RadA new adhesin of *Ruminococcus gnavus E1*

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ABSTRACT

Ruminococcus gnavus E1 is a Gram positive strict anaerobic bacterium, isolated from the dominant microbiota of a healthy adult. A 6kb-long open reading fragment called *radA*, was identified on the strain E1 chromosome, close to the genetic clusters involved in the biosynthesis of bacteriocins RumA and RumC which are active against the pathogen *Clostridium perfringens*. *radA* has strong homologies with genes of *Staphylococcus aureus*, *Bacillus cereus* and *C. perfingens* encoding adhesins proteins of the family MSCRAMMs. The portion of *radA* gene encoding the 218 amino acids located at the N-terminal extremity of the putative mature protein was cloned in the vector pGEXT4, expressed in *Escherichia coli*. Tests performed by ELISA method showed that fusion protein GST-RadA218 present the adhesion to some components of the extracellular matrix: type 2 and 3mucus, the laminin, type I and IV collagen. Interaction with Caco2, HT29-MTX cells and the basal lamina was detected. The results suggest that RadA could play an important role in the colonization of the digestive ecosystem.

Key words: Microbiota, MSCRAMMs, adhesion, colization factor, adhesin

INTRODUCTION

E1 strain is a coccus fusiform Gram positive, anaerobic strict bacterium, isolated from fecal dominant microbiota of a healthy adult. It belongs to the species *Ruminococcus gnavus*, one of the components of the phylogenetic cluster *C. coccoides* [1]. Interesting fact is that this strain is able to produce active bacteriocins Ruminococcin A (Rum A) and Ruminococcin C (RumC) against the pathogen *Clostridium perfringens*. These two substances appear to have an important role in protecting the host. The colonization of the digestive ecosystem may involve several mechanisms such as competition for a substrate or ecological niche, but there are other strategies such as bacterial adhesion. The various components of the extracellular matrix (ECM) represent a major gateway to pathogens and commensal bacteria. Through these components, bacteria can reach the target cell or tissue [2]. It is present at all levels of the organism, its abundance and composition varies depending on tissues. The main ECM macromolecules are polysaccharides (glycosaminoglycans and proteoglycans) and fibrous proteins, of structure (collagen and elastin) or adhesion (Fibronectin and Laminin) which play an important role in cell-cell or cell-ECM interactions and bacteria-cell [3]. ECM-bacterium interactions involve specific proteins called "adhesin". Each bacterium is capable of expressing one or more adhesins on its surface, and each adhesin is specific of a component. Via this phenomenon of adhesion, pathogenic bacteria adhere and invade cells, which constitute the first step of the infection. This fixation can lead to adverse effects on the host cell, as in the case of toxin production. In some non-pathogenic, this adhesion allows colonization and the implantation of bacteria, as well as the protection of a target tissue such as the production of antimicrobial substances. The colonization is a mandatory step for the vast majority of pathogenic bacteria of the intestine. Colonization of an organ, such

as the intestine or colon, is especially dependent on bacterial adhesins because pathogenic bacteria must overcome the barrier effect exerted by saprophytic flora. Thus, the first contact with the eukaryotic cell can activate certain genes involved in pathogenicity. Several species of gram-negative bacilli (*enteropathogenic E. coli, Salmonella, Shigella, Yersinia spp, Moraxella catarrhalis, Haemophilus influenzae, Haemophilus ducreyi, Xanthomonas oryzae, Neisseria meningitidis and Bartonella henselae*) and can synthesize proteins that will interact with the components of ECM to reach their target tissues [4]. These bacteria belong to the group of Proteobacteria. In proteobacteria, several adhesins have been identified [4]: YadA (Yersinia spp), UspA1 and A2 (*Moraxella catarrhalis*), Hia and Hsf (*Haemophilus influenzae*) DSRA (*Haemophilus ducreyi*) Eib (*E. coli*), XadA (*Xanthomonas oryzae*), NadA (*Neisseria meningitidis*) and BadA (Bartonella henselae). All adhesins of this new group form a trimetric complex of a pacifier shape. An N-terminal part (the head), a central or intermediate part (rod) and a C-terminal "tail" [5]. Some adhesins expressed on the surface of Gram-positive bacteria are classified in a separate group as a new family of adhesion proteins called: Microbial Surface Components Recognizing Adhesives Matrix Molecules (MSCRAMMS) [6]. The MSCRAMMS are cell surface adhesins that recognize and specifically bind to different components of the extracellular matrix. These adhesion proteins are found and characterized only in Gram-positive bacteria [7]. They are for the most embedded in the bacterial peptidoglycan by covalent bonds [8]. Among the best characterized we may mention the model protein CNA of *S. aureus* involved in the binding to collagen, and other adhesins of *S. aureus* as FnB for (Fibronectin-binding protein) binding protein to fibronectin [9], CLF (fibrinogen binding protein), EbpS (elastin binding protein) or CNE of *Streptococcus equi* which binds to collagen [10])

