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Research Article

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In vitro detection of porphyrin-producing wound bacteria with real-time fluorescence imaging

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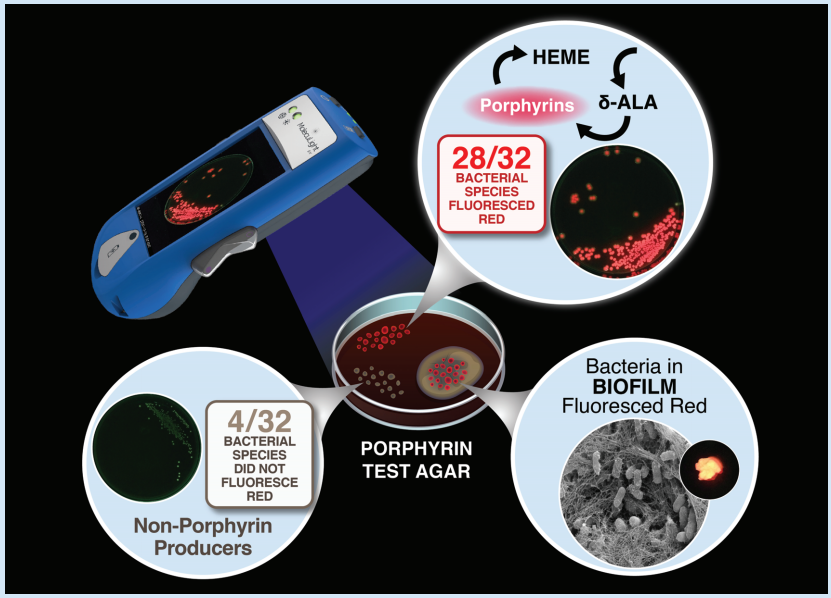
Aim: Fluorescence imaging can visualize polymicrobial populations in chronic and acute wounds based on porphyrin fluorescence. We investigated the fluorescent properties of specific wound pathogens and the fluorescence detected from bacteria in biofilm. **Methods:** Utilizing Remel Porphyrin Test Agar, 32 bacterial and four yeast species were examined for red fluorescence under 405 nm violet light illumination. Polymicrobial biofilms, supplemented with δ-aminolevulinic acid, were investigated similarly. **Results:** A total of 28/32 bacteria, 1/4 yeast species and polymicrobial biofilms produced red fluorescence, in agreement with their known porphyrin production abilities. **Conclusion:** These results identify common wound pathogens capable of producing porphyrin-specific fluorescence and support clinical observations using fluorescence imaging to detect pathogenic bacteria in chronic wounds.

Graphical abstract:



The porphyrin production and red fluorescing capabilities of 32 bacterial species were investigated using the MolecuLight i:X imaging device. A variety of bacteria species (32) commonly found in wounds were plated on porphyrin test agar, which includes ALA, a precursor of porphyrin. Under violet light illumination, 28/32 bacterial species fluoresced red while 4 known non-porphyrin producing species did not fluoresce red. Red fluorescence was also observed from porphyrin-producing bacterial species grown in a biofilm (as confirmed by SEM) when supplemented with nutrients to support porphyrin production and illuminated with violet light.

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Keywords: ALA • bacteria • biofilm • fluorescence • fluorescence imaging • heme • porphyrins • wounds

Wound care is a major clinical challenge which presents a burden to healthcare worldwide [1]. The presence of significant loads of bacteria in acute and chronic wounds can result in infection, which may delay or prevent wound healing [1–3]. Microbiological sampling is typically only performed when clinical signs and symptoms of infection are present and bacterial species and sensitivities need to be identified [4]. However, clinical signs and symptoms can be subjective and wounds harboring heavy levels of bacteria may appear asymptomatic despite delayed healing [5,6].

The development of bacterial biofilms may pose an additional challenge to treatment [7]. Biofilms contain sessile, polymicrobial communities of microorganisms encased in an exo-polysaccharide (EPS) layer [8,9]. This EPS serves multiple purposes, including promoting strong adherence and protection from environmental factors such as desiccation, immune targeting and antibiotic treatments [8,9]. The bacteria in these biofilms can be up to 1000-times more resistant to antimicrobials than planktonic (free-floating) bacteria [10]. The polymicrobial nature of wound infections [2] and biofilms [8,9] promotes synergy to accelerate their growth, reduce susceptibility to antimicrobials and is associated with worse patient prognoses [8,11]. Several *in vitro* and *in vivo* polymicrobial biofilm assays have been developed to investigate these interactions [11,12].

Fluorescence imaging has emerged as a method to visualize bacterial fluorescence in chronic wounds [13–16]. Clinical studies indicate that the handheld MolecuLight ***i:X*** fluorescence imaging device (MolecuLight, Inc., Toronto, Canada) visualizes bacterial fluorescence in real time based on the intrinsic production of red fluorescent porphyrins [17]. In multiple clinical trials, the presence of red fluorescence in wounds correlated with moderate-to-heavy bacterial loads [13–16]. A positive predictive value of 100% for detecting bacteria at these loads was observed in a multicenter clinical trial assessing red fluorescence in wounds [15]. Corresponding microbiology reports identified eight distinct bacterial species in areas of red fluorescence [15]. However, it is unclear which of the bacterial species in polymicrobial wounds contributes to the red fluorescence observed.

Bacterial autofluorescence is well established in the literature, largely attributed to the intrinsic production of red-fluorescent porphyrins [18,19], naturally-occurring intermediates in the heme biosynthetic pathway of bacteria and mammalian cells [20]. In order to synthesize heme, bacteria convert charged glutamyl-tRNA to δ-aminolevulinic acid (ALA) [20]. ALA is the nonfluorescing universal precursor to heme and represents the commitment step in the pathway [20]. Synthesis progresses through other intermediates including ringed porphyrin molecules before the incorporation of iron, resulting in heme [20]. The fluorescent properties of these ringed porphyrins are well established; excitation of porphyrins with ultraviolet or violet light results in the emission of light in the red wavelength region of the light spectrum [21,22].

