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Evaluating the effect of local pH on fluorescence emissions from oral bacteria of the genus *Prevotella*

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Abstract. A number of anaerobic oral bacteria, notably *Prevotellaceae*, exhibit red fluorescence when excited by short-wavelength visible light due to their accumulation of porphyrins, particularly protoporphyrin IX. pH affects the fluorescence of abiotic preparations of porphyrins due to transformations in speciation between monomers, higher aggregates, and dimers. To elucidate whether the porphyrin speciation phenomenon could be manifested within a microbiological system, suspensions of *Prevotella intermedia* and *Prevotella nigrescens* were examined by fluorescence spectrophotometry while being titrated against NaOH. The initial pH of the samples was <6, which was then raised toward the maximum found within a diseased periodontal pocket, being ~pH 8.7. The intensity of the fluorescence emissions increased between 600 and 650 nm with increasing pH. Peak fluorescence emissions occurred at 635 $\pm$ 1 nm with a second emission peak developing with increasing pH at 622 nm. A linear relationship was demonstrated between pH and the $\log_{10}$ ratio of 635:622 nm excitation fluorescence intensities. These findings suggest that the pH range found within the oral cavity could affect the fluorescence of oral bacteria *in vivo*, which may in turn have connotations for any clinical diagnoses that may be inferred from dental plaque fluorescence. [DOI: 10.1117/1.JBO.21.8.084003]

Keywords: protoporphyrin IX; *Prevotella*; bacteria; fluorescence spectrophotometry.

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1 Introduction

Quantitative light-induced fluorescence-digital (QLF-D) is a photographic method of plaque assessment that is being developed to inform and improve oral health. QLF-D utilizes the intrinsic red fluorescence of a contingent of oral bacteria at 405 nm excitation produced by light-emitting diodes to visualize dental plaque.[1] Research is currently being undertaken to correlate plaque fluorescence with the progression of oral diseases, such as dental caries.[2–5] The three possible mechanisms by which changes in antecedent growth conditions and the local physicochemical environment (i.e., pH) could affect plaque's fluorescent emissions are as follows.

1. By eliciting a shift in the composition of the microbial community[6] that would in turn alter the plaque metabolome and, consequently, the fluorophore complement of the plaque either directly or indirectly through mutual metabolic relationships.[7]
2. By altering fluorophore metabolism/accumulation within individual bacteria.[8]
3. By affecting the photochemical/photophysical mechanisms of molecular fluorescence[9] independently of the aforementioned changes in community dynamics and fluorophore metabolism.

These three proposed mechanisms affecting fluorescence could be manifested simultaneously within dental plaque and it would be very difficult to isolate and quantify their effects either *in vivo* or within a multispecies *in vitro* model of oral biofilm.[10]

A number of anaerobic oral bacteria metabolize heme from blood to produce fluorescent porphyrins, such as protoporphyrin IX (PPIX),[11] water-soluble uroporphyrin, and coproporphyrin.[12] Other biomolecules such as flavin adenine dinucleotide and nicotinamide adenine dinucleotide can also contribute toward autofluorescence in bacteria.[13] The fluorescent qualities of a number of anaerobes have been noted to change as a function of time.[14] Although the mechanism responsible for the observed changes in fluorescence with colony age was not speculated upon, it was likely to be due to porphyrins undergoing a transition from their monomeric form to oligomers, particularly dimers, in response to local pH or concentration,[15] which affected their fluorescent properties.[16] The effects of pH upon the fluorescence of porphyrins has already been studied,[17] but this has not yet been demonstrated in a bacterial system.

The bacteria *Prevotella intermedia* and *Prevotella nigrescens* are members of the "orange complex" of oral microorganisms,[18] which indicates that they are significantly associated with the progression of periodontal disease. These organisms accumulate relatively large amounts of PPIX.[12] A series of *in vitro* experiments were planned to isolate the possible effects of pH upon the photochemistry of bacterial fluorescence (mechanism #3) using single-species cultures of *Prevotella* to obviate the effects of a shift in the microbial population (mechanism #1) or porphyrin metabolism during growth (mechanism #2). The results of these experiments were then considered with respect to the potential impact that pH might have upon plaque imaging technologies such as QLF-D.