Adhesins belonging to the family of MSCRAMMS have also been identified in other bacteria such as *Enterococcus faecium* [11], *Streptococcus mutans* [12], *Erysipelothrix rhusiopathiae* [13] and *Bacillus anthracis* [8]. Mature adhesins share an organization in several functional domains: a signal sequence at the N terminal extremity, region a rich in hydrophobic amino acids, which comprise a specific binding site of fixation and interaction with components of the ECM [8]. It is followed by repeated domains B whose number differs from an adhesion to another. At C-terminal position, an anchor is characterized by LPXTG motif or NPQTN which is cleaved by sortase which is cleaved by sortase which allows the anchoring of the protein at peptidoglycan level [7, 14]. Bacterial adhesion is generally regarded as a property directly related to the ability to colonize a given ecosystem. Very often adhesins have been characterized in pathogenic bacteria. Many studies have shown their importance in the infectivity, and in these organisms, they are considered as a pathogenicity factors. Few of them have been characterized in commensal gut bacteria. Few studies have been conducted on the role of bacterial adhesion in the beneficial effects of intestinal microbiota strains on human health with the exception of bifidobacteria and lactobacilli. The MUB protein (mucus-binding protein) is the first adhesion protein to mucus described in *Lactobacillus reuteri*, which was classified in the family of MSCRAMMS [15]. *Clostridium coccoides* group is one of the most important in the human microbiota. It represents 25-60% of the intestinal microbiota and includes genera such as *Butyrivibrio, Clostridium, Coprococcus, Dorea, Ruminococcus Eubacterium* [16, 17]. Upstream of cluster RumA, *radA* gene expression (6Kb) under specific conditions of the digestive tract (*in vivo*) was determined [18]. According to the bioinformatic study RadA protein has an organization in different domains identical to the domains identified in MSCRAMMS (Figure 01). Signal peptide consisting of 32 amino acids followed by A region with 414 hydrophobic amino acides , eight dominates Cna and at the end an anchor that has LPXTP motif different from those identified in other adhesins (LPXTG), the LPXTP would be a new anchoring motif [18]. The aim of the study was to demonstrate the functionality of RadA by determining its specific site of interaction with some ECM components: Mucus type I, II, III, collagen type I and IV, laminin, as well as Caco-2 and HT29 cells and the basal lamina. To locate the site involved in the adhesion of RadA to the ECM of a very accurately a portion located in the N-terminal position corresponding to a portion of the region A of *radA* gene is named RadA 218 (218 for the number of amino acids constituting this part of the RadA protein).

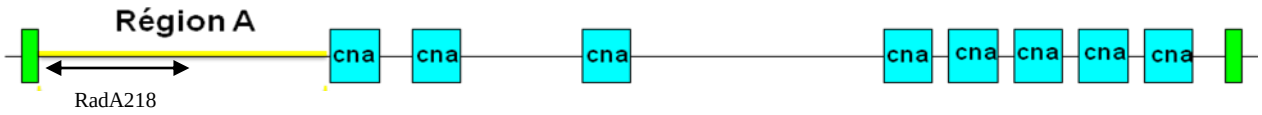

Fig 01 **:** General structure of RadA. : Hélices trasmembranaires , : Domaines Cna .[18]

MATERIALS AND METHODS

E1 strain is cultured anaerobically in a chamber type 'Freter 'in controlled atmosphere (85% N2, 10% H2, 5% CO2). They are incubated at 37 °C on BHI medium supplemented with yeast extract and hemin (BHI-YH). *E. coli* DH5 are cultivated aerobically at 37°C on Lauria-Bertani medium (LB DifcoTM Broth, Miller) agar or liquid at 37°C under agitation. For the selection of plasmids, the medium was LB supplemented with ampicillin at a concentration of 50mg/mL