Heme, obtained either through direct uptake or biosynthesis derived from porphyrin production, is important for the virulence of pathogenic bacteria [20]. In an analysis of 474 bacterial species, only 4% of these species exclusively uptake heme, while 82% performed biosynthesis or both methods [23] and thus would produce fluorescent porphyrins. However, these results are based on bioinformatic analysis of sequenced bacterial genomes and are not inclusive of all the most clinically relevant bacterial species. Indeed, there is limited literature characterizing the heme–porphyrin biosynthesis pathway of many bacterial species.

Thus, while there is clinical evidence to support the visualization of bacterial fluorescence in wounds excited by violet light illumination, we sought to expand our knowledge by characterizing the *in vitro* porphyrin-producing capability of many of the most prevalent bacterial species present in chronic wounds and determining if these bacteria, when present in a biofilm *in vitro*, continue to produce visible red porphyrin fluorescence. These data demonstrate the ability of the MolecuLight ***i:X*** to visualize porphyrin production in 28 of 32 bacterial species tested and one out of four yeast species. Furthermore, red fluorescence was detected in polymicrobial biofilms, suggesting that bacteria within biofilms can be visualized using this fluorescence imaging device.

Materials & methods

Bacterial & yeast strains

Bacterial and yeast strains were obtained from Mt. Sinai Clinical Microbiology or Texas Tech University. When possible, reference ATCC strains were selected; if unavailable, clinically isolated strains were substituted. Strain reference numbers can be found in Table 1 or in the Supplementary data.

Table 1. List of the 32 bacterial and four yeast species plated on Remel Porphyrin Test Agar and tested for red fluorescence production.

Species	Reference number	Aerobe/anaerobe	Gram	Red intensity at 40 h (aerobe) or 120 h (anaerobe/yeast) (± STDEV)	Red FL
Staphylococcus aureus	ATCC 29213	Aerobe	+	0.987 ± 0.007	Yes
Staphylococcus epidermidis	ATCC 12228	Aerobe	+	0.804 ± 0.143	Yes
Staphylococcus capitis	ATCC 35661	Aerobe	+	0.889 ± 0.080	Yes
Staphylococcus lugdunensis	ATCC 700328	Aerobe	+	0.982 ± 0.002	Yes
Pseudomonas aeruginosa	ATCC 27853	Aerobe	–	0.639 ± 0.049	Yes
Pseudomonas putida	MIFF-F03-BC729-3-7	Aerobe	–	0.678 ± 0.051	Yes
Enterococcus faecalis	ATCC 12386	Aerobe	+	0.0002 ± 0.0003	No
Escherichia coli	ATCC 25922	Aerobe	–	0.905 ± 0.041	Yes
Corynebacterium striatum	ATCC 1293	Aerobe	+	0.907 ± 0.027	Yes
Finegoldia magna	ATCC 29328	Anaerobe	+	0.0002 ± 0.0003	No
Proteus mirabilis	ATCC 12453	Aerobe	–	0.845 ± 0.018	Yes
Proteus vulgaris	ATCC 13315	Aerobe	–	0.718 ± 0.052	Yes
Enterobacter cloacae	ATCC 13047	Aerobe	–	0.882 ± 0.047	Yes
Serratia marcescens	ATCC 13880	Aerobe	–	0.780 ± 0.069	Yes
Acinetobacter baumannii	ATCC 19606	Aerobe	–	0.814 ± 0.081	Yes
Streptococcus agalactia	ATCC 12386	Aerobe	+	0 ± 0	No
Streptococcus mitis	ATCC 49456	Aerobe	+	0.002 ± 0.001	No
Klebsiella pneumoniae	ATCC 1705	Aerobe	–	0.582 ± 0.019	Yes
Klebsiella oxytoca	ATCC 700324	Aerobe	–	0.804 ± 0.049	Yes
Morganella morganii	MIFF-F03-BC707-1-6	Aerobe	–	0.706 ± 0.116	Yes
Propionibacterium acnes	MIFF-F03-BC713-4-4	Anaerobe	+	0.975 ± 0.014	Yes
Stenotrophomonas maltophilia	ATCC 17666	Aerobe	–	0.874 ± 0.091	Yes
Bacteroides fragilis	ATCC25285	Anaerobe	–	0.966 ± 0.017	Yes
Aeromonas hydrophila	ATCC 35654	Aerobe	–	0.778 ± 0.059	Yes
Alcaligenes faecalis	ATCC 35655	Aerobe	–	0.769 ± 0.148	Yes
Bacillus cereus	MIFF-F03-BC717-5-1	Aerobe	+	0.578 ± 0.077	Yes
Citrobacter koseri	ATCC 27156	Aerobe	–	0.984 ± 0.010	Yes
Citrobacter freundii	MIFF-F03-BC728-3-7	Aerobe	–	0.779 ± 0.105	Yes
Clostridium perfringens	ATCC 13124	Anaerobe	+	0.998 ± 0.001	Yes
Listeria monocytogenes	MIFF-F03-BC712-3-4	Aerobe	+	0.720 ± 0.232	Yes
Peptostreptococcus anaerobius	ATCC 27337	Anaerobe	+	0.941 ± 0.016	Yes
Veillonella parvula	MIFF-F03-BC728-8-5	Anaerobe	–	0.856 ± 0.112	Yes
Candida albicans	ATCC 10231	Yeast		0 ± 0	No
Candida guilliermondii	MICA-A01-LPTM1-3-7	Yeast		0.346 ± 0.024	Yes
Cryptococcus neoformans	ATCC 76484	Yeast		0.029 ± 0.051	No
Torulopsis glabrata	MIFF-F01-PHL35-1-6	Yeast		0.0003 ± 0.0005	No

Red fluorescence intensity was measured at 40 (aerobe) or 120 (anaerobe) h. Values are reported as averages with standard deviation. n = 3 per species.
FL: fluorescence; STDEV: standard deviation.

Bacteria & yeast preparation & plating

All bacteria and yeast were subcultured in suspension from frozen isolates. A 1:100 dilution of a 0.5 McFarland suspension was prepared. Agar plates were inoculated in triplicate and streaked using the four-quadrant method to obtain isolated colonies. Noninoculated plates were included as negative controls. Plates were incubated at 37°C either in oxygenated or anaerobic conditions and imaged at 24, 40 or 120 h post-plating (as indicated). Bimicrobial bacteria cultures were subcultured independently and combined at a 1:1 or 4:1 ratio as indicated immediately prior to plating.