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2 Materials and Methods

Two species of *Prevotella* were used in these experiments; *P. intermedia* (ATCC 25611) and a wild-type *P. nigrescens*, as identified by matrix-assisted laser desorption/ionization-time of flight,[19] that was isolated from the oropharynx of an individual suffering from a sore throat. Stocks of these bacteria were maintained on anaerobic basal agar (Oxoid, Basingstoke, UK), supplemented with 5% defibrinated horse blood (TCS Biosciences, Botolph Claydon, UK), and incubated in an anaerobic chamber (80% N_2, 10% CO_2, 10% H_2) (Don Whitley, Shipley, UK) at 37°C. After 24 h incubation, colonial growth was swabbed from the agar and suspended in unbuffered saline (Oxoid) before being adjusted to an optical density of 0.1 units at 650 nm (Model 2021, Cecil Instruments, Cambridge, UK) with additional saline. Next, 3 ml of the bacterial suspensions were transferred to UV-grade optical methacrylate cuvettes (Kartell, Noviglio, Italy) and loaded into a fluorescence spectrophotometer (Cary Eclipse, Agilent, Stockport, UK).

With the cuvette *in situ* within the fluorescence spectrophotometer, a pH microelectrode (Beetrode™, World Precision Instruments, Sarasota, FL) was located in the upper portion of the suspension, away from the light path and the pH recorded. The scan parameters used were as follows; excitation 398 nm, emission 600 to 650 nm, excitation slit 5 nm, emission slit 5 nm, scan rate 120 nm/ min, averaging time 0.5 s, and emission data interval 1 nm. Following the baseline capture of pH and the emission spectrum, 0.5 μl of 50-mM NaOH (Sigma) was added to the cuvette while it was *in situ* within the fluorescence spectrophotometer. Next, the suspension was mixed by repeated pipetting via a 1-ml tip before the pH was measured again along with the fluorescence emission spectrum. This process was repeated until the pH reached 8.7; a value that is commensurate with that found within a diseased periodontal pocket.[20] The fluorescence emission spectral data were exported to a spreadsheet program (Microsoft Excel) for analysis.

3 Results

The baseline pH of *P. intermedia* was 5.78; whereas *P. nigrescens* was 5.01. The average increase in pH observed in the bacterial suspensions following the addition of a 0.5-μl aliquot of 50-mM NaOH was 0.26 ± 0.07 for *P. intermedia* and 0.45 ± 0.18 for *P. nigrescens* (±95% confidence intervals).

When described as either peak fluorescence (measured at 635 nm) or integrated fluorescence (sum of measurements 600 to 650 nm), increasing the pH elicited a corresponding increase in the intensity of fluorescence emissions (Fig. 1). Statistical analyses revealed strong quadratic polynomial relationships between pH and either of the methods of interpreting fluorescence for the two species tested, with a minimum R^2 of 0.986. Using a comparative pH range between the two bacteria of $\Delta pH = 2.94$ (5.78 to 8.72) in *P. intermedia* and $\Delta pH = 2.90$ (5.84 to 8.74) in *P. nigrescens*; the integrated fluorescence increased 2.31 times in *P. intermedia* and 2.68 times in the case of *P. nigrescens*.

Spectral analysis of the fluorescence emissions revealed peak fluorescence occurred at 635 ± 1 nm with a second peak at 622 nm, which became more pronounced with the increasing pH. Normalizing the emission data to the peak value at 635 ± 1 nm showed that the rates of increase in fluorescence emissions between the peaks at 635 and 622 nm were not equivalent (Fig. 2) with the intensity of the 622-nm peak increasing at a greater rate than at 635 nm. A linear relationship was demonstrated between pH and the $\log_{10}$ of the ratio of 635:622 nm emissions (Fig. 3).

4 Discussion

Prevotella spp. were chosen for these experiments since this genus produces relatively large amounts of PPIX on the cell surface in the monomeric form[21] along other water-soluble porphyrins, such as coproporphyrin and uroporphyrin,[12] and because they fluoresce well under QLF-D lighting conditions when grown on blood-containing agars.[22] Using a single species of bacteria within a titration model ensured that any shifts observed in fluorescence were due to the changes in local pH that were instigated at the point of data acquisition and not as a result of the effects that antecedent growth conditions had upon porphyrin metabolism and/or community dynamics.

