strains. Interestingly both IG1 and MP-10 have the same primer sequence (Forward primer –F 5'-CAGAAAATTCCGGGATCTCA-3' and Reverse primer –R 5'-TGAGCTGTTCGACAAGTTGG-3') (Table 2). In silico PCR indicates that FnBP of L. pentosus KCA1 has 215 base pairs (bp) product, while the other pentosus and plantarum strains have 241 bp and 220 bp respectively (Figure 2). This suggests that FnBP is a potential marker for distinguishing these strains.

All the FnBP predicted had the same protein family identification Pfam ID (PF05833) with N terminus of the FnpA region occurring between 4-441 as shown in Figure 3 (green highlight) along the amino acid sequences [12]. The Domain of unknown function (DUF814) appears to occur in the region of 450-535 (yellow highlight) belonging to the Pfam ID05670 [22]. This domain contains a conserved motif D/E-X-W/Y-X-H that may be functionally important. However, all the FnBP predicted in these organisms belong to an atypical group of FnBP, which lack the secretion and cell-wall anchoring-LPXTG motif sequences present in other characterized FnBP [23]. Staphylococcus aureus MN8 was used for comparison, although this strain has 973 amino acid residues with two domain regions (780-818 and 819-850) for fibronectin binding. This strain is known to bind to fibronectin [24]. and it has only 10.2113% identity to the FnBP of L. pentosus KCA1.The chromosomal region of the focus gene (Fibronecin-binding protein) was compared with four similar organisms in the RAST subsystem. The graphic is centered on the focus gene, which is red and numbered 1 as shown in Figure 4. Sets of genes with similar sequence are grouped with the same number and color. Genes whose relative position is conserved in at least four other species are functionally coupled and share gray background boxes. The gene cassette containing fibronectin-binding protein in L. pentosus KCA1 has in close proximity a transcription regulator (KCA1_1549|Transcriptional regulator, MarR family), ABC transporter permease protein (KCA1_1547|ABC transporter, permease protein) and ATP-binding protein (KCA1_1546|ABC transporter, ATP-binding protein) similar to L. plantarum WCFS1 [25]

The protParam bioinformatic tool revealed individual amino acid frequency utilization in the biosynthesis of the FnBPs from the selected strains. For example, FnBP of L. pentosus KCA1 contains 56 leucine, 36 valine residues, which is higher when compared with other selected strains (Table 3). Also the number of carbon C (2862) and hydrogen atoms (4551) were slightly higher than other strains, thus suggesting the uniqueness of the KCA1 FnBP. However, only one tryptophan residue is utilized in all the strains, while there is absence of cysteine. The number of positively charged amino acid residues (Arginine-R and Lysine-K) is higher than the negatively charged residues (Aspartic acid-D and Glutamic acid-E) in all the strains. All the selected FnBP are predicted to be stable as shown by the instability index. It appears FnBP of L. pentosus strains are more stable than L. plantarum strains. A protein whose instability index is smaller than 40 is predicted as stable, a value above 40 predicts that the protein may be unstable [26].

The 3-D structure of FnBP was based on SWISS-MODEL Workspace [20], using a template protein ID-3doaA and a residue range 1-278 for the tested strains. FnBP of L. pentosus KCA1 has 32.74% sequence identity to the template, while IG1, MP-10, and WCFS1 have 32.74%, 32.38%, and 32.74% respectively. The model generated a QMEAN Z-score of -2.33, -2.74, -2.67, and -2.77 for KCA1, IG1, MP-10 and WCFS1 respectively (Figure 5). The QMEAN Z-score is based on a composite score consisting of four statistical potential terms involving carbon beta interaction energy, atom pair-wise energy, solvation and torsion angle energy [27].

The fibronectin/fibrinogen-binding protein FbpA (KCA1_1548) may be involved in adherence mechanisms in the vagina. A similar study in Lactobacillus iners AB-1 has demonstrated that its FnBP is a key mediator for colonization and persistence in the

vagina[10]. We determined in vitro the fibronectin-binding capability of L. pentosus KCA1 using a fibronectin-binding assay (Figure 6) and showed that L. pentosus KCA1 can effectively bind to anti-human fibronectin antibody.

Conclusion

The FnBP, which is a surface-associated protein, predicted in L. pentosus KCA1 has revealed unique genetic fingerprint that may be utilized for differentiation between other pentosus and plantarum strains. It may be involved in processes involving degradation and uptake of nutrients, communication with other vaginal microbes including the host cells and binding to substrates of the vaginal mucosa. The FnBP present in the genome of L. pentosus strain KCA1 suggests that this strain utilizes it, in addition to other adhesion molecules to adapt and persist in the vaginal environment, for maintaining urogenital health.

ACKNOWLEDGEMENTS

The research of Anukam K.C., is partly funded by the Third World Academy of Sciences (TWAS) under the RESEARCH GRANT AGREEMENT (RGA) No.09-017RG/BIO/AF/AC_G-UNESCOFR:3240230312.