To determine the appropriate *in vitro* conditions required for porphyrin production by bacteria, model bacterial species *Staphylococcus aureus* was plated on four different solid agar plates in triplicate and imaged under violet

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light after 24 and 40 h (Supplementary Figure 1). The agar plates chosen for analysis were: tryptic soy agar (TSA, a general-purpose bacterial growth media), blood agar (TSA with defibrinated sheep's blood), chocolate agar (blood agar with lysed blood cells) and Remel Porphyrin Test Agar (PTA; Thermo Fisher Scientific Microbiology Division, Oxoid Inc, Nepean, Canada), a commercially available agar comprised of a chocolate agar base supplemented with ALA to support porphyrin metabolism [24]. Red fluorescence was only observed when *S. aureus* was grown on the PTA plates, confirming the specificity of porphyrins as the source of red fluorescence. TSA possesses a strong intrinsic autofluorescence which appears bright green when imaged under violet light (Supplementary Figure 1), making it unsuitable for *in vitro* bacterial fluorescence imaging.

To correlate red fluorescence intensity with bacterial colony counts, *S. aureus* and *Escherichia coli* strains were subcultured, titered to 100 colony-forming unit (CFU) per plate and plated on Remel PTA. The inoculated plates were incubated for 12–48 h at 37°C. At the determined time point, replicates were imaged using the MolecuLight ***i:X*** and enumerated to obtain colony counts at these time points.

Biofilm procedure

As previously described [11,12], bacterial species were grown up and standardized to 10^8 CFU/ml. Done in seven replicates, 100 µl of each bacterial inoculate (10^7 CFU) was added to 7 ml of media (50% plasma, 50% Bolton's broth) with a P200 pipette tip to act as scaffold. Bacteria were incubated for 4 days at 37°C with shaking. On day 3, ALA (5 mM) was added to six biofilm cultures with one biofilm acting as a negative control. After 24 h of ALA incubation, the biofilms were removed from the culture, washed to remove any residual planktonic bacteria and finally removed from the scaffold. Standard and fluorescent images were taken at each stage of this process. Five biofilm cultures were imaged using a scanning electron microscope (SEM) to observe bacteria and EPS interactions. These samples were fixed and mounted onto imaging SEM sample mounts and then sputter coated with a thin layer of Au/Pd alloy for conductivity. SEM images were taken at four different magnifications (400, 3.5K, 6K and 10K) at each of four locations (12, 3, 6 and 9 o'clock) per sample using a Hitachi S/N 4300 field emission scanning electron microscope. The remaining biofilm was homogenized and plated on selective and differential media to obtain the final concentrations of each bacterial species.

Standard & fluorescence imaging

All standard and fluorescence images were taken using the MolecuLight ***i:X***. This device consists of a camera sensor, a fluorescence emission optical filter and two narrow-band 405-nm LEDs which produce violet light illumination. To capture fluorescence images, the room was darkened and the fluorescence mode (violet LEDs illuminating field of view) was switched on. The device includes a range finder and ambient light indicators which confirm an acceptable distance from the plates (8–12 cm) and suitable darkness for fluorescence imaging, respectively.

Image analysis

Raw color images were converted from the red, green, blue (RSV) color space to hue, saturation, value (HSV) format. The noise was removed and colonies were clustered using a *k*-means clustering algorithm [25]. Within these clustered colonies, the sum of the intensity of the red pixels (320–34°) was normalized to the sum of the intensity values of all clustered pixels. The triplicated values were averaged and plotted with error bars representing the standard deviation. A threshold of 0.2 was applied to distinguish between species that were considered to fluoresce red and those that did not (Table 1, Figures 1 & 4, Supplementary Figures 2, 3 & 6). Red fluorescence intensity over time was analyzed in the same manner but only the average intensity of the red pixels was reported (Figure 2).

Results

In vitro imaging of bacterial cultures

We examined the fluorescent properties of 32 of the most prevalent bacterial species in chronic wounds using a fluorescence imaging device (MolecuLight ***i:X***). The diverse genera of bacterial species included in our analysis were plated, in triplicate, on Remel PTA plates and incubated at 37°C in either oxygenated (aerobes) or anaerobic (anaerobes) conditions for up to 120 h. The bacteria were imaged for fluorescence under violet light illumination at 24 h (aerobes only), 40 and/or 120 h (anaerobes only; Figure 1, Supplementary Figures 2 & 3). An automated image analysis algorithm was used to determine the relative red fluorescent intensity of each species (Figure 1C & D, Supplementary Figures 2 & 3). A normalized red fluorescent intensity of greater than 0.2 was considered positive for red fluorescence (Table 1, Supplementary Figure 4).

