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Influence of culture conditions on porphyrin production in *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*

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ABSTRACT

Background: Increasing antibiotic resistance among pathogens has raised the demands for new treatment methods such as antimicrobial photodynamic therapy (aPDT) and phototherapy (PT). Experiments for investigating the effects of these methods are often performed *in vitro*, but the procedures for cultivation of microbes vary between different studies. The aim of this study has been to elucidate how the profile of endogenously produced porphyrins differs by changing the variables of bacteria culturing conditions.
Methods: Two oral pathogens, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, were selected as model organisms. The contents of porphyrins and heme in the bacteria were analysed with liquid chromatography–tandem mass spectrometry when bacteria was cultivated for different lengths of time (3–9 days), upon passaging as well as when growth medium were supplemented with or without horse blood.
Results: Both porphyrin and heme content in *A. actinomycetemcomitans* are highly affected by the age of the culture, and that the porphyrin profiles changes during cultivation. When cultivated colonies of *A. actinomycetemcomitans* were passaged onto a new, fresh growth medium a large change in porphyrin content occurred. Additional porphyrins were detected; uroporphyrin and 7-carboxylporphyrin, and the total porphyrin content increased up to 28 times. When *P. gingivalis* was grown on blood containing medium higher concentrations of protoporphyrin IX (2.5 times) and heme (5.4 times) were quantified compared to bacteria grown without blood.
Conclusions: This study demonstrate that there is a need for more standardized culturing protocols when performing aPDT and PT experiments *in vitro* to avoid large variations in porphyrin profiles and concentrations, the aPDT/PT target compounds, depending on the culturing conditions.

1. Introduction

Dental biofilm is defined as the community of microorganisms, predominantly bacteria, found on the teeth surfaces. This community is embedded in a matrix of polymers of bacterial and salivary origin [29]. Periodontal diseases (gingivitis and periodontitis) range from inflammation of the gingiva to serious inflammation of the supporting tissues of the teeth. The pathogenesis of periodontitis is characterized as an immuno-inflammatory process triggered by the accumulation of dental biofilm, which lead to destruction of periodontal ligament and alveolar bone [9,3]. It has been shown that the presence of "red-complex" pathogens *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* can interfere with host defense mechanisms and cause homeostasis breakdown in the dental biofilm [44,7]. *Aggregatibacter actinomycetemcomitans* is another key pathogen that can interfere with host defense by producing a powerful leukotoxin (LtxA) that specifically affects human circulating leucocytes [17]. Recent studies have demonstrated that both A. *actinomycetemcomitans* and *P. gingivalis* can be inactivated by irradiation with blue light at wavelengths in the region of 405–460 nm, and it has been suggested that endogenous porphyrins are involved in the mechanism [4,19]. This is of great interest since there is an increasing demand to find new efficient treatment modalities

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of periodontal diseases and this has raised an increased interest towards the role of porphyrins.

Emerging treatments of periodontal disease are phototherapy (PT) and antimicrobial photodynamic therapy (aPDT). Photodynamic therapy has been successfully used in treatment of cancer as well as acne [41]. In aPDT a molecule, a photosensitizer (PS), is illuminated by light of a certain wavelength in the visible spectrum. The ground state (S_0) PS absorbs one photon and changes to a higher energetically excited state (S_1). The S_1 PS returns to S_0 by emitting one photon (*i.e.* fluorescence), or it can enter an excited triplet state (T_1) by intersystem crossing. If oxygen is present, the T_1 PS can react in two different ways called type I and type II. In type I the T_1 PS transfers the charge to a substrate or oxygen that can produce reactive oxygen species such as oxygen radicals and hydrogen peroxide. In type II reaction, the T_1 PS transfers the energy to oxygen in its triplet ground state and creates excited singlet oxygen. In both reactions the PS go back to the S_0 state. Singlet oxygen can oxidize biomolecules, leading to cytotoxicity, and is probably the major damaging species in aPDT [41,5]. Porphyrins are frequently used as photosensitizers. They are either added in their native form directly to the site of treatment, or by induced production of endogenous porphyrins by addition of 5-aminolaevulinic acid. This compound is a porphyrin precursor that bypasses the feedback control of porphyrin synthesis in the cell, resulting in high porphyrin concentrations [41,24,37]. It has been shown that certain oral bacteria, *e.g. P. gingivalis* and *A. actinomycetemcomitans,* are affected by PT without exogenous addition of a photosensitizer [4,42,18,11]. Henry and coworkers have shown that the effect of laser irradiation on black pigmented bacteria decreases with the time of culturing (0–2 d), and photosensitivity could not be correlated to the amount of protoporphyrin IX (PPIX) in the bacteria [18]. Photosensitivity has not been correlated to the total amount of porphyrins present in bacteria, suggesting that the porphyrins are not the sole determinants of photosensitivity [42]. Cieplik et al. [4] have suggested that flavins could be another main cause for photosensitivity in *A. actinomycetemcomitans.* However, it could also be that not only the total amount of porphyrins is important; the photosensitizing efficiency of the various porphyrin species differs [42]. In a review article by Dai et al. [6] the authors argue that the significant variation in microbial killing efficiency between different illumination studies could partly be explained by differences in experimental setup such as different culture conditions. In most illumination studies the culturing of microbes is performed in liquid media [4,2,27], but solid agar media have also been used in some studies [42,11]. There are also variations in how long time the microbes are cultured before illumination is performed, from less than a day [4,28] to several days [21,14]. There is little work done investigating how the porphyrin profile varies in microbes just by changing the culturing conditions. It is well known that different culture conditions can trigger complex adaptive responses such as physiological, behavioral and genetic changes [43] which also could have an effect on the bacterial production of porphyrins. Kjeldstad et al. [23] investigated the influence of different pH in the growth medium on porphyrin production in *Propionibacterium acnes* at different days of incubation (4–15 d), and they could see that the amount as well as the pattern of porphyrins varied. Hamblin et al. [16] saw that cultures of *Helicobacter pylori* and *P. acnes* had a higher degree of photosensitivity when the bacteria were grown for 96 h compared to 24 h and they stated that this is due to the differences in porphyrin concentration.