PCR

PCRs were carried out on pure genomic DNA of *R. gnavus* E1 and on purified genetic constructions. 2 µM of specific primers 214Eco- 218Xho (Table 1), 2.5 mM of dNTPs, 5 U of Taq GoldR Ampli (Applied Biosystems) in the appropriate buffer. PCR reactions were performed in a thermocycler Master cycler personal (Eppendorf) under the following conditions: an activation step of the Ampli Taq (10min at 94°C), a denaturation step (30 sec at 94°C), a hybridization step (30 sec) of the primers to the matrix, an elongation step at 72°C (1 min). These cycles were followed by a post elongation step (10 min at 72°C). The chosen primers for PCR allows the amplification only of the region radA218 therefore without the signal peptide, which correspond to a fragment of 654pb. The PCR products were verified by sequencing.

Table 01: Primers used for PCR

Primers	Sequences
414Eco	5'-CCGGAATTCTTAGAACAGTCAGAGAATAAAGCG-3'
218Xho	5'-CCGCTCGAGTAATAAATATTCTCCGGTAAGATCACCCGG3'

Fusion protein construction

The amplification product of 654pb has been cloned in the vector pGEX-4T- 1 who has a GST label, followed by a thrombin site. The use of this plasmid allows the production of a fusion protein which will be easily purified. The cloning is a directed cloning, and the plasmid is digested with the two restriction enzymes *EcoRI* and *XhoI*. *radA218*, framed by the same restriction sites, was amplified with the primer pair 218Eco-218Xho. The ligation was realized using T4 DNA ligase under standard conditions. The transformation was performed in DH5α competent cells (In Vitrogen). The genetic constructs were verified by sequencing.

Recombinant proteins induction

Induction was performed in competent *E. coli* BL21DE3 (InVitrogen). Treated proteins are fusion proteins having a GST tag.

preculture

A transformed colony was resuspended in 2 ml tube containing LB supplemented with ampicillin and incubated at 37°C under agitation at 250 rpm overnight.

culture

In a 250 ml Erlenmeyer flask, 50 mL of LB-ampicillin medium was inoculated with 500µL of pre-culture incubated at 37° C with gentle agitationt until reaching an OD of approximately 0,6. When the OD is reached, 50 µL of IPTG (1M) were added, and the culture was incubated at 17°C under gentle agitation

overnight.

Extraction and purification of recombinant proteins

Purification kit

The supernatant is collected and the proteins are purified with **Novagen GST.Bind Kits TM** whose the principle is based on the use of an affinity column with a precast with a resin (GST-Bind Resin); The supernatant is contacted with the resin and the GST tagged proteins fixed on the beads. To remove impurities, successive washings were performed with washing buffer (GST / Bind / Wash Buffer: NaHPO4 43mm; 14.7 mM KH2PO4, 27 mM KHCl; pH 7.3). Bound proteins are detached with the elution buffer (Buffer GST Elution: 100mM reduced Glutathione dissolved in Reconstitution Buffer).

Protein assay according to Bradford

Protein concentration is determined by the method described by Bradford (1976) using bovine serum albumin (BSA) as stallion protein. Proteins were diluted to 1/200th in the Biorad protein assay solution. The proteins are assayed with a spectrophotometer (280 nm).

Electrophoresis on polyacrylamide gel in the presence of SDS and immunoblot

The total protein of each culture are separated by polyacrylamide gel electrophoresis under denaturing conditions, that is to say in the presence of SDS (SDS-PAGE). The resolving gel preparation is made with distilled water, 30% Acrylamide, 1M Tris (pH 8.8), 10% SDS, 10% APS and 0,008µl TEMED are added the last to polymerize gel. Distilled water and ethanol are added in order to balance the gel. The concentration gel was prepared in the same conditions with 1M Tris (pH 6.8), then poured over the gel so that the proteins are at the same level before being separated. After polymerization, the gel is placed in the electrophoresis apparatus with Tris / glycine/ SDS 1X buffer. Samples containing denaturation buffer (25µL Sample Buffer with SDS and a reducing agent, DTT - Di-Thio-threitol) were heated at 100°C for 5 min. Samples and the size marker 'Dual Color Standards' (BIO-RAD') are deposited on the gel and subjected to 150V DC for one hour. After migration of the proteins, the gel is placed directly in a staining solution (0.2% Coomassie Blue, 40% methanol, 10% acetic acid) for. It is then decolorized (10% acetic acid, 20% ethanol).