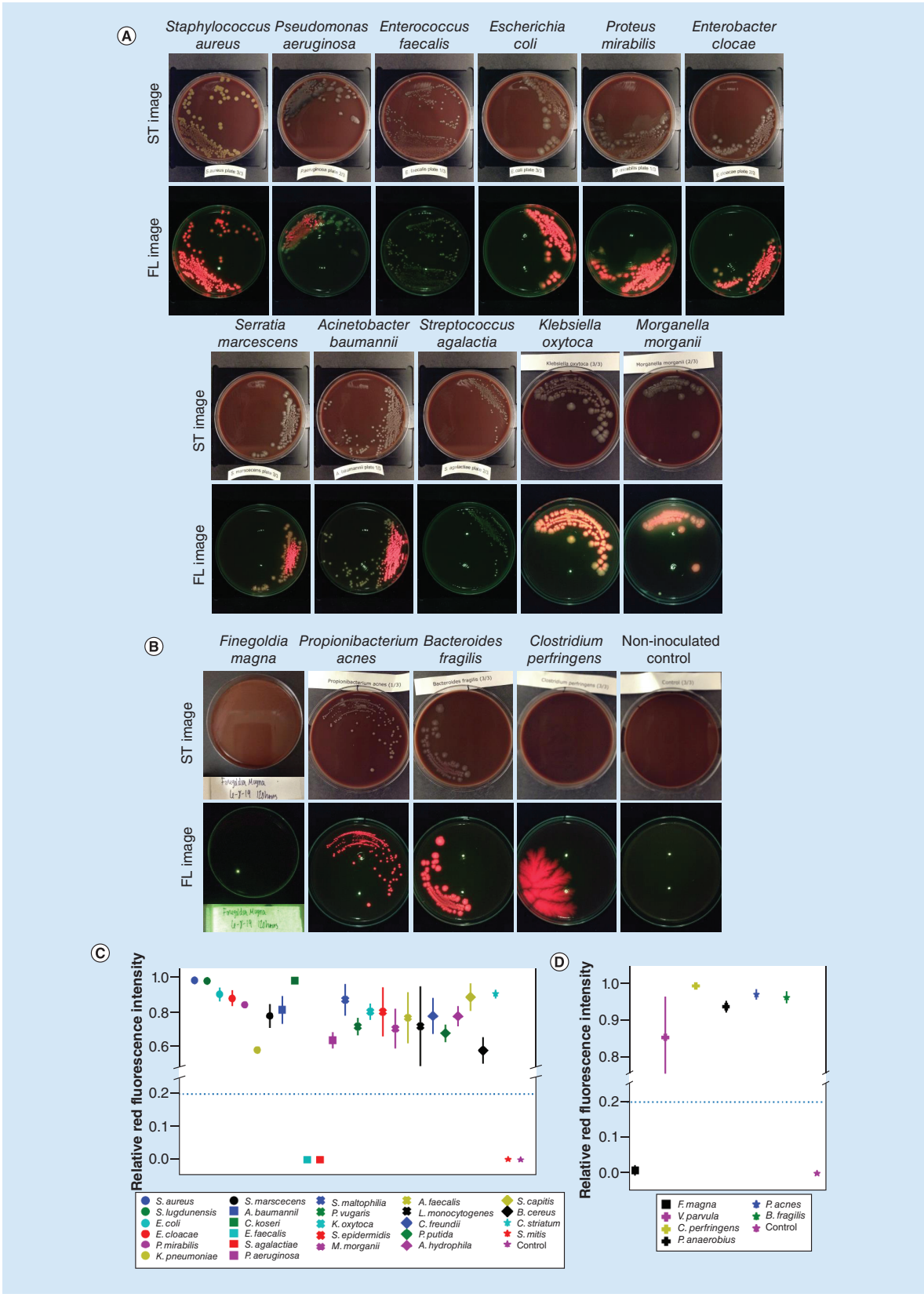

Figure 1. Porphyrin-producing aerobic and anaerobic bacteria emit red fluorescence under violet light illumination. Representative images of aerobic **(A)** and anaerobic **(B)** bacterial species plated in triplicate on Remel PTA. Bacteria were imaged under violet light after 40 h at 37°C in oxygenated **(A)** or 120 h in anaerobic conditions **(B)**. Noninoculated control plates are seen in the bottom right. Relative red fluorescence intensity of all aerobic species at 40 h **(C)** and anaerobic species at 120 h **(D)** was determined using an automated image analysis algorithm. Mean values are presented with error bars representing standard deviation. Values above 0.2 (blue dashed line) were considered positive for red fluorescence. (n = 3 plates per species)
PTA: Porphyrin Test Agar.