The aim of this study was to elucidate how the porphyrin profile differs by changing the culturing conditions, such as time of culturing, passaging and addition of blood to the growth medium. The hypothesis was that changing these conditions would yield differences in porphyrin profiles and that would highlight the importance of standardization in culture protocols when performing aPDT and PT experiments *in vitro*. In the present study *A. actinomycetemcomitans* and *P. gingivalis* were selected as model oral pathogens, since they are periodontopathogenic, non-fastidious anaerobic bacteria and in addition *A. actinomycetemcomitans* has all the necessary enzymes required for the *de novo* synthesis of porphyrins and heme, while *P. gingivalis* lacks important enzymes in the porphyrin synthesis [36].

2. Methods

2.1. Chemicals

A porphyrin acid chromatographic marker kit (CMK-1A) containing 10 ± 1 nmol of each of six porphyrins (mesoporphyrin IX, coproporphyrin I, 5-carboxylporphyrin I, 6-carboxylporphyrin I, 7-carboxylporphyrin I and uroporphyrin I) was obtained from Frontier Scientific Inc (Logan, UT, USA). Separate standards of protoporphyrin IX (purity $\geq$ 95%) and hemin from bovine (purity $\geq$ 90%) were obtained from Sigma Aldrich (Schnelldorf, Germany). Formic acid ($\geq$98%) and acetic acid (99.8%) were also products of Sigma Aldrich. HPLC-grade methanol and acetonitrile were purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland) and analytical-grade hydrochloric acid (37%) from Riedel-de Haën (Seelze, Germany).Ammonium acetate (pro analysi grade) was obtained from Merck (Damstadt, Germany). Synergy 185 water purification system from Millipore (Molsheim, France) was used to produce deionized water at 18 Ω.

2.2. Strains and culture conditions

2.2.1. Aggregatibacter actinomycetemcomitans

A. actinomycetemcomitans strain ATCC® 33384™ serotype c was obtained from the Department of Clinical Bacteriology at the University of Gothenburg, Sweden.

Culturing of *A. actinomycetemcomitans* was made on Columbia agar with 5% defibrinated horse blood (Substrate unit, Karolinska University Hospital, Huddinge, Sweden). Cultures were grown at the Department of Odontology, Karolinska Institutet using a CO_2 incubator (Model T303, Assab Medicin, Sweden) with 4.9% CO_2 atmosphere. Temperature was set at 37.1 °C. At the day of harvest each sample was prepared by collecting and pooling all visible colonies of bacteria from 3 to 5 agar plates. A plastic cell scraper was used for collection of the colonies, which were transferred to a 1.5 mL Eppendorf vial containing 500 μL of 0.4% (w/v) NaCl. Each vial was pre-weighed to allow gravimetric measurement of sample size.

A time study was performed where *A. actinomycetemcomitans* was allowed to grow for up to 9 days, and cultures from day 3 to day 9 were harvested and analysed with respect to both porphyrin and heme content.

Investigation of the effect of passage was made by incubation of *A. actinomycetemcomitans* for 4 days on blood agar plates followed by transfer to fresh blood agar plates and additional culturing for 4 and 7 days.

The bacterial weight of *A. actinomycetemcomitans* was 88.8 mg on average for all experiments.

2.2.2. Porphyromonas gingivalis

Two strains of the gram-negative black-pigmented anaerobe bacterium *P. gingivalis*, ATCC® 33277™ and ATCC® BAA-308™ (LGC Standards, Borås, Sweden) were grown on Brucella agar with 5% defibrinated horse blood and Tryptic Soy Agar plates (TSA) in an incubator (Model T303, Assab Medicin, Sweden) at 37.1 °C under anaerobic conditions obtained by using a GasPak EZ Gas Generating Container System (Becton, Dickinson and Company, Franklin Lakes,

NJ). The GasPak system gave a CO_2 concentration ≥13% and an oxygen concentration ≤1% during cultivation. Both the Brucella blood agar and TSA agar were supplemented with 5 mg/L of hemin and 10 and 1 mg/L respectively of vitamin K (Substrate unit, Karolinska University Hospital, Huddinge, Sweden).

A time trend study was performed where both strains of *P. gingivalis* grown for 6 days on Brucella agar and then passaged to new Brucella agar were they were allowed to grow for additional 4, 7 and 11 days.

For the experiments with blood and blood free agar *P. gingivalis* was incubated for 6 days on Brucella agar then passaged to both TSA agar without blood and Brucella agar supplemented with blood. *P. gingivalis* and grown for additional 8 days.

The colonies of *P. gingivalis* were collected in the same manner as been described for *A. actinomycetemcomitans.* The bacterial weight of *P. gingivalis* was 105.7 mg on average for all experiments.

2.3. Sample preparation

A validated method for the extraction and analysis of porphyrins from bacteria has been developed by Fyrestam et al. [13] and the method has been used in this present study. The procedure is explained briefly below. After harvest, porphyrins were extracted from the bacteria using a combination of Tris/EDTA buffer and ultrasonication, followed by clean-up using C18 solid phase extraction (SPE). In brief, the samples were put in Tris/EDTA buffer (5 mM Tris-HCl, 10 mM EDTA, pH 7.2) for 1 h. Formic acid was added to precipitate proteins and to keep pH low, and the cells were further lysed with ultrasonication for 1 min using an ultrasonication rod (Sonics vibra-cell, Newton, CT, USA) and centrifuged at 2500*g* to form a pellet of bacterial debris. The supernatant containing the porphyrins was put on a pre-conditioned C18 SPE cartridge, washed with ammonium acetate, and eluted with acetone:formic acid 9:1 (v/v). The eluate was evaporated to dryness using a gentle stream of N_2 at a temperature of 70 °C, where after the analytes were resuspended in 500 μL of 6 M formic acid. Samples containing very high concentrations of porphyrins and heme were further diluted with 6 M formic acid.

