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Studies on fusobacteria associated with periodontal diseases

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Abstract

The physiological and metabolic characteristics of representative isolates of the various subspecies of *Fusobacterium nucleatum* were investigated by growing them in continuous culture in chemically-defined media. Behaving almost identically, these organisms were found to obtain energy from the fermentation of simple carbohydrates such as glucose or fructose or from the fermentation of certain amino acids, free or in the form of small peptides. The latter can be attacked by aminopeptidase activity which was shown to be essential for the growth of the organism in an environment lacking fermentable carbohydrate and free amino acids but replete with small peptides. This metabolic versatility may explain the presence of *F. nucleatum* in both supra- and sub-gingival dental plaque and why it is often found together with organisms such as *Porphyromonas gingivalis* which display powerful endopeptidase activities. Using the technique of allozyme electrophoresis, the current subspeciation of *F. nucleatum* was shown to be of doubtful validity and evidence, based upon physiological and metabolic properties, for differences in pathogenicity between isolates was not detected. While this organism is a member of various bacterial consortia associated with periodontal diseases, its contribution to the disease process remains unclear.

Key words: *Fusobacterium nucleatum,* growth characteristics, metabolism, periodontal diseases.

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Introduction

The bacterial species *Fusobacterium nucleatum* is one of the most frequently detected cultivable organisms in subgingival dental plaque from both inactive and active gingivitis and periodontitis sites.[1-4] Moore and Moore concluded that *F. nucleatum* is the most frequent cause of gingival inflammation that initiates periodontal disease and that it is the most common predominant pathogen in subsequent periodontal destruction.[4] Moreover, it is found in a number of extra-oral sites where, together with other organisms, it causes polymicrobial infections.[5]

Despite some confusion surrounding their validity, the heterogeneous collection of bacteria characterized as *F. nucleatum* has been divided into a number of subspecies; namely, subspecies *nucleatum, vincentii, polymorphum, fusiforme* and *animalis,* the first two of which are believed to be associated with sites of periodontal disease.[6-8] In an attempt to explain differences in pathogenicity, studies in this laboratory have accordingly focused on various aspects of the physiology and metabolism of the Type strain and a clinical isolate from within each of the putative sub-species.

The growth and metabolism of *Fusobacterium nucleatum*

The growth and nutritional aspects of the metabolism of the various strains of *F. nucleatum* were studied by growing them under continuous culture conditions in a chemostat using methods described previously.[9] Briefly, the growth medium was a filter-sterilized chemically-defined medium (CDM). It contained a range of amino acids, a number of vitamins, nucleotides, salts, trace elements and a fermentable carbohydrate such as glucose or fructose.[10] Tween 80 was added to aid cell dispersion, as was thioglycollic acid to maintain a low redox potential. The growth temperature was 37°C, the pH controlled by the automatic addition of either 2 mol/L KOH or 2 mol/L HCl and anaerobic conditions maintained by gassing both the culture vessel and medium reservoir with a N_2/CO_2 (90:10) mixture. Under varying conditions of growth rate and pH, growth parameters such as biomass – as measured by cell dry mass and protein content – and metabolic end-products were determined.[9] All strains showed similar physiological and metabolic properties. For example, they grew well in various CDMs, with or without added carbohydrate, over a pH range of about 6 to 8, the optimum being

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between 7.0 and 8.0. In the absence of fermentable carbohydrate – most likely to occur in the subgingival environment – energy and carbon were obtained from the fermentation of the amino acids glutamate (Glu), histidine (His), lysine (Lys) and serine (Ser). Most strains appeared to be auxotrophic for His and some for Lys. The end-products of fermentation were acetate:butyrate:formate (3:2:1), irrespective of growth rate. It is worth noting here that butyrate was shown to be a potent *in vitro* inhibitor of gingival fibroblast proliferation and could, therefore, be a virulence factor.[11] In terms of interbacterial nutritional co-operation – a key to explaining the co-existence of dental plaque bacteria – the formate produced by *F. nucleatum* could act as an electron donor for *Wolinella recta* with acetate performing the same function for Eubacterium species.

