SHORT COMMUNICATION

O-2. INVOLVEMENT OF RESPIRATORY CHAIN IN BIOFILM FORMATION IN *PORPHYROMONAS GINGIVALIS*

J. Leclerc, B. Martin, Z. Tamanai-Shacoori, L. Le Pottier, H. Guyodo, V. Meuric and M. Bonnaure-Mallet.

EA1254 “Microbiologie – Risques infectieux”, Université Rennes 1, 2 avenue Prof. L. Bernard, Rennes, France. julia.leclerc@etu.univ-rouen.fr

Key words
Porphyromonas gingivalis - periodontal disease - ΔcydAB - Δfnr - biofilm formation

Introduction
Porphyromonas gingivalis (P.gingivalis) is a gram-negative strict-anaerobe bacterium and constitutes one of the main pathogen responsible for periodontal diseases (2). Periodontal diseases are chronic inflammatory infections induced by bacteria organised in biofilm. More than 500 bacteria species take part in this biofilm including P. gingivalis (3).
Although strict-anaerobe, P. gingivalis is able to endure the presence of low oxygen concentration. Previous in silico studies comparing on P. gingivalis and Escherichia coli metabolisms showed that some genes like cydAB (encoding for cytochrome bd) or fnr (fumarate nitrate reduction regulator) were involved in aerobic respiratory and aero-anaerobic switch respectively (1).
The present work is based on the creation of P. gingivalis mutants for the operon cydAB and the gene fnr and their characterization using various approaches: gene expression, growth studies and biofilm formation.

Materials and Methods
Strains and culture conditions:
P. gingivalis ATCC 33277 and its isogenic mutants were grown in anaerobic chamber (10 % CO2, 10 % H2 and 80 % N2). P. gingivalis and Streptococcus gordonii DL1 pCM18 strain (GFPmut3*) were grown in enriched brain heart infusion (BHI) medium and on Columbia 3 agar with horse blood enriched by yeast extract, hemin and menadione at 37°C.
Mutants construction:
The central part of the cydAB operon or the fnr gene was deleted and replaced by insertion of the Erythromycin cassette via double homologous recombination of the suicide vector pSK-erm.
Gene expression and polar effect:
Reverse transcription-PCR was used to amplify the different mutated genes and their flanking genes on total RNA extracted from P. gingivalis wild-type and mutants strains.
Growth:
The growth of each P. gingivalis strain was studied in anaerobic chamber at 37°C by measure of Optic Density at 600 nm over time.
Formation of static biofilm:
Formation of mono-bacterial (P. gingivalis) and bi-bacterial (P. gingivalis and S. gordonii) biofilms was studied using Biofilm Ring Test® method (Biofilm Control, France).
Formation of dynamic biofilm:
Formation of bi-bacterial biofilm formation with S. gordonii-GFP and different strains of P. gingivalis was analyzed under continuous flow on glass cover-slips (Ludin Chamber® Life Imaging Services). To identify P. gingivalis a specific immuno-staining was used. Biofilms were visualised by confocal microscopy (Leica DMI 6000 CS, Sp5) and images analysed and quantified with Image J and Comstat 2 associate with Matlab softwares.

Results
Gene expression studies showed that when fnr gene and cydAB operon were deleted, flanking genes bordering were still expressed

in the same way in the mutants as in the wild-type strains. Growth of mutant strains was not modified by the mutations as compared to the wild-type strain. Analysis of static bi-bacterial biofilm with P. gingivalis and S. gordonii did not show any difference between mutant strains and wild-type strain: biofilms were completely formed after 3 hours. Analysis of static mono-bacterial biofilm showed that mutant strains induced the formation of a biofilm after 5 hours whereas wild-type strain needed 7 hours. However, the biofilm formed by Δfnr strain seemed to start losing its structure after 7 hours. Quantification of P. gingivalis in dynamic biofilm (Table) showed that the Δfnr strain formed a thicker biofilm with higher biomass that wild type strain. ΔcydAB mutant strain seemed to have a similar behaviour to wild-type strain.

	WT-1	WT-2	WT-3	Cyd-1	Cyd-2	Cyd-3	Fnr-1	Fnr-2	Fnr-3
Total biomass ($\mu m^3/\mu m^2$)	6,5	2,9	7,1	5,6	5,8	7,0	4,2	12,2	14,1
Maximum thickness (μm)	33,2	30,2	37,3	34,2	29,2	40,3	49,3	74,5	130,9

Table: Quantification of P. gingivalis dynamic biofilm studied under flow conditions.
Biomasses attributed to P. gingivalis and maximal biofilm thicknesses were shown for wild-type and both mutants strains (ΔcydAB and Δfnr) in three different confocal image acquisitions after 24 hours of biofilm formation. Biofilms were fixed by 3.7% formaldehyde before observation.

Discussion

Both mutants were constructed and confirmed by RT-PCR and there was no polar effect on genes flanking the mutation. The mutations had no effect on bacterial growth in anaerobic condition as expected. However, these genes may be involved in bacterial growth under variable nutrients or in the presence of few quantity of oxygen. Thus future experiment will be performed in different conditions like oxygen presence and in modified culture media.

In static biofilm studies, the acceleration of biofilm production for mutant strains could be explained by an influence of oxido-reduction status of the bacterium that could modify gene expression implicated in virulence such as adhesion capacities (a mechanism already shown for fimbriae for example (4)). To be closer to in vivo conditions we used the dynamic biofilm model studies and showed that in the presence of S. gordonii (a primary coloniser in periodontal biofilms) Δfnr strain made a thicker biofilm. The putative influence of this transcriptional regulator Fnr on gene involved in P. gingivalis respiration and growth could explain the higher volume found in Δfnr multi-bacteria biofilms. For the ΔcydAB strain, there is no observable difference on dynamic biofilm formation with wild-type strain.

Conclusion

As Fnr is a transcriptional factor, the effect of its mutation seems to be more pleiotropic compared to the mutation of cydAB, which is only involved in oxygen metabolism. These preliminary results give us a better understanding of factors involved in biofilm formation during periodontal diseases depending on P. gingivalis. These preliminary results should be repeated and require further studies in order to understand the precise role of genes in P. gingivalis metabolism.

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