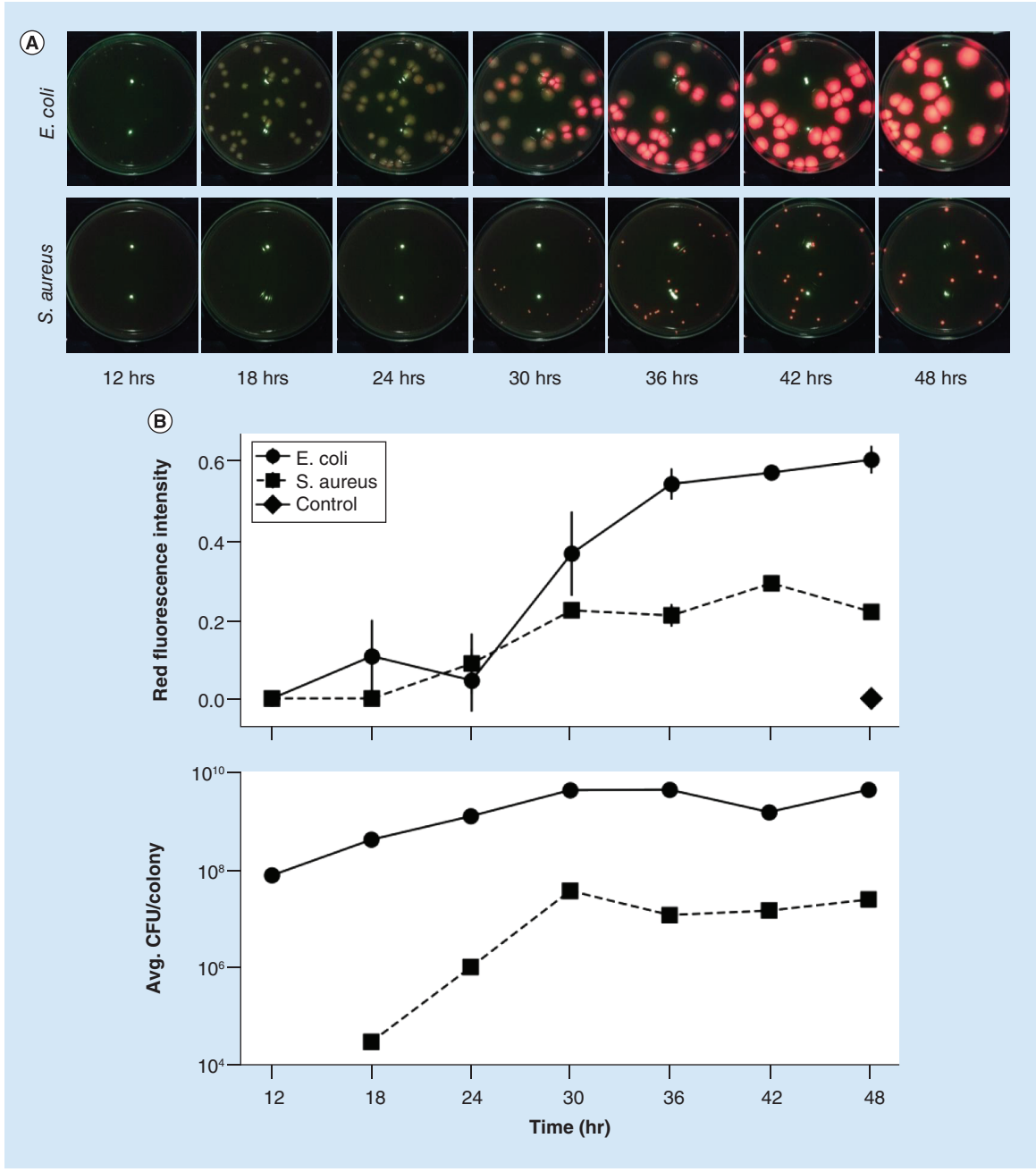

Figure 2. Red fluorescence emitted from *Escherichia coli* and *Staphylococcus aureus* over a 48-h period. To correlate red fluorescence intensity with bacterial counts, *Escherichia coli* **(A, top row)** and *Staphylococcus aureus* **(A, bottom row)** were plated on Remel PTA and incubated at 37°C for up to 48 h. Fluorescence images were captured every 6 h starting at 12 h and ending at 48 h using the MolecuLight ***i:X*** imaging device. Red fluorescence intensity of the bacterial colonies was determined using an automated image analysis algorithm. Uninoculated plates served as a negative control. Average CFU of each species were determined for each time point. Values are plotted as average and standard deviations (n = 3) for each time point.
CFU: Colony-forming unit; PTA: Porphyrin Test Agar.

Overall, 28/32 (87.5%) bacterial species displayed porphyrin-specific red fluorescence when illuminated with violet light after 40 h of incubation. After 40 h of incubation and under violet light illumination, 23 of the 26 aerobic bacterial species produced detectable red fluorescence, but three species (*Streptococcus agalactiae, Streptococcus mitis* and *E.faecalis*) failed to produce detectable red fluorescence above the 0.2 threshold (Figure 1A & C, Table 1).

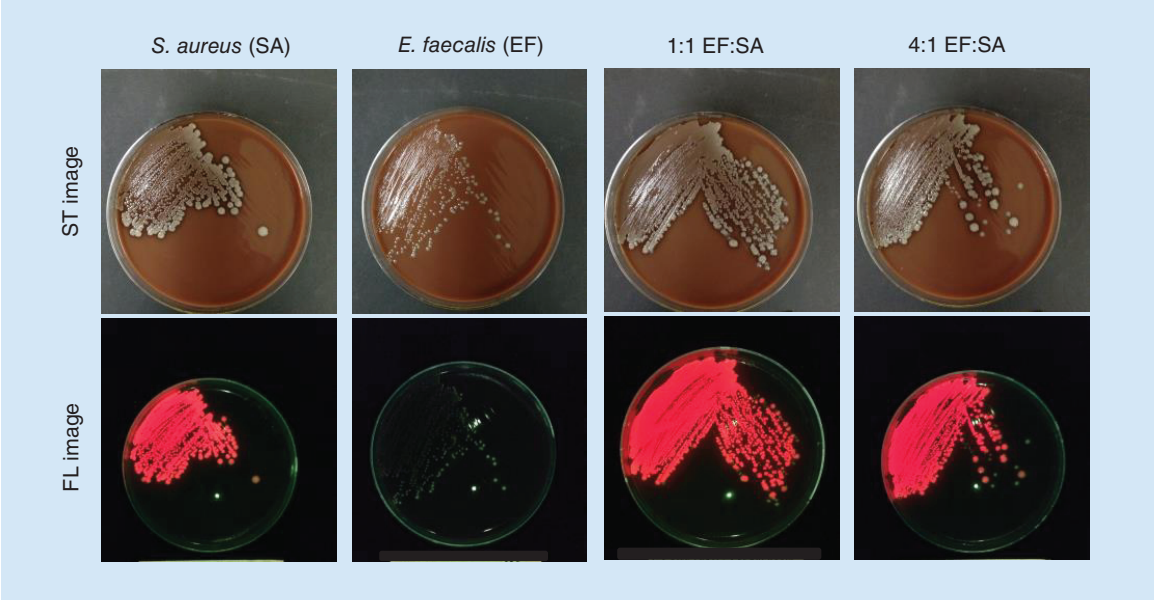

Figure 3. Red fluorescence is detectable from polymicrobial bacterial cultures consisting of porphyrin- and nonporphyrin-producing species. *Staphylococcus aureus* (porphyrin-producing species) and *Enterococcus faecalis* (nonporphyrin-producing species) were plated either individually or at a 1:1 or a 4:1 ratio of *E. faecalis:S. aureus* on Remel PTA, incubated at 37°C and imaged after 24 and 40 h. Representative images were captured at 40 h. From left to right: *S. aureus* alone, *E. faecalis* alone, 1:1 *E. faecalis:S. aureus* and 4:1 *E. faecalis:S. aureus*. (n = 3 plates per condition).
PTA: Porphyrin Test Agar.

At 24 h, most of the aerobic species displayed some red fluorescence, however, the fluorescence intensity was quite variable between the species (Supplementary Figure 2). While red fluorescence was detected at 40 h in *Pseudomonas spp, Stenotrophomonas maltophilia* and *Klebsiella pneumoniae*, red fluorescence was at or below the threshold at 24 h for these species (Supplementary Figures 2 & 4). This may be attributable to differences in growth, ALA uptake and porphyrin metabolism between bacterial species.

Due to the slower growth rate of anaerobic bacteria and challenges with their growth conditions, six anaerobic species were imaged under violet light after 40 and 120 h. Red fluorescence was observed in all anaerobic species except *Finegoldia magna* at 40 and 120 h (Figure 1B & D, Supplementary Figure 3). The intensity of the red fluorescence increased over time, as did the size of the bacterial colonies, similar to results from the aerobes. Uninoculated plates, which served as negative controls, did not display red fluorescence at any time point (Figure 1B, Supplementary Figure 4). Based on these results, the ideal time to investigate the red fluorescent properties of bacteria is 40 h post plating for aerobic bacteria and 120 h for anaerobic bacteria, though this is reliant on differences in bacterial growth dynamics. A complete table summarizing the fluorescent intensities of all bacterial species at all timepoints imaged is found in Supplementary Table 1; corresponding representative images of the bacterial species are displayed in Figure 1, Supplementary Figure 2 & 3. These results indicate that the majority of clinically relevant common wound pathogens investigated here produce red fluorescence when illuminated with violet light using the MolecuLight ***i:X*** imaging device.