REFERENCES

1. Collado MC, Surono IS, Meriluoto J, Salminen S. Potential probiotic characteristics of Lactobacillus and Enterococcus strains isolated from traditional dadih fermented milk against pathogen intestinal colonization. J Food Prot, 2007; 70: 700-705.

2. Ofek I, Hasty DL, Doyle RJ. Bacterial Adhesion to Animal Cells and Tissues. ASM Press, 2003; Washington, DC.

3. Sha BE, Zariffard MR, Wang QJ, Chen HY, Bremer J, Cohen MH, Spear GT. Female genital-tract HIV load correlates inversely with Lactobacillus species but positively with bacterial vaginosis and Mycoplasma hominis. J Infect Dis, 2005; 191:25–32.

4. Reid G, Bruce AW. Urogenital infections in women: can probiotics help? Postgrad Med J, 2003; 79:428–432.

5. Lebeer S, Vanderleyden J, De Keersmaecker SC. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol, 2010; 8:171–184.

6. Reid G, Burton J. Use of Lactobacillus to prevent infection by pathogenic bacteria. Microbes Infect, 2002; 4:319–324.

7. Pankov R, Yamada KM. Fibronectin at a glance. J Cell Sci, 2002; 115: 3861–3863

8. Henderson B, Nair S, Pallas J, Williams MA. Fibronectin: a multidomain host adhesin targeted by bacterial fibronectin-binding proteins. FEMS Microbiol Rev, 2011; 35, 147–200.

9. Joh D, Wann ER, Kreikemeyer B, Speziale P, Höök M. Role of fibronectin-binding MSCRAMMs in bacterial adherence and entry into mammalian cells. Matrix Biol, 1999; 18: 211–223

10. McMillan A, Macklaim J, Burton JP, Reid G. Adhesion of Lactobacillus iners AB-1 to human fibronectin: a key mediator for persistence in the vagina? Reprod. Sci, 2012; Nov 30.

11. Schwarz-Linek U, Höök M, Potts JR. The molecular basis of fibronectin-mediated bacterial adherence to host cells. Mol Microbiol, 2004; 52 (3): 631–641.

12. Christie J, McNab R, Jenkinson HF. Expression of fibronectin- binding protein FbpA modulates adhesion in Streptococcus gordonii. Microbiol, 2002; 148: 1615-1625

13. Anukam K, Macklaim JM, Gloor GB, Reid G, Boekhorst J, Renckens B, van Hijum SA, Siezen RJ. Genome sequence of Lactobacillus pentosus KCA1: vaginal isolate from a healthy premenopausal woman. PLoS One, 2013; 8:e59239.

14. Isono K, McIninch JD, Borodovsky M. Characteristic features of the nucleotide

sequences of yeast mitochondrial ribosomal protein genes as analyzed by computer program GeneMark. DNA Res, 1994; 1: 263–269.

15. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. Bioinformatics, 2007; 23:2947–2948

16. Aziz R, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST server:Rapid annotations using subsystems technology. BMC Genomics, 2008; 9: 75.

17. Gasteiger E., Hoogland C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D., Bairoch A.;(In) John M. Walker (ed): The Proteomics Protocols Handbook, Humana Press 2005); 571-607.

18. McGuffin LJ, Bryson K, Jones DT. The PSIPRED protein structure prediction server, Bioinformatics, 2000; 16: 404-405.

19. Cole C, Barber JD, Barton GJ (2008). The Jpred 3 secondary structure prediction server, Nucleic Acids Research, 2008; 36: W197-W201.

20. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. Bioinformatics, 2006; 22:195-201.

21. Kleerebezem M, Hols P, Bernard E, Rolain T, Zhou M, et al. The extracellular biology of the lactobacilli. FEMS Microbiol Rev, 2010; 34: 199–230.

22. Courtney HS, Li Y, Dale JB, Hasty DL. Cloning, sequencing, and expression of a fibronectin/fibrinogen-binding protein from group A streptococci. Infect Immun 1994;62:3937-3946

23. Jedrzejas MJ. Unveiling molecular mechanisms of bacterial surface proteins: Streptococcus pneumoniae as a model organism for structural studies. Cell Mol Life Sci, 2007; 64: 2799-2822

24. Rennermalm A, Li YH, Bohaufs L, Jarstrand C, Brauner A, Brennan FR, Flock JI. Antibodies against a truncated Staphylococcus aureus fibronectin-binding protein protect against dissemination of infection in the rat. Vaccine, 2001; 19:3376-3383

25. Siezen RJ, Francke C, Renckens B, Boekhorst J, Wels M, et al. Complete resequencing and reannotation of the Lactobacillus plantarum WCFS1 genome. J Bacteriol, 2012; 194(1): 195

26. Guruprasad K, Reddy BVB, Pandit MW. Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence. Protein Eng, 1990; 4:155-161

27. Benkert P, Biasini M, Schwede T. Toward the estimation of the absolute quality of individual protein structure models." Bioinformatics, 2011;27(3):343-50.