Studies on the Stress Response in *Fusobacterium nucleatum*.

A Thesis submitted in fulfillment of the requirements for admission to the degree of Doctor of Philosophy

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Errata sheet for PhD thesis entitled, Studies on the Stress Response in *Fusobacterium nucleatum*.

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ABSTRACT

Fusobacterium nucleatum is a saccharolytic Gram-negative anaerobic organism belonging to the so-called 'orange complex' which is believed to play an important role in the microbial succession associated with the pathogenesis of periodontal disease. Its genome contains niche-specific genes shared with the other inhabitants of dental plaque, which may help to explain its ability to survive and grow in the changing environmental conditions experienced in the gingival sulcus during the progression from health to disease. The pH of the gingival sulcus increases during the development of periodontitis and is thought to occur by the metabolism of nutrients supplied by gingival crevicular fluid. Studies have shown that *F. nucleatum* is partly responsible for the rise in pH and have concluded that in comparison to other plaque inhabitants, *F. nucleatum* has the greatest ability to neutralise acidic environments. In common with a number of other oral bacteria, *F. nucleatum* has also been shown to produce intracellular polyglucose (IP) from simple sugars such as glucose, galactose and fructose. Its response and adaptation to stressful environmental conditions such as pH is unknown. The overall aim of this study was, therefore, to determine how *F. nucleatum* copes with environmental stresses induced by pH changes.

F. nucleatum was grown by continuous culture in a chemically defined medium at a growth rate corresponding to those measured *in vivo*. The effect on protein expression, and IP synthesis was examined during steady-state growth at high (>7.2<7.8) or low pH (pH 6.4). The present study also investigated the response of *F. nucleatum* to growth at pH 8.2. It was found that the organism grew as a biofilm and this corresponded with an increase in cellular hydrophobicity and decreased IP levels.

Optimal growth pH's differed between the different sub-species used in this study. In response to pH stress, *F. nucleatum* changed its amino acid and glucose utilisation and increased IP synthesis at the expense of cell numbers. Pulsing the chemostat with glutamic acid or serine produced an increase in IP synthesis and the pattern of end-products observed was dependent upon the amino acid being fermented. The effect on IP synthesis in response to increased levels of exogenous fermentable amino acids was also compared during concomitant fructose or glucose fermentation. Growth media containing fermentable amino acids and supplemented with fructose produced higher cell numbers and non-detectable levels of IP compared to media containing glucose.

The differential expression of cytoplasmic- and cell envelope-proteins induced by changes in pH were identified by two-dimensional gel electrophoresis. The results represent the first proteomic investigation of *F. nucleatum*. Twenty-two cytoplasmic proteins were found to have altered expression in response to external pH. At low (sub-optimal) pH, proteins associated with the generation of ATP and ammonia were up-regulated, the latter contributing to the alkalinisation of the gingival sulcus. Conversely, neutral to alkaline pH conditions led to the up-regulation of enzymes involved in energy storage. The study also identified several proteins associated with iron limitation and fatty acid synthesis which might not otherwise have been identified as part of the pH-dependent response.

In response to growth at pH 7.8, 14 cell envelope proteins were identified as having significantly altered expression. Down-regulated proteins included those associated with uptake of C4 di-carboxylates and phosphorus, a potential membrane protease and an enzyme associated with amino acid fermentation. The up-regulation of a transcriptional regulator linked to the repression of sugar metabolism was also reported along with proteins linked to the transport of

iron. The periplasmic chaperone, peptidyl prolyl cis trans isomerase, which is responsible for the folding of outer membrane proteins, was also found to be up-regulated.

In conclusion, the proteomic investigation of protein expression by *F. nucleatum* identified gene products which form part of the organism's coordinated stress response to changes in environmental pH. In addition to these, the physiological based studies also presented help to explain the organism's persistence during the transition from health to disease *in vivo*.

SIGNED STATEMENT

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* Growth pH and transient increases in amino acid availability influence polyglucose synthesis by *Fusobacterium nucleatum*. Zilm, P.S. Gully, N.J. and Rogers, A.H. FEMS Microbiol Letts. **215** (2002) 203-208.

Changes in growth and polyglucose synthesis in response to fructose metabolism by *Fusobacterium nucleatum* grown in continuous culture. Zilm, P.S., Gully, N.J. and Rogers, A.H. Oral Micro. Immunol. **18** (2003) 260-262.

The Proteomic profile of *Fusobacterium nucleatum* is regulated by growth pH. P.S. Zilm, C.J. Bagley, A. H. Rogers, I.R. Milne and N. J. Gully. Microbiology. 2007, **153**:148-159

Co-adhesion and biofilm formation by Fusobacterium nucleatum in response to growth pH. P.S Zilm and A.H Rogers. (2007) Anaerobe **13:**146-152

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