2.4. HPLC–MS/MS analysis

Two different HPLC systems were used for the analyses. Analysis of *A. actinomycetemcomitans* extracts were made using a Perkin Elmer 200 series binary solvent delivery system consisting of two micro pumps, an autosampler equipped with a 5 μL loop and a vacuum degasser (Perkin Elmer, Norwalk, CT, USA). For *P. gingivalis* the analyses were made on an Agilent HPLC system consisting of an 1100 binary pump solvent delivery system, an 1100 degasser and an 1100 autosampler (Agilent, Wilmington, DE, USA). An ACE 3 C18-PFP column (75 × 2.1 mm, d_p = 3 μm, Advanced Chromatography Technologies Ltd, Aberdeen, Scotland) was used for the separation with a gradient elution using water and acetonitrile as mobile phase. A Perkin Elmer Sciex API 2000 triple quadrupole mass spectrometer (Toronto, Canada) with a TurboIon Interface for electrospray ionization was used for both HPLC systems. Detection of porphyrins was made using positive electrospray mode (ESI+) and selected reaction monitoring (SRM) with three compound specific transitions for each porphyrin, all used both as quantifier and qualifier ions. Ion source parameters were as follows: ion source temperature 200 °C, capillary voltage 5 kV, nebulizer gas 35 psi and curtain gas 20 psi.

Due to the tendency of porphyrins and heme to aggregate in polar solvents [20], only single injection of each sample was possible to ensure the accuracy of the analysis. The cycle time for one single analysis with the HPLC–MS/MS instrument is approx. 1 h. To analyze the time trend study for *A. actinomycetemcomitans*, using seven individual samples, standards, blank samples, and performing separate porphyrin and heme analyses, the total run time is over 24 h. Within that time, porphyrins and heme have started to aggregate and it is not possible to do repeated analysis of the samples with satisfactory results. In the evaluation of the method [13] it has been shown that the accuracy of the method is high and that intraday precision of the instrument is ≤5% for all porphyrins. The precision was checked during the analysis by triplicate injections of a standard porphyrin mixture.

3. Results and discussion

3.1. Growth time study of microorganisms

The HPLC–MS/MS analyses revealed differences in the porphyrin patterns when *A.actinomycetemcomitans* was incubated at different times of growth. At all the investigated days coproporphyrin I (CPI), coproporphyrin III (CPIII) and PPIX were identified and quantified. Chromatograms showing the porphyrin content of a sample at incubation day 4 and 7 are shown in Fig. 1.

Coproporphyrin III, PPIX and CPI were detected at day 3 with 80%, 16% and 4% respectively of molar total porphyrin content (TPC). The porphyrin profile changed with time of culturing. At day 9 PPIX had increased to be the most abundant porphyrin with 85% of TPC, while CPIII had decreased to 15% and CPI was below limit of quantification (LOQ). Coproporphyrin III was the dominating porphyrin species at day 3 but decreased during incubation to have its lowest abundance at day 6, a level that was kept to the final day of growth (Fig. 2). Protoporphyrin IX showed the opposite pattern. It had its lowest abundance at day 3, after which it increased during cultivation and stabilized at a level of around 90% from day 6. Coproporphyrin I was the porphyrin with the lowest abundance of those detected in the growth time study.

The pattern seen in Fig. 1, where CPIII is decreasing, PPIX is increasing and CPI is stable at the beginning of the growth time study, can be explained by the enzymatic pathways of heme biosynthesis. Heme biosynthesis starts with 5-aminolevulinic acid, which in bacteria is formed through the so called C-5 pathway from glutamate-tRNA [12]. 5-Aminolevulinic acid is enzymatically converted to a number of porphyrinogens to the end product of heme. Porphyrinogens differs from porphyrins by the pyrrole rings being linked together by methane rather than methine bridges. None of the porphyrinogens are fluorescent, but oxidation of the porphyrinogens can occur and form the corresponding fluorescent porphyrin [25]. From four porphobilinogen molecules, formation of hydroxymethylbilane is catalysed by hydroxymethylbilane synthase and by rearrangement and cyclization, uroporphyrinogen III is formed by uroporphyrinogen III synthase. In the absence of this enzyme uroporphyrinogen I is formed spontaneously. Uroporphyrinogen decarboxylase, the enzyme that converts uroporphyrinogen to coproporphyrinogen is not isomer specific, resulting in two isomers of CP; CPI and CPIII. However, coproporphyrinogen oxidase that converts coproporphyrinogen to protoporphyrinogen IX by decarboxylation of the propionic acid groups into vinyl groups is highly specific to the coproporphyrinogen III isomer, so coporporphyrinogen I isomer will not be further metabolized [25]. Coproporphyrin I concentrations were however decreasing during bacterial growth and are probable due to excretion from the cell [8]. The only porphyrin present in the actual pathway is PPIX which is formed through oxidation of the corresponding porphyrinogen. The last step in the heme synthesis is insertion of iron to PPIX by the enzyme ferrochelatase, Quantified molar concentrations of porphyrins in *A.actinomycetemcomitans* in the time trend study are summarised in Table 1.