Red fluorescence increases in intensity over time

Having observed an increase in red fluorescence intensity over time for most bacterial species (Supplementary Figure 2 & 3), we sought to investigate the relationships between time and bacterial growth on red fluorescence intensity. *S. aureus* and *E. coli* were plated as individual colonies and imaged every 6 h beginning at 12 h and ending at 48 h (Figure 2). Over the 48-h period, an increase in red fluorescence intensity and colony counts was observed in both bacterial species (Figure 2B). However, the increase in red fluorescence intensity was not directly proportional to bacteria count. More so than bacterial load, time appears to be a major factor affecting the intensity of the red fluorescence. In both cases, 24–30 h was required for the bacteria to take up the ALA and produce detectable red fluorescence, which appeared to plateau after 36 h. We observed intrinsic differences between the bacterial species,

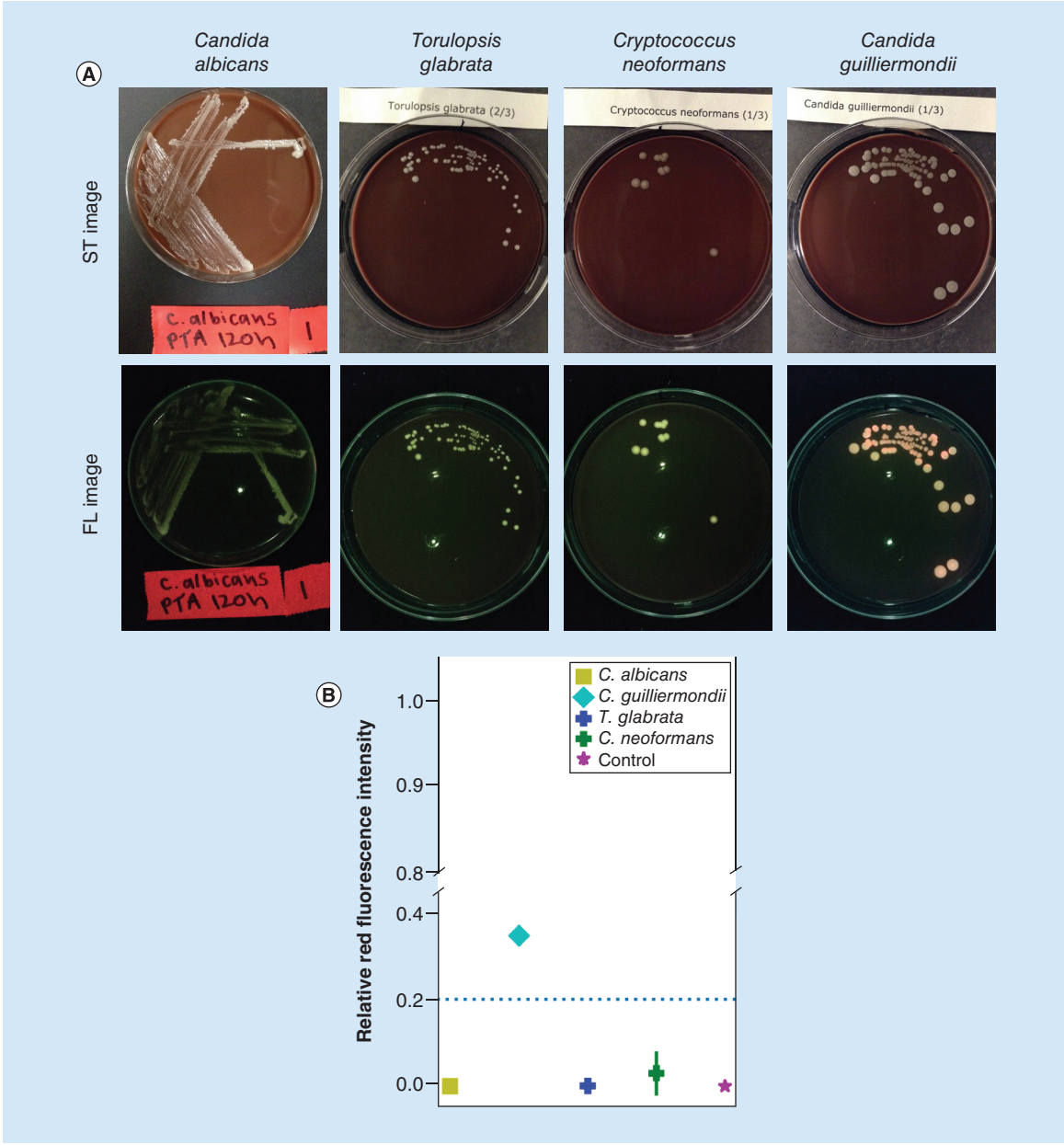

Figure 4. Most yeast species do not produce detectable red fluorescence under violet light illumination. Four yeast species were plated on Remel PTA, incubated at 37°C and imaged under standard and fluorescent light at 40 and 120 h post-plating. Representative images after 120 h are displayed **(A)**. Relative red fluorescence intensity was determined using an automated image analysis algorithm **(B)**. Mean values are presented with error bars representing standard deviation. Values above 0.2 were considered positive for red fluorescence. (n = 3 plates per species).
PTA: Porphyrin Test Agar.

both in terms of bacterial growth dynamics (*E. coli* 2-log growth vs *S. aureus* 3-log growth) and the intensity of the red fluorescence (*E. coli* 0.60 vs *S. aureus* 0.22 at 48 h). Thus, it appears that the variations in red fluorescence intensity are more dependent on the bacterial species than bacterial load in these experiments.