When grown under a variety of physiological conditions in CDM containing carbohydrate (glucose) as well as amino acids, individual strains behaved somewhat differently. For example, one strain utilized 30-80 per cent of the available glucose, converting it to lactate as well as the acids noted above, but converting little to intracellular polyglucose (IP). Another strain, depending upon growth pH, utilized 40-65 per cent of the glucose, converting a significant proportion to IP. When grown in a CDM in which glucose was the primary energy source, all strains responded in a similar fashion; that is, most of the glucose was consumed and converted principally to lactate and butyrate with virtually no IP production. The significance of IP production and the conditions under which it is produced were investigated in more detail (see below).

Useful insights into the efficiency with which various substrates provide energy for biomass production can be obtained by measuring the rate of substrate consumption over a range of dilution (growth) rates. Various kinetic parameters were calculated by the methods of Pirt[12] and Herbert and Kornberg,[13] after determining the specific rate of glucose or amino acid consumption (q_s) in terms of mmol of substrate consumed/mg dry mass of cells/hour. The best-fit line of q_s versus D (dilution rate, mL/h) was plotted by regression analysis; the reciprocal of the gradient is equivalent to the maximum growth yield (Y_{max}) and the intercept is the maintenance energy coefficient (*m*). Maintenance energy is required for specific maintenance functions of the cell; for example, turnover of cell materials and osmotic work. The values of Y_{max} and *m* for cells grown on amino acids were about 20 g dry mass/mol and 0.80 mmol/g dry mass/h, respectively. Corresponding values for cells grown on glucose were about 65 g dry mass/mol and 0.40 mmol/g dry mass/h respectively. The data in terms of maximum growth yields (Y_{max}) accurately reflect the fact that the fermentation of amino acids yields only 1 ATP/mol whereas the mixed-acid fermentation of glucose under carbohydrate-limiting conditions yields 3 ATP/mol of substrate consumed.[14]

The breakdown and utilization of peptides

The low levels of free amino acids usually present in the oral environment would probably be insufficient to sustain the growth of Gram-negative anaerobes, including *F. nucleatum*, that obtain energy from the fermentation of amino acids. Since it lacks endopeptidase activities, *F. nucleatum* will not grow on proteins such as casein or albumin,[9] but organisms such as *Porphyromonas gingivalis*, which is often found together with *F. nucleatum* in active disease sites, does possess such activities.[15] Provided that they contained the appropriate residues, resultant peptides would thus be potential energy sources for *F. nucleatum*. Accordingly, the ability of resting cells of *F. nucleatum* to attack unsubstituted peptides containing the appropriate residues – present C- or N-terminally or buried in the peptides – was investigated. The ability of growing cells to utilize an essentially amino acid-free peptide fraction prepared from a commercial peptone was also studied. Resting cells were found to cleave all of the 19 tested peptides, containing 3-6 residues, and the four key energy-yielding amino acids – Glu, His, Ser and Lys – were rapidly taken up. Moreover, the peptone-derived hydrophilic peptide fraction, rich in Glu and Asp, promoted growth and peptides smaller than 1 kDa were rapidly utilized. The cleaved residues metabolized were those previously shown to limit growth in CDM, namely Glu, Ser, His and Lys.[16] Findings such as these may partly explain why *P. gingivalis* and *F. nucleatum* frequently co-exist in periodontally-diseased sites.[17]

The isolation, purification and properties of an (amino) peptidase from F. nucleatum

The fact that *F. nucleatum* can cleave small peptides and utilize the released residues for growth, indicates that exo (amino) peptidase activities are of paramount importance from a nutritional viewpoint. Accordingly, attempts were made to isolate, purify and characterize such activities. The organism was grown in a medium rich in various peptones in the chemostat at a dilution rate of 0.08 h^{-1} under anaerobic conditions as described above. Cells were collected from the overflow at 4°C and then stored at -80°C. After thawing, cells were disrupted in a French Pressure Cell and then centrifuged to remove unbroken cells and cell débris. The supernatant (crude extract) was then freeze-dried and stored dry at 4°C. After treatment of the crude