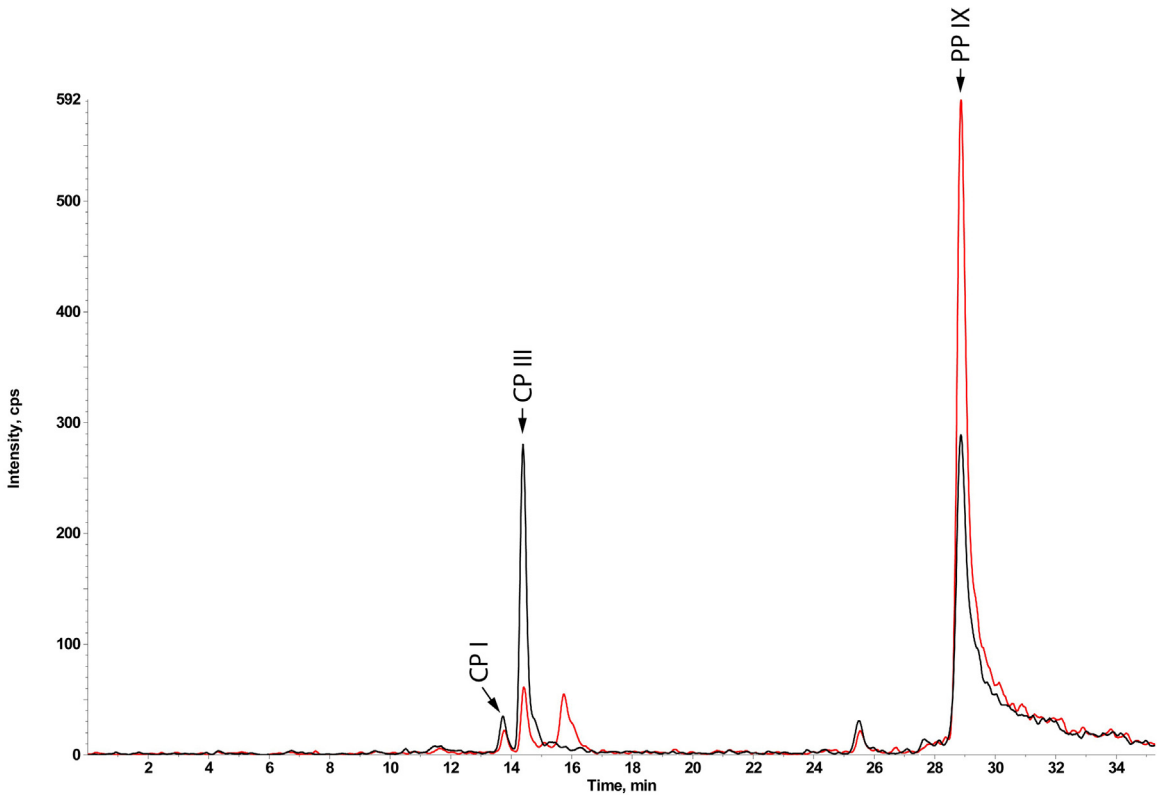

Fig. 1. Chromatogram of *A. actinomycetemcomitans* incubated for 4 days (black chromatogram) and 7 days (red chromatogram). Sample sizes are close to equal (Day 4: 51.7 mg Day 7: 45.3 mg) making the chromatograms directly comparable. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

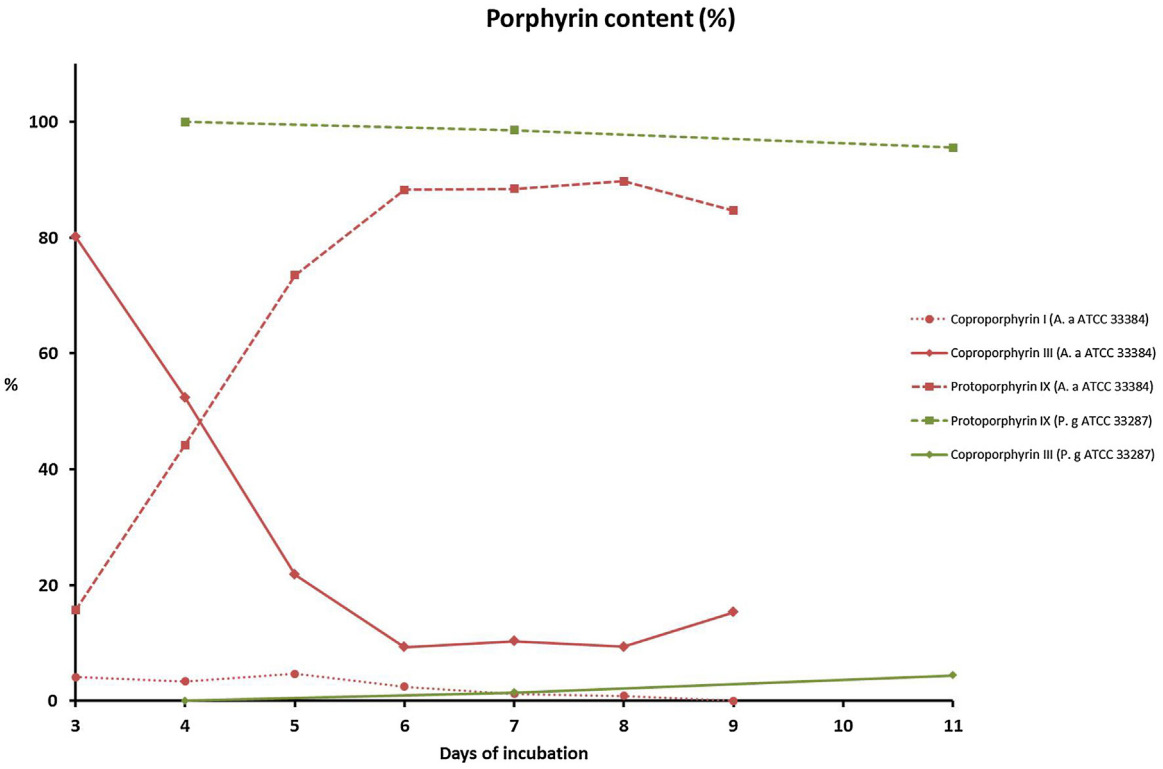

Fig. 2. Total Porphyrin Content expressed as% of quantified porphyrins during different days of culturing in *P. gingivalis* ATCC 33277 (green lines) and *A. actinomycetemcomitans* ATCC 33384 (red lines). Data points for each day are a single injection of a pooled sample, consisting of all visible bacteria from 3 to 5 agar plates. Dotted line = CPI, solid line = CPIII and dashed line = PPIX. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Molar concentrations (pmol/g) of porphyrins quantified in *A. actinomycetemcomitans* at different days of cultivation.