Adhesion Test: ELISA (Enzyme Linked ImmunoSorbent Assay)

Pure proteins Coating

The proteins used for adhesion tests: Mucus 1, 2, 3, collagen I and IV, laminin (Sigma).

The pure proteins (1 mg/mL) were diluted to obtain a final concentration of 40 µM in carbonate buffer (pH 9). 100 µL were deposited per well in 96-well plates MAXISORP (4 p mol per well). The coating was performed overnight at 4°C. The wells were subjected to three washes with PBS + 0.05% Tween

cell line

Two eukaryotic cell lines were used: Caco-2 (Human epithelial colorectal adenocarcinoma cells) is epithelial cell derived from a human colon carcinoma. In culture this line is organized in a monolayer. HT29-MTX: intestinal tumor cells. Basically the HT29-MTX are enterocytes which have been treated with methotrexate (MTX) to become goblet cells secreting mucus. The culture medium is DMEM glutamax (25 mM glucose) of GIBCO supplemented with 10% fetal calf serum (FCS) filtered at 0.2 microns with 1% of penicillin and streptomycin. Incubation of cells is performed under a humid atmosphere at 37°C in the presence of 5 to 10% CO2. Cells were seeded onto 96 wells (at 5000 cells / well) and cultured for 3-4 weeks to obtain differentiated cells. Aspiration of wells and cells fixation by 4% PFA for 20 min at room temperature. Cells underwent three PBS washes. Saturation or permeabilisassions cells was by PBS + BSA or gelatin 2% and Triton X100 0.1% (30 minutes).

Obtaining of basal lamina of HT29-MTX or Caco-2 human intestinal cells

Cells were seeded onto 96 wells (at 5000 cells / well) and cultured for 3-4 weeks. Cells stall in PBS (without

calcium) + X100 Triton 0.1% for 4h. Three PBS washes.

Detecting of the interaction Rad218 - pure protein / cell / basal blade

The wells were saturated with PBS and gelatin ,1h, at room temperature. Three washes with PBS + Tween 0.05% (250 uL). Incubation with Rad218 (0-40 pmol per well 100uL = 0 to 400 µM) for 2 h at 37°C. 3 washes PBS + Tween 0.05% (250µL). Incubation with primary antibodies (Rb) anti-GST 1: 200, 1h, room temperature, 100 µL diluted in PBS + BSA 2%. Three washes PBS + Tween 0.05% (250µL). incubation with secondary antibodies (Goat) anti-Rb IgG conjugated HRP 1: 5000, 1 h, room temperature, 100 uL diluted in PBS + BSA2%. Six washes PBS + Tween 0.05% (250µL). Adding HRP substrate (OPD, sigma) 100 uL per well, 30 minutes at room temperature. Stopping the reaction by adding 50 µL of H2SO4. DO reading at 490 nm.

RadA218 MUC2-competition with soluble proteins

Muc2 (10pmole) was coated and incubated for one hour with 40 pmol (400 µM) of soluble proteins at 37°C. Then Rad218 was coated at Muc2.

RadA218-MUC2 competition with lectins

Muc2 (10pmole) was coated and incubated with 10 ug of lectin (100 µg/mL, 100 uL) for 1h at 37°C before adding rad218 (2.5 pmole).

RESULTS

Verification of protein of interest production

Protein purification was performed using the Novagen GST.Bind TM Kit. The protein expected has an MMthéo ≈ 48.18 kDa. After analysis on SDS-PAGE gel, the presence of a band of about 48 kDa was detected, this band corresponds to the fusion protein RadA 218 (Figure 02)

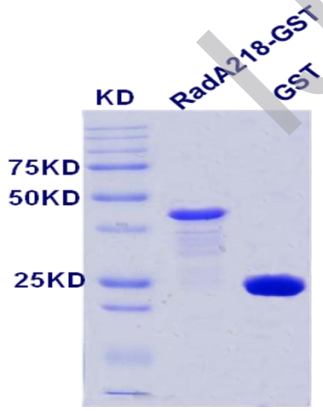

Fig 02 : Purification RadA218 affinity column

The peptide sequence Analysis

After migration, the fusion protein RadA218 was cut on SDS-PAGE gel and sent for analysis at the Pasteur Institute (Paris). The proteins are digested by the endolysin and the resulting peptides were separated by HPLC on DEAE-C18 column the entire peptide is sequenced and after the lysine no other amino acid is detected. Peptide sequencing allowed the obtaining of the following sequence:

SDLVPRGSPEFLEQSENK

↑ ↑

End of **GST** Beginning of **RadA**

Rad218 - Pure Protein Interaction

The OD measured is proportional to the specific interaction of RadA218 protein with different matrix constituents. The results indicate a significant signal in RadA218 reflecting its preferential adhesion in the following order: mucus type 2, laminin, mucus type 3, type IV collagen and collagen type I. no adhesion was observed for mucus type 1 .At the GST which is the negative control, no interaction was observed which proves that the GST is not involved in the fusion protein adhesion (Figure 03).

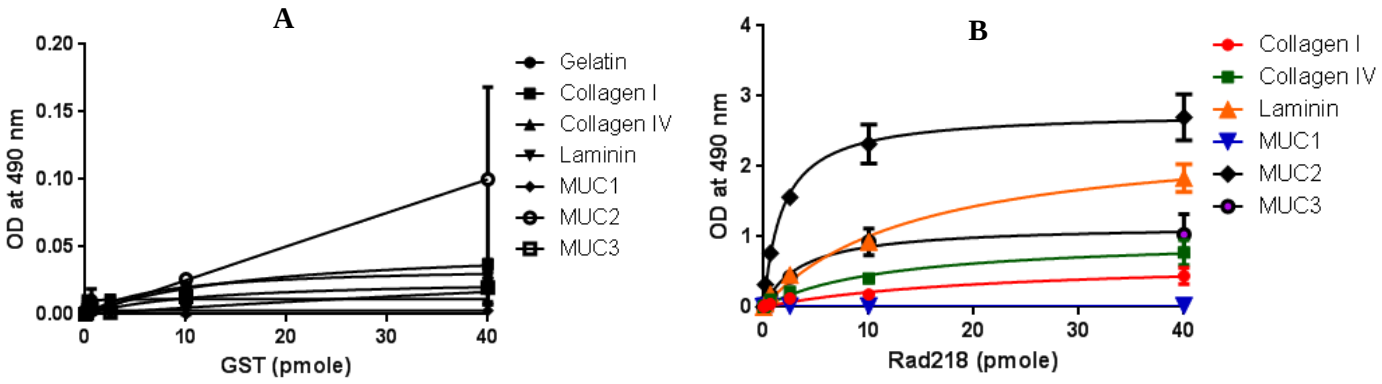

Fig03 : **(A)** : Interaction GST-ECM, **(B) :** Interaction RadA218-ECM. MUC1, 2, 3 : Mucus de type 1, 2, 3

As type I and IV collagen are proteins widely distributed in the body, which often were described as a gateways to the infectious process of Gram-positive pathogens [8], it remains weakly present in the intestine compared to mucus which is distributed on epithelial cells of the intestine offering a binding and colonization site with commensal bacteria as confirm the results (Figure 03). In the works of Zong and collaborators in 2005, who carried out the search for specific adhesion site of CAN, the A region was divided into three domains "N1, N2, N3." Constructing three fusions proteins was performed "CNA31-531, CNA31-344, CNA141-531" (Figure 04). Adhesion tests on these three proteins have revealed a low adhesion and CNA31-531 CNA141-531. However, CNA31-344 showed strong affinity for collagen. Thus, only a part of the region A interacts with collagen type I.

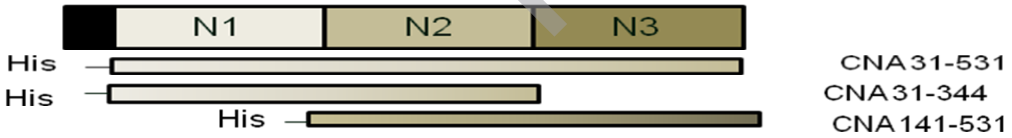

Fig 04: Schematic representation of the arrangement of the different constructions carriend in the region A [8].