extract with protamine sulphate to remove nucleic acids, the (amino) peptidase activity (AP) was partially purified by ammonium sulphate fractionation followed by hydrophobic interactive chomatography, ion exchange chromatography and isoelectric focusing. The AP was found to have a molecular mass of about 54 kDa, a pI of 5.1 and displayed optimal activity at pH 7.5 against a wide range of small peptides. It was cobalt-activated and completely inhibited by the metal ion chelators EDTA and 1, 10-phenanthroline and by bestatin; the enzyme thus appears to be a typical metallo (amino) peptidase. It was isolated from *F. nucleatum* strains ATCC 10953 and ATCC 25586. Preliminary studies on the AP from the former strain indicated a high degree of N-terminal amino acid sequence homology with Pep M from S*almonella typhimurium.* Growth of *F. nucleatum* in a peptone-based medium, containing low levels of free amino acids, would depend upon AP activity while growth in the carbohydrate-free CDM would not. Bestatin, already shown to be a potent inhibitor of the AP activity, was indeed found to inhibit the growth of both *F. nucleatum* isolates in the former but not in the latter medium. This supports the notion that AP activities play a key role in the nutrition of *F. nucleatum* in environments, such as the gingival sulcus, in which little or no free amino acids or fermentable carbohydrate are available. Compounds such as bestatin might prove useful in periodontal therapy in the sense that they can combat the growth of asaccharolytic Gram-negative anaerobes, such as the putative periodontopathogen *P. gingivalis*.

The significance of intracellular polyglucose (IP) production

The microbial communities of which dental plaque is comprised are frequently nutrient limited and must be able to survive feast or famine or fast or famine conditions.[18] One of the factors involved in this complex survival phenomenon is the possession of reserve materials which may aid starvation-survival by acting as reserves of carbon and energy.[19] In common with a number of other oral bacteria, *F. nucleatum* produces, under suitable growth conditions, intracellular glycogen-like polyglucans (IP) from various simple sugars.[9] The organism was grown at a dilution rate of D=0.065 h^{-1} in a CDM containing 20 mmol/L glucose, under which conditions it produced large amounts of IP. Aliquots of this culture were starved under anaerobic conditions and assayed at various times for IP content and viability. Treated in the same fashion were cells grown in a CDM lacking fermentable carbohydrate and in which the organism produced no IP. Both cultures had 50 per cent survival times of about 1.5h and were not eliminated even after 32 h of starvation. From these data it was concluded that IP does not influence starvation-survival. However, cell history was also shown to be important since cells grown at D=0.12h^{-1} were completely eliminated after 8-16 h of starvation while cells grown at the slower rate of D=0.048 h^{-1} were not completely eliminated even after 32 h.[20]

Concurrent with the above experiments, the influence of environmental factors, notably pH, on IP formation was also investigated. The organism was grown in a CDM containing 20 mmol/L glucose at pHs of 5.8, 6.7 and 7.8 and at a e_{rel}=0.5; that is, at a dilution rate equivalent to 50 per cent of e_{max}. At steady-state conditions, culture samples were removed; cells assayed for protein and IP content and culture filtrates assayed for acidic end-products and residual glucose. In a separate set of experiments, cultures growing at pH 6.7 were exposed to transient excesses ('pulses') of either Glu or Ser. At various time intervals following the pulse, culture samples were removed and the above parameters assayed.