Porphyrin	*A. actinomycetemcomitans* [pmol/g]						
	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Coproporphyrin I	26	17	11	13	6	3	<LOQ
Coproporphyrin III	510	269	54	50	51	35	54
Protoporphyrin IX	100	227	181	477	438	330	299

<LOQ = Detected, but below limit of quantification.

Table 2
Molar concentrations [nmol/g] of porphyrins quantified in *P. gingivalis* at different days of cultivation.

Porphyrin	*P. gingivalis* [nmol/g]		
	Day 4	Day 7	Day 11
Coproporphyrin I	n.d	n.d	n.d
Coproporphyrin III	<LOQ	0.1	0.2
Protoporphyrin IX	9	6	17
n.d = Not detected			

<LOQ = Detected, but below limit of quantification.

When analysing extracts from *P. gingivalis* only CPIII and PPIX were detected and quantified (Table 2). The TPC for *P. gingivalis* showed a porphyrin profile that was similar between the days of cultivation (Fig. 2). Protoporphyrin IX was the most abundant porphyrin with 96–100% TPC at the investigated days. Both coproporphyrin and PPIX have been detected in *P. gingivalis* previously [42,18,13].The porphyrin content during growth of the *P.gingivalis* strain ATCC BAA-308 was investigated in the same way as described above and it exhibited a TPC profile during growth similar to strain ATCC 33277 (data not shown).

The TPC profile of *P. gingivalis* differs from *A. actinomycetemcomitans*. For the former, CPI was not detected and the profiles of CPIII and PPIX were more stable throughout the time trend study. These differences in profiles could be explained by the fact that *P. gingivalis* is a bacterium that lacks genes required for the *de novo* synthesis of PPIX and heme, and is therefore dependent on external sources of these compounds to be able to grow [32]. *P. gingivalis* lacks the enzymes for biosynthesis of 5-aminolevulinic acid, uroporphyrinogen, coproporphyrinogen and PPIX, but it does have the enzyme for conversion of coproporphyrinogen to protoporphyrinogen as well as ferrochelatase for formation of heme [36]. *P. gingivalis* has specific membrane proteins on its outer membrane that recognize, bind and transport compounds with porphyrin macrocyclic structures into the cell. The reason for why we can find any porphyrins at all in *P. gingivalis* is most likely due to the uptake of these compounds from the growth medium [32].

Due to the size of experiment it should be mentioned that we have only performed the whole time trend study experiments once, but the porphyrin profile has been confirmed by performing single experiments on day 4 and 7.

The results from the studies of *A. actinomycetemcomitans* and *P. gingivalis* above demonstrate that there is a need for a more standardized protocol for cultivation times in order to be able to compare the results from different studies. Extensive research has been performed on *in vitro* experiments with aPDT and PT, and a number of these studies use exogenous photosensitizers added to the bacteria, while others have used endogenously produced photosensitizers. In these cases the conclusion have been that porphyrins are the main contribution to the bacterial sensitivity to blue light [4,2,28,16]. There are lot of variations in the literature on how the bacteria are grown, and in many cases there is a lack of information about passage number, growth times and other culturing conditions. If porphyrins are the major contribution to the photosensitivity of microbes, it is of great interest to investigate which porphyrin species is the more effective to work as a photosensitizer, as well as what is the porphyrin profile of the microbe. As can be seen from the *in vitro* growth experiments presented here, there are large differences in the profile caused by the culturing conditions for the microbes. This could give different outcomes of microbial photosensitivity when performing aPDT/PT experiments on *A. actinomycetemcomitans* at day 3, when PPIX constitutes only 16% of the TPC, compared to day 9 were when PPIX constitutes >85% of the TPC and has a 3–5 times higher concentration. However, for *P. gingivalis*, bacteria lacking the enzymes for porphyrin synthesis the day of incubation that the illumination is performed could have no or very small effect on results from aPDT/PT experiments. To have a more standardized method when performing aPDT/PT experiments *in vitro,* could solve this problem when dealing with bacteria producing endogenous porphyrins.

3.2. Analysis of heme

To investigate the content of heme (PPIX-Fe^{2+}) in bacterial extracts, hemin (PPIX-Fe^{3+}) was used as a standard for identification. Retention times and product ion scans of the ion m/z 616 (consistent with protonated heme) in an extract of *A.actinomycetemcomitans* and a hemin standard solution is shown in Fig. 3. The hemin spectrum is dominated by cleavage of the two propionic acid substituents, giving rise to the ions m/z 557 and m/z 498 respectively. Additional loss of a CH_3 can be seen at m/z 483. This fragmentation is consistent with what has been described by Gorchein et al. [15]. When compared to heme in the *A. actinomycetemcomitans* extract from day 9, the product ion scan and retention times are identical. The heme concentration is of interest in order to widen the understanding of iron acquisition mechanisms in the investigated bacteria. However, heme is highly unstable in polar solvents since it easily forms aggregates by π-π stacking mechanisms [20]. This makes heme hard to quantify with sufficient accuracy in bacterial extracts, and to our knowledge no validated analytical method utilizing HPLC-MS/MS is available. A sample extract of baker's yeast, *Saccharomyces cerevisiae*, was used as a model organism during validation experiments. It was put through the sample preparation steps described above and analysed by HPLC-MS/MS with repeated injections during 11 h. In this case the heme decreased by a factor of 8.4 compared to CPIII. However, since the clean-up procedure as well as the time frame for the analysis was identical for all samples, the relative concentrations are comparable to each other to investigate the trends during cultivation. This revealed that the heme concentration increased exponentially with time of culturing. From day 3 to day 9 the concentration increased 16-fold (Fig. 4).