Both N1 and N2 domains were cristallographied, and several amino acids have been identified as being necessary for this interaction. Initially, the collagen interacts with tyrosine (Y175) and Phenylalanine (F 191) of the domain N2; secondly, the Y88 and P108 of N1 domain bind to the M180 and P182 of the N2domain leading to a ring formation where collagen will be trapped. An almost similar organization to CNA was observed in RadA 218. Indeed, the positioning of amino acids is conserved (Figure 05).

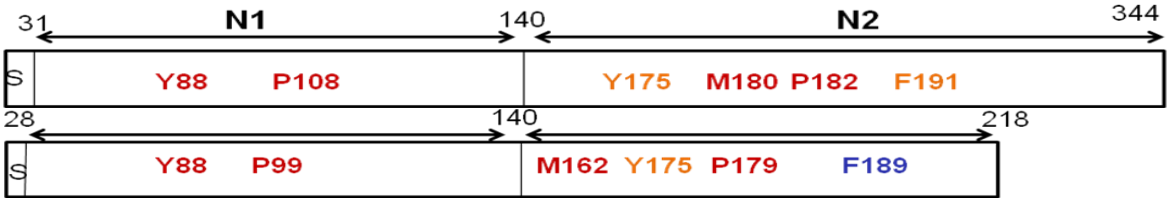

Fig 05: Comparison of the arrangement of amino acids in the two regions A of CNA and RadA. **S** : peptide signal.

CAN fixe collagen via a protein-protein interaction, the results obtained suggest the possibility that RadA218 interact equally with collagen by protein protein recognition.

Interaction Rad218 - Basal lamina of human intestinal cells:

RadA218 has an identical affinity for the basal lamina of the two types of cells human intestinal Caco-2 and HT29MTX. The GST was used as a negative control of adhesion (Figure 06).

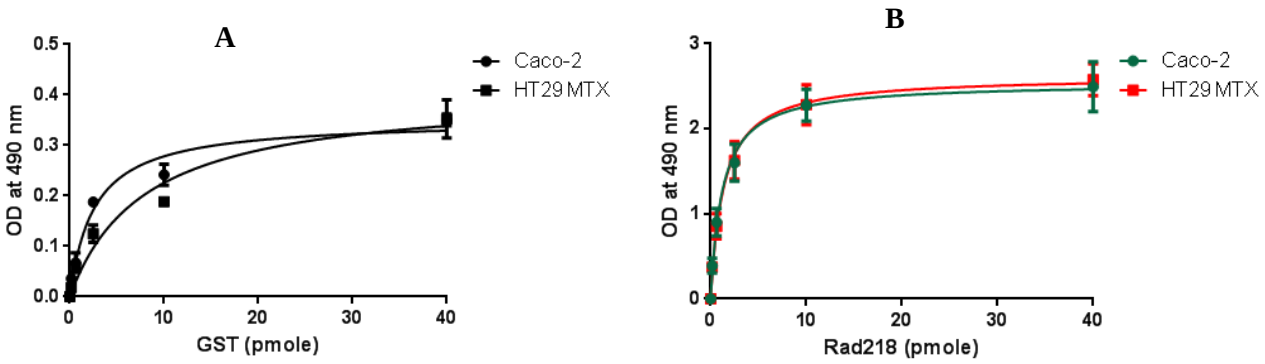

Fig 06: (**A**) : interaction GST –lame basale, (**B**) : interaction RadA218- lame

Rad218 - human intestinal cells interaction

From the graph (Figure 07), RadA218 interact preferentially with Caco-2 cells compared to HT29 cells. The GST was used as a negative control of interaction. Inhibition of the interaction-RadA218 MUC2