Although the fluorescence spectrophotometer used in this study was capable of conducting sequential scans at multiple excitation wavelengths, it was considered prudent to utilize a single excitation wavelength to better represent the potential

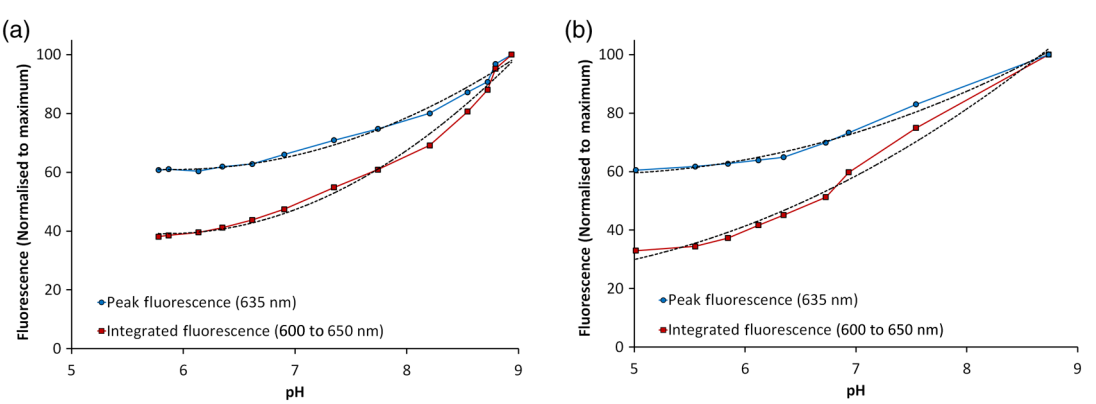

Fig. 1 Analysis of fluorescence emissions with increasing pH in suspensions of *Prevotellaceae*. "peak fluorescence" refers to a single measurement at 635 nm, while "integrated fluorescence" is the sum of all fluorescence values between 600 and 650 nm. The data were normalized to 100 at their maximum value and the dashed black lines represent quadratic regression lines of best fit. (a) *P. intermedia* (peak fluorescence; $R^2 = 0.990$, pH $p \leq 0.01$, pH^2 $p \leq 0.01$) (integrated fluorescence; $R^2 = 0.992$, pH $p \leq 0.01$, pH^2 $p \leq 0.01$). (b) *P. nigrescens* (peak fluorescence; $R^2 = 0.990$, pH $p \leq 0.01$, pH^2 $p \leq 0.01$) (integrated fluorescence; $R^2 = 0.986$, pH $p = 0.110$, pH^2 $p = 0.01$).

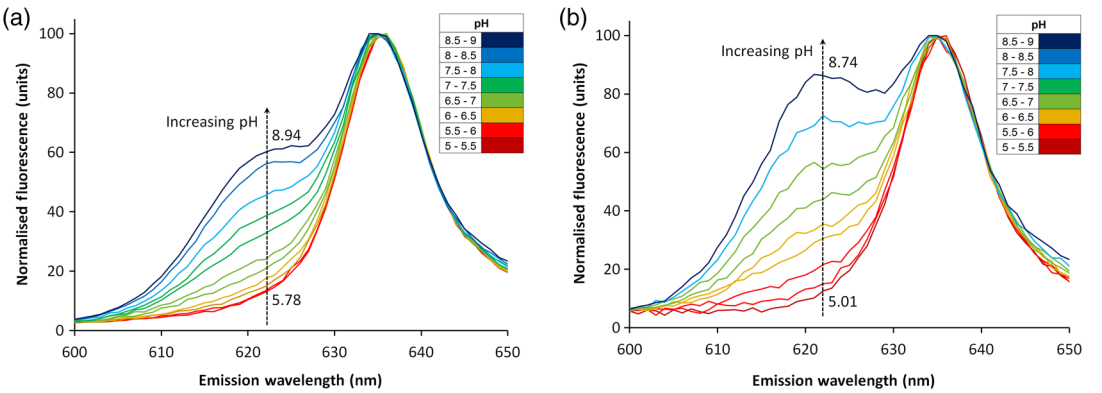

Fig. 2 Fluorescence emission spectra for suspensions of (a) *P. intermedia* and (b) *P. nigrescens* as the pH was increased by the sequential addition of 0.5-μl aliquots of 50-mM NaOH. Fluorescence values shown were normalized to 100 at the peak value (635 $\pm$ 1 nm).