Heme is toxic to the bacteria in high concentrations due to its high redox potential [1] and the biosynthesis of heme is regulated by a negative feedback control. Our results show that the investigated bacteria were accumulating heme in an exponentially increasing concentration during growth, while at the same time no decrease in porphyrin concentration could be observed. This may suggest that *A. actinomycetemcomitans* utilize heme from its surroundings, *e.g.* from hemoglobin present in the Columbia agar containing 5% horse blood, which is consistent with previously studies of *A. actinomycetemcomitans* iron acquisition properties [22,31]. Porphyrins are however intermediates in heme biosynthesis and the fact that these intermediates were detected is an indication of that the bacteria also could synthesize heme endogenously since there is no known synthetic pathway that converts heme to CPIII/CPI [16]. The property of *A. actinomycetemcomitans* and other bacteria to both be able to synthesize as well as acquire heme is poorly understood, and if *A. actinomycetemcomitans* prefer endogenously or exogenously synthesized heme is still

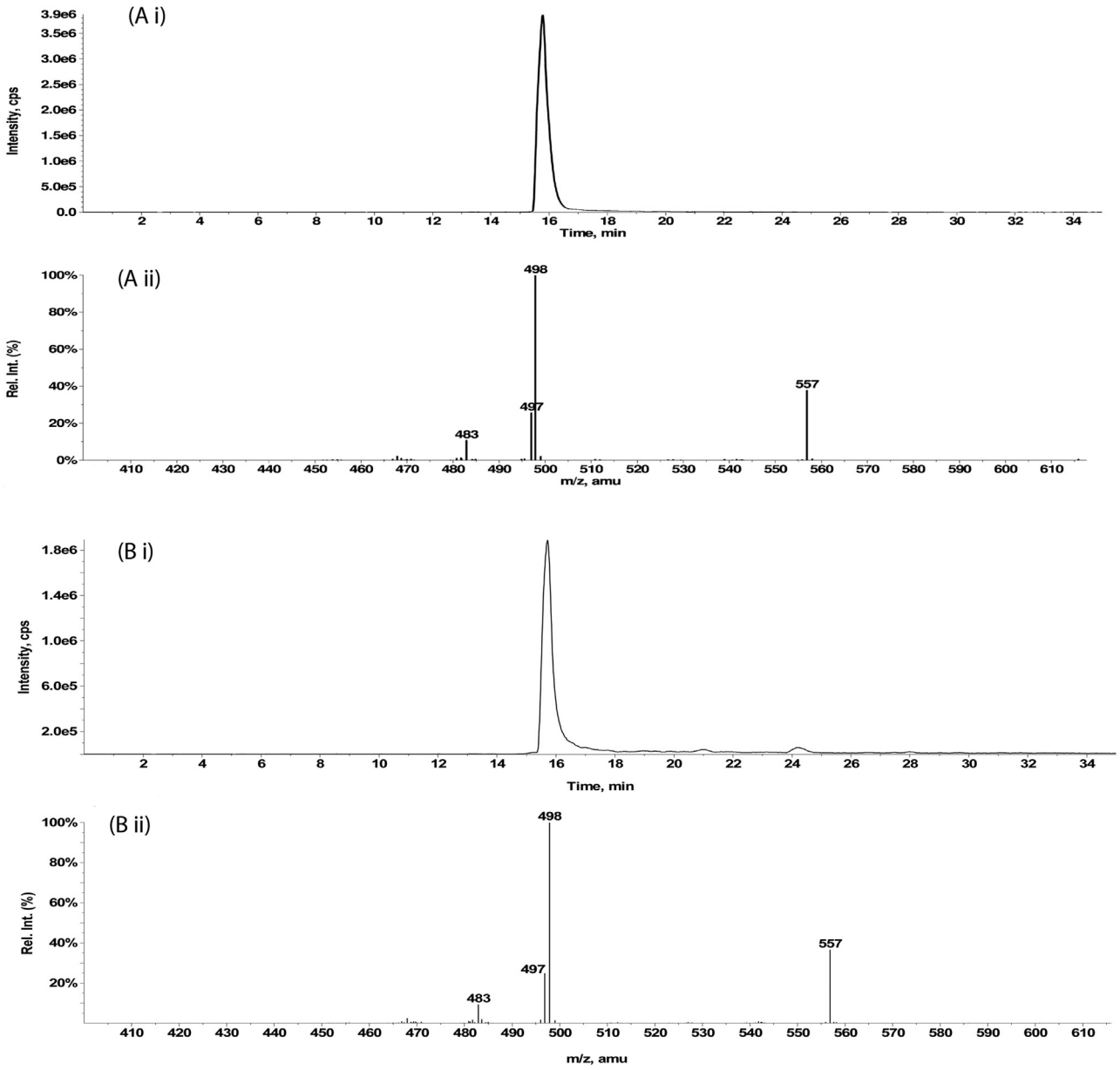

Fig. 3. HPLC-MS/MS chromatogram (i) and product ion scan (ii) for a standard solution of hemin (A) and extract of cultivated *A. actinomycetemcomitans* at day 9 of incubation (B).

unknown. One hypothesis is that a bacterium that is capable of both synthesis and acquisition choose the most energetically favorable method for iron acquisition. The systems for extraction of heme from hemoglobin are energetically costly and when the bacteria are in an iron rich environment they will synthesize heme, while in an iron deficient environment they acquire exogenous heme [1,33].

3.3. *Passage of*A. actinomycetemcomitans

Passage typically leads to more viable and mature bacterial culture as new nutrients are provided to the bacteria. Serial passage *in vitro* of clinical isolates of *A. actinomycetemcomitans* has shown that the bacteria transforms from rough, adherent colonies to smooth and non-adherent colonies [10]. It can thus be suspected that the porphyrin and heme patterns also can be influenced by passage number. Chemical analysis of passaged *A. actinomycetemcomitans* harvested after 4 and 7 days showed the presence of heme, PPIX, CPI and CPIII in both samples, similar to when no passage were performed. In addition, uroporphyrin (UP) and 7-carboxylporphyrin (7P) were detected in samples from passaged cultures at day 7 (Fig. 5). The reason for these porphyrins to appear upon passaging could be that when the bacteria are cultured using specific culture conditions, it will activate genes coding for the synthesis of different proteins and enzymes optimal for that environment, *e.g.* enzymes involved in the biosynthesis of heme or membrane proteins associated with uptake of heme from the growth medium [34,35]. Upon passage to a new agar plate, with the same nutrient composition, these genes are already activated and the bacteria start synthesizing heme earlier compared to non-passaged bacteria. The total amount of porphyrins is increasing upon passaging (Fig. 6). At day 4 of incubation there was a small, non-significant difference in TPC between bacteria that had been passaged (0.56 nmol/g) and non passaged bacteria (0.51 nmol/g). However, when the bacteria were left to grow for 7 days, a large difference in TPC was observed between passaged and non passaged bacteria. The TPC increased by a factor of 28, from 0.50 to