The biomass at the growth pH of 5.8 was only about 4 per cent of that obtained at pH 6.7; only 15 per cent of the available glucose was utilized and the proportion of lactate, compared with the major end-products acetate and butyrate, was greatly reduced. The IP level (per unit biomass) was, however, increased ten-fold. At the high growth pH of 7.8 the biomass was reduced to about 80 per cent of that obtained at pH 6.7; some 65 per cent of the available glucose was dissimilated and although the relative amounts of acetate, butyrate and formate were almost identical to those produced during growth at pH 6.7, the proportion of lactate was more than doubled. The IP level, as at pH 5.8, was increased ten-fold. None of these cultures was limited for glucose but they were amino acid-limited, as evidenced by the changes produced following pulsing with either Ser or Glu. For example, the pulse with Ser produced a two-fold biomass increase in 5 h. The IP level also increased 4- to 5-fold with a concomitant fall in lactate production. Eventually, the IP was catabolized and this resulted in increased lactate production. As a result of both IP and Ser metabolism, the other acidic end-products also increased, returning to steady-state levels about 80 h post-pulsing. The data indicated that the organism was stressed at growth pHs of 5.8 and 7.8. While the consumption of glucose was unaffected at high pH, it was greatly reduced at low pH. However, in both instances, there was a marked increase in IP, reflecting a conversion of glucose-6-phosphate to glucose-1-phosphate and subsequent IP synthesis. At pH 7.8, the elevated lactate level indicates that glucose is channelled through the glycolytic pathway to produce energy as well as conversion to IP. The results of pulsing experiments indicate that when the

amino acid (energy) limitation is relieved by the addition of either Ser or Glu, there is a marked biomass increase due mainly to the fermentation of these substrates and this appears to 'release' glucose for IP synthesis. As the added amino acid is used up, subsequent IP catabolism makes energy available through glycolysis. Overall, it may be concluded that glucose can be diverted into IP synthesis by stress in the form of unfavourable environmental pH or by the transient relief of (fermentable) amino acid limitation. While the synthesis of IP may thus be a response to stress, this storage does not, as detailed above, appear to aid starvation-survival.

The validity of the current sub-speciation of F. nucleatum

As already noted, isolates characterized as *F. nucleatum* show a high degree of heterogeneity as revealed by techniques such as pyrolysis mass spectrometry[21] and ribosomal RNA gene restriction patterns.[22] While at least four subspecies have been proposed, there is confusion surrounding such subspeciation. For this reason, a number of Type strains and clinical isolates of *F. nucleatum* were subjected to the biochemical technique of allozyme electrophoresis (multilocus enzyme electrophoresis) which has successfully been used to type bacterial isolates and to determine the evolutionary relationships between other bacterial species and subspecies.[23] Briefly, the organisms were grown anaerobically at 37°C on blood agar for 2-3 days and the harvested cells stored at -80°C. After the addition of lysis buffer to the thawed cells, these suspensions were ultrasonically disrupted, centrifuged and the supernatants (cell lysates) loaded onto a cellulose acetate support medium. After electrophoresis, enzymes were stained and up to 21 of them exhibited sufficient staining intensity, resolution and separation to enable reliable genetic interpretation.[24] Forty-four isolates were eventually examined and three distinct genetic clusters were identified; one cluster consisted exclusively of extra-oral isolates, a second cluster consisted predominantly of oral isolates, and the third cluster consisted of a single human oral isolate. A number of anomalous results were obtained; for example, isolates Fev-1, ATCC 10953,^{T} ATCC 49256,^{T} 10446 and 10772 all belonged to a single genetic cluster. However, according to previous classification,[6-8] the first three of these isolates belong to the subspecies *nucleatum, polymorphum* and *vincentii,* respectively and strains ATCC 10953 and ATCC 49256 are the Type strains of their respective subspecies. These results cast doubt on the validity of the current subspeciation and suggest that an extensive epidemiological study should be undertaken to redefine unequivocally the taxonomic divisions in the genus Fusobacterium.[25]

Conclusions

Most Gram-negative bacteria associated with periodontal diseases are asaccharolytic but *F. nucleatum* can obtain energy by the fermentation of simple sugars such as glucose or fructose but not sucrose; or from the fermentation of certain amino acids, free or in the form of small peptides. This metabolic versatility probably explains why the organism is found in both supra- and sub-gingival dental plaque. Its nutritional requirements could also explain why it is often found in periodontally-diseased sites together with organisms such as *P. gingivalis* which possesses powerful endopeptidase activities. The current subspeciation of *F. nucleatum* is of doubtful validity and evidence, based upon physiological and metabolic characteristics, for differences in pathogenicity between isolates has not been detected. While this organism is a member of various bacterial consortia associated with various forms of periodontal disease, its contribution to the disease processes remains unclear.

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