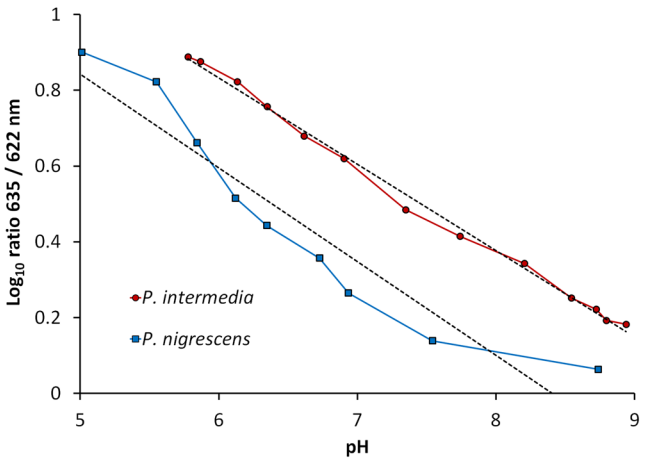

Fig. 3 Ratiometric analysis of fluorescence emissions in response to pH, dashed lines represent lines of best fit calculated by linear regression; *P. intermedia* ($R^2 = 0.996$, $p \leq 0.01$), *P. nigrescens* ($R^2 = 0.904$, $p \leq 0.01$).

light sources that could be easily incorporated into a photographic method of plaque visualization, such as QLF-D. With respect to the two methods of describing the fluorescence data in Fig. 1, "integrated fluorescence" is more representative of the information that would be captured by digital photography, incorporating a wide bandpass filter to isolate the red portion of the spectrum. The two emission peaks at 635 and 622 nm have different excitation maxima, 410 and 398 nm, respectively (data not shown). The experiments described herein focused upon the 622-nm emission since this appeared to be the most responsive to changes in pH, which is the reason why 398 nm was chosen as the excitation wavelength. A previous study[23] into the fluorescence of dental calculus was also able to resolve two excitation peaks at 398 and 405 nm, which in this instance was attributed to the presence of more than one porphyrin derivative as would indeed be expected in either of the two strains used in the present study.[12] A particularly pertinent paper discussed two fluorescence emissions from PPIX in cancer of glial tissue (brain, spine) at 620 and 634 nm, which were attributed to the effects of pH upon PPIX.[24]

The fluorescence intensity values presented in Fig. 1 are unlikely to be reproducible with respect to dental plaque due to differences in operating parameters (i.e., camera equipment and settings, ambient lighting conditions, positioning) and biological variations between patients and their respective oral microflora and plaque accumulation. Undertaking a ratiometric analysis of the fluorescence emission data at 622 and 635 nm could potentially obviate this problem by effectively constituting a form of internal calibration.

The mechanism responsible for the observed changes in fluorescence was not determined in this study, but may be inferred from similar studies that looked at the effects of pH upon abiotic porphyrin mixtures.[17,25,26] These studies suggested that porphyrins can undergo a transition from their monomeric form to higher aggregates and oligomers, particularly dimers, in response to the increasing pH.[27] The concentration of a porphyrin can also affect its fluorescence characteristics due to a very similar phenomenon.[28] The effects of differences in concentration were essentially fixed in this study since the dilution caused by the pH adjustments (i.e., 0.5 μl into 3 ml) were <0.02% per aliquot of NaOH.

The findings of this study suggests that plaque pH at the point of observation may affect red fluorescence emissions as captured by imaging techniques such as QLF-D. The ratiometric analysis of specific emission wavelengths could potentially be used to estimate the pH of a suspension of *Prevotella* spp. and perhaps other microbial systems, which contain fluorescent porphyrins. With further work, it may also be possible to apply this technique to dental plaque, which could be used to help diagnose oral diseases such as caries (i.e., low plaque pH) or periodontal disease (i.e., high plaque pH). Future studies are required to elucidate the importance of this reported phenomenon in relation to changes in fluorescence due to different plaque microflora and porphyrin accumulation.

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