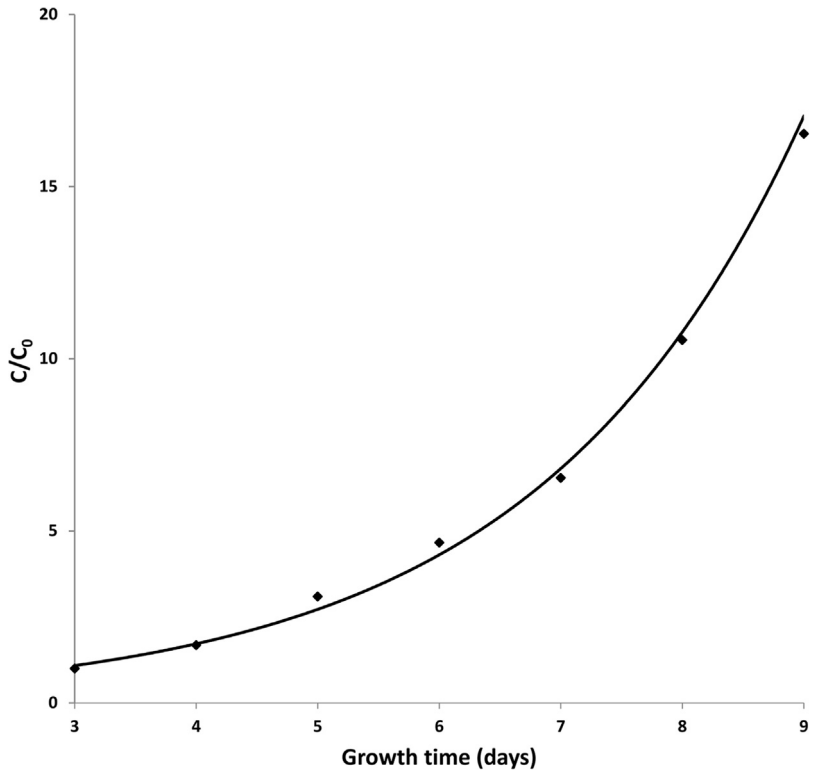

Fig. 4. Relative heme concentrations in *A. actinomycetemcomitans* during time trend study. C is heme concentration at given day and C_0 is the initial heme concentration at day 3. Data points for each day are a single injection of a pooled sample, consisting of all visible bacteria from 3 to 5 agar plates.

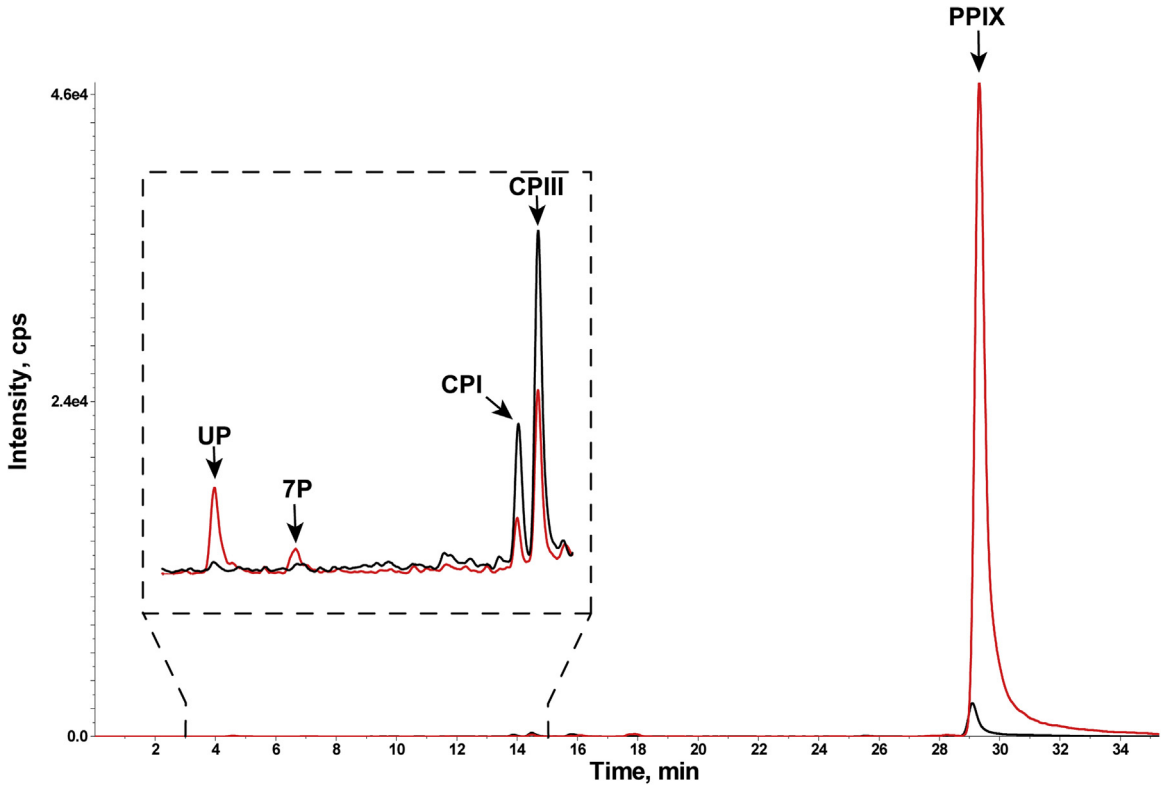

Fig. 5. Comparison of porphyrin profile between passaged (red curve) and non passaged (black curve) cultivated *A. actinomycetemcomitans* at day 7 of incubation. Sample sizes were 104.9 mg for passaged and 45.3 mg for non passaged bacteria. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