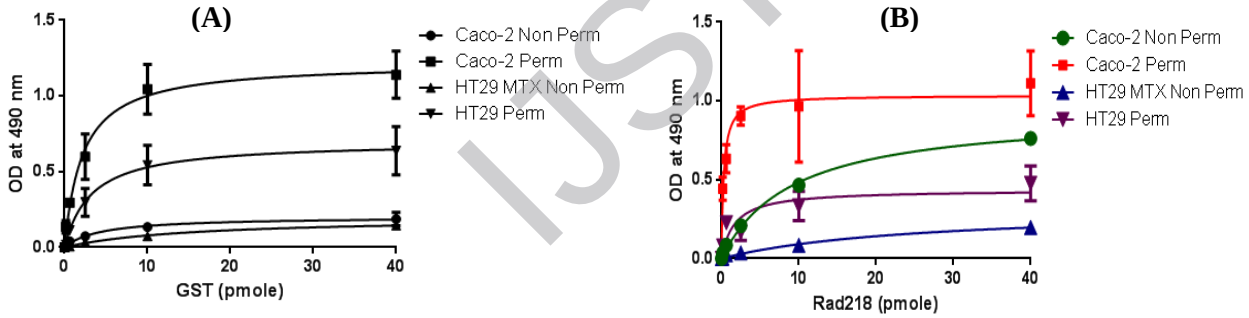

Fig 07: (**A**): interaction GST – permeabilized cells and not permeabilized, (**B**): interaction RadA218- permeabilized cells and not permeabilized

The inhibition of the adhesion Rad218-MUC2 by soluble proteins indicates a weak interaction of RadA218with the MEC in order: MUC2, Laminin, MUC3, Collagen I, and Collagen IV (Figure 08)

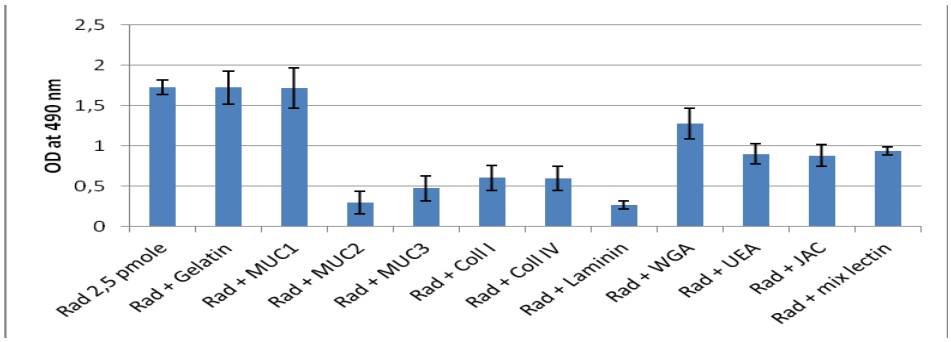

Fig 08: Inhibition by soluble protein or lectin binding RadA218 (2.5 pmol)-MUC2. UEA: Agglutinie of Ulex, JAC: Jacline lectine, WGA : Wheat germ agglutinin

Inhibition by lectins UEA is similar to those of JAC lectins and the lectins mixture. UEA has an affinity for the fucose, primary liaison specificity Fucα (1,2) Galβ and JAC lectin fixe the galactose (α pattern / /βGal), WGA has an N-acetyl-Glucosamin affinity. The results suggest the possibility that the RadA218 fixe fucos or galactose pattern.