Detection of nonporphyrin producing bacteria in polymicrobial contexts

Only four of the 32 species assessed (from the *Enterococcus, Streptococcus* and *Finegoldia* genera) did not produce red fluorescence. Though nonporphyrin producing, non-red fluorescing bacterial species are rarely found in isolation [2] as most chronic wounds are polymicrobial, they may be the predominate pathogen in a wound. Thus,

we next aimed to determine if red fluorescence would be detectable if a nonporphyrin-producing bacterial species was the predominate species in a poly-microbial setting. To examine this, we plated *E. faecalis* (nonporphyrin producing) and *S. aureus* (porphyrin producing) on Remel PTA plates in a 1:1 and a 4:1 *Enterococcus:Staphylococcus* ratio and imaged them under violet light after 24 and 40 h (Figure 3, Supplementary Figure 4). In both ratios, red fluorescence was observed, confirming that red porphyrin fluorescence is detectable in situations where porphyrin-producing bacteria represent the minority of the bacterial population.

In vitro imaging of yeast cultures

Porphyrin production has long been associated with bacteria [18,21], but many yeast species contain the heme/porphyrin pathway [26]. In a clinical trial examining chronic wounds with fluorescence imaging, yeast (specifically *Candida albicans*) was identified by microbiological analysis from areas of red fluorescence in combination with other bacterial species [15]. To investigate whether yeast species might also produce red fluorescent signatures, four yeast species (*Candida albicans, Torulopsis glabrata, Cryptococcus neoformans* and *Candida guilliermondii*) were plated on Remel PTA, incubated at 37°C and imaged at 40 and 120 h. Of the four yeast species evaluated, only *Candida guilliermondii* produced detectable red fluorescence (Figure 4). After 120 h and under violet light illumination, a light pink fluorescence was observed in this species; no detectable fluorescence was observed at 40 h. The fluorescence detected was much lighter than the cherry red fluorescence observed in most of the bacterial species. These results suggest that the level of porphyrin production in most yeast species is not sufficient to produce detectable red fluorescence using the MolecuLight ***i:X***.

Imaging bacteria in biofilm

To assess the effects of biofilm on the visualization of red fluorescence, a polymicrobial culture comprised of a 1:1:1 ratio of *S. aureus:E. coli:Enterobacter cloacae* was grown in media based on an established biofilm assay [11,12]. Scanning electron microscopy (SEM) imaging was used to confirm presence of biofilm (Figure 5C). EPS matrix with closely associated rod and cocci bacteria were observed in SEM images. In the absence of host elements (e.g., blood) in the growth media, the presence of matrix surrounding these bacteria supports the presence of biofilm, consistent with others who utilize SEM to determine the presence of biofilm in wounds and *in vitro* wound models [12,27–30].

Under violet light illumination, red fluorescence was detected in all biofilms supplemented with ALA (Figure 5A). In the absence of ALA, the control biofilm did not produce red fluorescence (Figure 5B), suggesting that ALA and subsequent porphyrin production is required for the emission of red fluorescence.

The polymicrobial nature of the biofilms was confirmed through selective and differential plating of the homogenized biofilms that were not sent for SEM analysis. The final mean bacterial concentrations were *S. aureus* (5.0×10^4 CFU), *E. coli* (3.1×10^7 CFU) and *E. cloacae* (2.3×10^4 CFU); these species correspond with the rods and cocci observed in the SEM images. These data indicate that the excitation violet light and emitted red fluorescence can penetrate the EPS matrix surrounding these biofilms and the porphyrin producing bacteria found within can be detected via fluorescence imaging.

Discussion

Our results confirm that porphyrin metabolism in bacteria results in production of red fluorescence when illuminated with violet light. Here, we clearly demonstrate the capacity of porphyrin-producing bacteria, including gram-positive and gram-negative, aerobic and anaerobic species to emit red fluorescence when illuminated by violet light *in vitro* (Table 1). When grown on plates supplemented with ALA, we observed red fluorescence from 28/32 bacterial and 1/4 yeast species (Figures 1 & 4, Supplementary Figures 2 & 3). In addition, we observed red fluorescence from porphyrin-producing bacteria grown in biofilms when supplemented with ALA (Figure 5). Addition of ALA was necessary in both the PTA plates and the biofilm media in order to elicit this red fluorescence; in its absence, the bacteria did not fluoresce (Figure 1, Supplementary Figure 1).

Though we show that ALA supplementation is required to image bacteria under violet light *in vitro,* previous studies demonstrate it is not necessary for *in vivo* experiments [31] or clinically [13,15], as red fluorescence correlating to bacterial presence has been readily and repeatedly observed without the use of contrast agents [13–16]. The lack of red fluorescence from *Streptococcus* and *Enterococcus* genera was unsurprising as the *Streptococcus* and *Enterococcus* genera are well known to lack the ability to synthesize heme (and thus porphyrins) and instead rely solely on heme