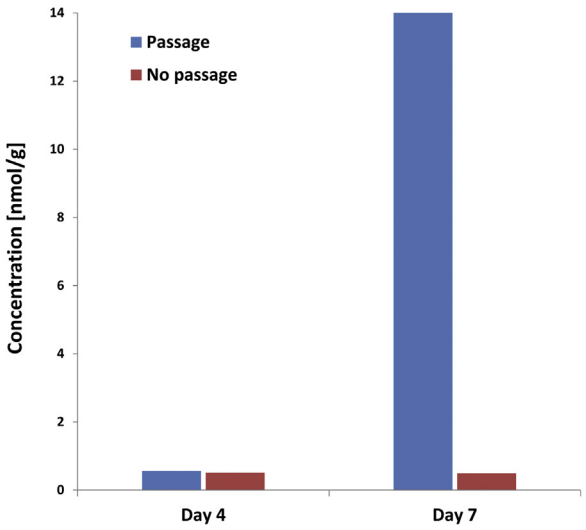

Fig. 6. Total porphyrin content, expressed as nmol/g, in passaged (blue, first bar on each day) and non passaged (red, second bar on each day) *A. actinomycetemcomitans* at day 4 and 7 of cultivation. Data points for each day are a single injection of a pooled sample, consisting of all visible bacteria from 3 to 5 agar plates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

13.8 nmol/g. All porphyrins that are intermediates in heme biosynthesis have strong photosensitive properties [30], *i.e.* UP and 7P may also contribute to the degree of photosensitivity of microbes. This large difference in TPC could thus have an impact on aPDT/PT experiments making bacteria with similar properties as *A. actinomycetemcomitans* more sensitive to light upon passaging.

Heme concentrations were also shown to be affected by passaging. When *A. actinomycetemcomitans* was subcultivated and grown for 4 and 7 days the concentration of heme increased by a factor 4 and 2 respectively, compared to non-passaged bacteria.

3.4 P. gingivalis *grown on agar with/without blood*

When the *P. gingivalis* strain ATCC BAA-308 were grown for 8 days on agar plates containing blood, the colonies were black pigmented, while bacteria grown for the same period of time without blood were transparent. The black pigment in *P. gingivalis* consists of μ-oxo dimer of heme $[Fe(III)PPIX]_2O$ and is suggested to act as an oxidative buffer [39]. $[Fe(III)PPIX]_2O$ is in equilibrium with heme and *P.gingivalis* grown with blood is suspected to also contain higher concentrations of heme than *P.gingivalis* grown without blood. In this study we found that the heme concentration in *P. gingivalis* was approximately 5 times higher in bacteria grown with blood compared to in the absence of blood, 49.8 and 9.3 nmol/g respectively. Smalley et al. [38] has shown that *P. gingivalis* grown in heme limited conditions accumulates less heme than those grown in the excess of heme. However, that study did not investigate changes in porphyrin profile. In the chemical analysis of cultivated *P. gingivalis* performed in our study, CPIII could not be identified in the sample without blood in growth medium, while when cultured on agar supplemented with blood CPIII reached a concentration of 0.2 nmol/g. Protoporphyrin IX were detected in both samples, but the sample supplemented with blood contained higher concentrations than the one without blood, 21.1 and 8.5 nmol/g respectively. Blood contains heme, PPIX and CPIII and the fact that *P. gingivalis* had higher concentrations of these porphyrins when grown on media containing blood, suggests that the bacterium has an uptake from the growth medium of these compounds. However, the presence of PPIX in *P. gingivalis* could also be due to ferrochelatase catalysed removal of iron from heme taken up from the growth medium by *P. gingivalis*. This reversed direction of ferrochelatase has been observed in *Haemophilus influenzae* [26].

During an infection in the gingival gum there is an access to blood from the host and the use of blood in the growth medium would be close to simulate a real environment when performing aPDT/PT experiments. Some previous studies uses broth without blood when doing aPDT/PT experiments [4,11,40] which has the advantage of decreasing unwanted absorption of light by hemoglobin in the blood and it is also simple to perform dilution series with a liquid. However, when taking the results obtained here in consideration, the use of blood free medium can have an impact on the results from *in vitro* aPDT and PT experiments, since the TPC change when using a blood containing medium.

4. Conclusion

Porphyrins that are synthesized by the bacteria themselves are often used as photosensitizers in PT experiments on bacteria *in vitro*. However, lack of standard procedures for culturing conditions (passage number, growth time *etc.*) makes it difficult to compare aPDT/PT results from different studies. The results presented here show that there are large differences in the porphyrin profile in the model organisms when different culture conditions are used. The subculture procedure and the incubation time have a strong impact on bacterial TPC when the bacteria have all necessary enzymes for biosynthesis of heme. Microorganisms lacking these enzymes are not as highly affected by the length of culturing. Passage numbers are affecting the bacterial TPC and upon passaging, more porphyrin species can be detected in *A. actinomycetemcomitans* and the TPC increased 28 fold at day 7 of incubation in these experiments. When agar plates were supplemented with horse blood, *P. gingivalis* produced higher concentrations of porphyrins.

The results presented here demonstrate the need for more standardized methods to be employed when performing aPDT/PT experiments *in vitro*. The reason for this conclusion is the large variation in porphyrin profiles and concentrations, the aPDT/PT target compounds, in the bacteria obtained by different culturing conditions. High passage number, longer time of incubation, and agar plates supplemented with blood should probably be closer to imitate the conditions in the oral cavity and *in vitro* results of PT and aPDT experiments from different studies could be easier to compare. Further investigations concerning porphyrin content in periodontopathogenic bacteria at different culture conditions may lead to standardization protocols in bacterial cultivation when performing aPDT and/or PT experiments.

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