DISCUSSION AND CONCLUSION

RadA is a putative adhesion protein of a *R. gnavus E1*, which comprises a very close structure to those of adhesins described in the literature. We have sought to highlight the functionality of RadA in determining its specific interaction with the ECM site. RadA is probably synthesized as a pre-protein. It features a 28 aa signal peptide at the N-terminal position which presents the pattern (Ala-X-Ala) [18] conserved in the exported Gram positive bacteria proteins and allows the addressing of the protein at the cytoplasmic membrane level. The N-terminal domain or A region is involved in the interaction with the ECM; cna repeated domains that are involved in the projection and the flexibility of the region A [6, 7, 10, 19]. The anchor located at the C-terminal position [20] is characterized by the presence of a pattern LPXTG recognized by type A sortase or a recognized pattern NPQTN not a type B sortase [22]. In the RadA, the anchor has been identified as a transmembrane helix located in C-terminal position which has a motif LPXTP. The collaboration with the laboratory of comparative genomics has allowed to identifie the presence of a gene encoding a B sortase downstream of radA, which seems surprising [18]. The mechanism of the interaction "collagen-adhesin" is done initially by hydrophobic bonds between one of the domains of the region A and its ligand. In RadA, we can assume that the collagen would bind with the Y88 (as CNA) and P99. The affinity that the collagen present to RadA may require a structural reorganization of the various domains of the region A leading to a conformation that would trap it . To answer this hypothesis, RadA218 could be divided into two domains (RadA218-140 / RadA141-218). The result would be obtained after adhesion test on collagen could provide information on the exact location of the adhesion site. It therefore becomes interesting to crystallize RadA28-140 and RadA141-218 in order to model the interaction of these two domains with collagen. Finally, site-directed mutagenesis on different residues "Y88, P99, Y175, M162, P179, F189" would confirm their role in the interaction of RadA218 with type I collagen. If the type I collagen is a protein widely distributed in the body, which has often been described as a gateway for the infectious process Gram positive pathogens [8], it remains weakly at the intestinal level compared to mucus which is distributed over the epithelial cells of the intestine offering a binding and colonization site with commensal bacteria. The results we obtained indicate that Rad218 interacts with the mucus, type 2 mucus,the laminin compared with collagen type I, IV and type 3 mucus. The results suggest that the recognition Rad218- MUC2 involves galactose and / or fucose residues, to confirm that a chemical deglycosylation results could highlight what are the carbohydrate patterns involved in the adhesion of the RadA218 to type 2mucus, laminin, basal lamina and cells. In *Staphylococcus aureus* a deglycosylation of laminin reduced the adhesive strength [22]. We would suggest that RadA would be synthesized in the cytoplasm as a preprotein then translocated through the cytoplasmic membrane, where the PS would be cleaved. The pattern LPXTP would then be recognized by B sortase which catalyze the hydrolysis of the peptide bond between Thr and Pro residues, generating an amide bond between Thr and the amine group of the Gly of peptidoglycan. Subsequently, the eight fields would project cna RadA414 to the ECM, allowing it to interact specifically with the latter. The specific site is located between $RadA_{28\text{-}140}$ and $RadA_{141\text{-}218}$. Initially ECM would bind specifically to $RadA_{141\text{-}218}$ by hydrophobic bonds, in a second time $RadA_{141\text{-}218}$ and $RadA_{28\text{-}140}$ interact together to form a loop where ECM is trapped (Figure 09).

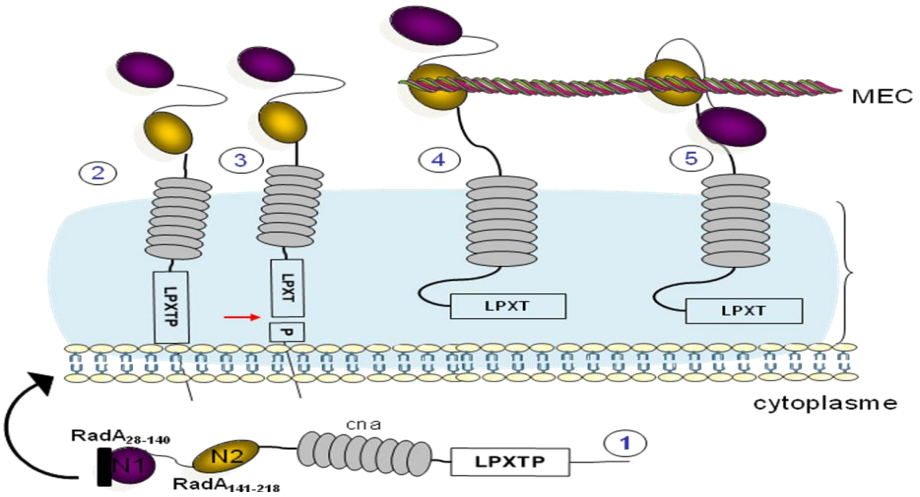

Fig 09**:** Interaction RadA with the ECM. (1) Synthesis of RadA, (2) Cleavage of PSet export RadA to the cytoplasmic membrane, (3): Action B sortase, (4) Anchorage peptidoglycan RadA141-218 and interaction with the ECM; (5): trapping RadA28-140 between ECM / RadA141-218

By analogy with the adhesins of the family MSCRAMMS expressed by pathogenic Gram-positive bacteria, it is likely that RadA actively involved in the colonizing ability of *R.gnavus E1*. Initially, *E1* express RadA, colonize the intestinal mucus, and at high cell density, express Ruma / RumC. The production of these antimicrobial substances could improve the ability of *R. gnavus* to colonize an ecological niche, than *E1* could compete for receptors on the intestinal epithelium cells surface which would prevent adhesion and therefore the entry of enteroinvasive pathogens bacteria within the epithelial cells. So the attachment to gastrointestinal surfaces allow them to exert their beneficial effect on the host's health. As the mucus is constantly renewed, and therefore the ability of bacteria to bind to the intestinal walls is generally considered an important factor in probiotic bacteria [15].

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