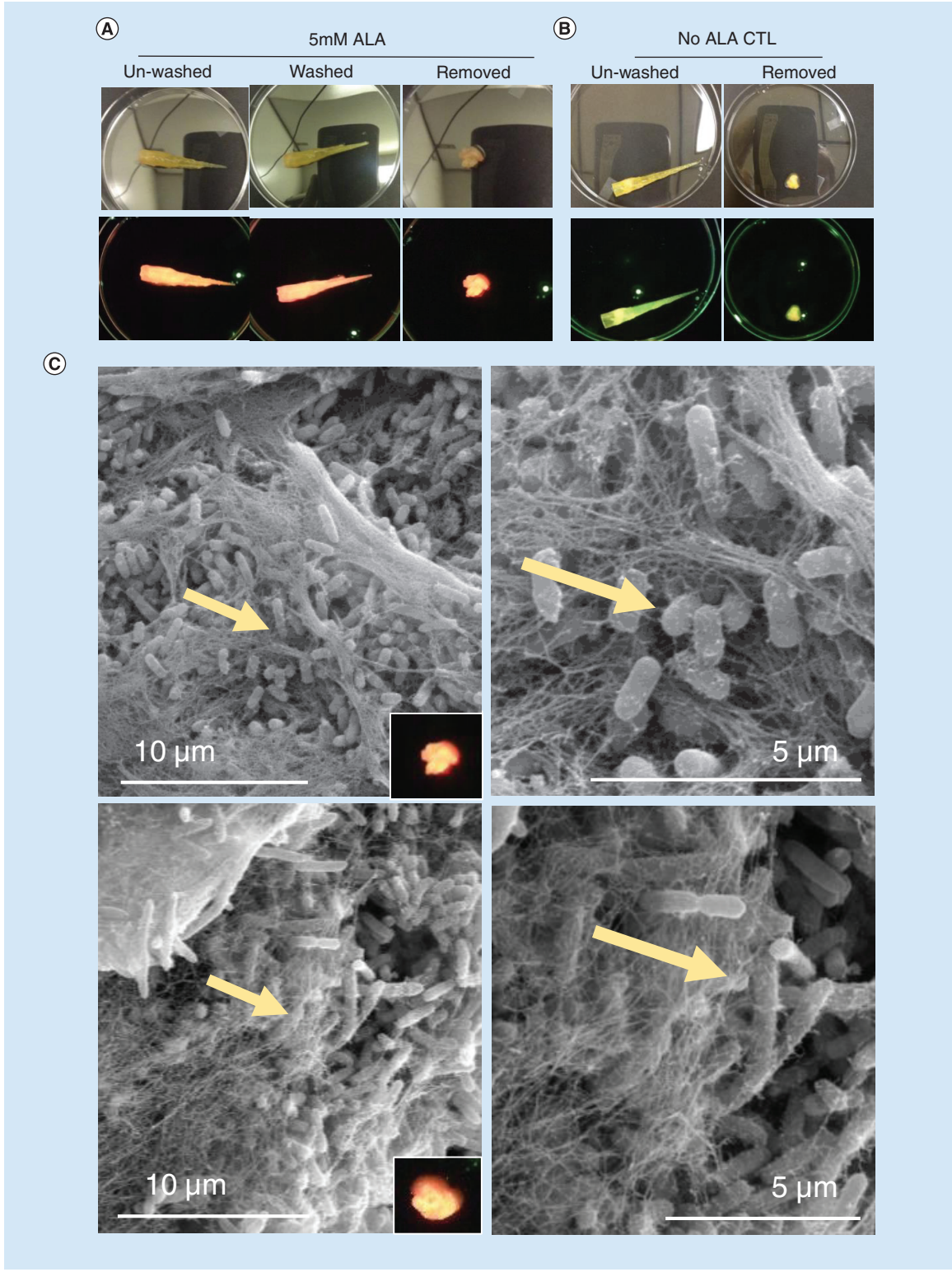

Figure 5. Polymicrobial biofilm cultures fluoresce red under violet light illumination in the presence of δ-aminolevulinic acid (ALA). Polymicrobial biofilms (1:1:1 ratio of *Staphylococcus aureus:Escherichia coli:Enterobacter cloacae*) were grown for 4 days in an established biofilm assay and incubated for 24 h with ALA. The biofilm and scaffold were removed from the media, washed to remove any planktonic bacteria and the biofilm was removed from the scaffold (n = 5). The same procedure was done for biofilm grown in the absence of ALA (negative control, n = 1). Representative standard and fluorescence images of unwashed, washed and removed biofilm grown in the presence of ALA **(A)** or absence of ALA **(B)** were taken using the MolecuLight ***i:X*** imaging device. SEM images of two representative washed samples are depicted in **(C)** at magnifications of 3500×, 10,000×, 3500× and 6000×, respectively (clockwise from top left panel). Yellow arrows point to regions of closely associated bacteria and EPS matrix.
ALA: δ-aminolevulinic acid; EPS: Exo-polysaccharide.

uptake [20,23]. The lack of red fluorescence detected in nonporphyrin-producing bacterial species further emphasizes porphyrin production as the source of the bacterial red fluorescence observed under violet light imaging.

A correlation between red fluorescence and predicted heme biosynthesis [23] was observed in all species tested except *Bacteroides fragilis. B. fragilis* displayed bright red fluorescence (Figure 1B & D), despite reports of insufficient genomic evidence of porphyrin production in this species [23]. However, KEGG pathway analysis has demonstrated the presence of many genes in the porphyrin/heme pathway. Multiple groups have suggested that heme uptake is required for growth of *B. fragilis,* a limitation that could not be overcome by the addition of ALA alone, suggesting an inability to produce heme [32]. Our results clearly demonstrate the ability of *B. fragilis* to produce red fluorescence, but it is important to note that porphyrin content was not directly assessed in this assay, merely inferred.

The presence of nonfluorescent bacterial species suggests a potential clinical limitation of this fluorescence imaging device. Though nonporphyrin-producing bacteria represent a small minority of species, *Enterococcus faecalis* and *Streptococcus agalactia* have been reported in between 6–72 and 1–39% of chronic wounds, respectively [33–36]. Similarly, *Finegoldia magna* is reported in up to 65.3% of wounds when measured using sequencing techniques [36] and may be underreported using culture analysis. However, in the largest study of wound microbiota to date (2963 wounds, analyzed via 16S rDNA pyrosequencing), these nonporphyrin-producing bacterial species appeared monomicrobially less than 1% of the time [2], as most chronic wounds are polymicrobial. Our bimicrobial investigations of *S. aureus* and *E. faecalis* (Figure 3) confirmed that red fluorescence is observed, even when nonporphyrin-producing bacteria are the predominant species. These *in vitro* results are further supported by clinical investigations where *E. faecalis* was detected in regions of red fluorescence in wounds imaged with the MolecuLight ***i:X*** [13,15]. In these studies, *E. faecalis* was detected along with other bacterial species [13,15], all of which were porphyrin-producing. In another clinical trial, red fluorescence was observed in a wound comprised of 46% nonporphyrin-producing species, confirmed using DNA pyrosequencing [16]. Together, these findings suggest that polymicrobial wounds may display red fluorescence even when nonporphyrin producing species are present or predominant.

The majority of yeast investigated, including *Candida albicans* (the most common yeast in chronic wounds [37,38]), did not emit red fluorescence under violet light illumination. However, red fluorescence was observed from *Candida guilliermondii,* which may represent a yeast capable of high porphyrin production. Studies on photodynamic damage of yeast have demonstrated lethal effects on *C. guilliermondii* with lower light irradiation power and a lack of exogenous sensitizers [26,39], compared with typical photodynamic treatments of other fungal infections [40]. The clinical significance of the ability of *C. guilliermondii* to emit red fluorescence is presently unclear, as this yeast does not appear to be particularly prevalent in chronic wounds [37,38].

Using established SEM imaging methods to confirm presence of biofilm, we show that bacteria within an EPS matrix can exhibit red fluorescence when supplemented with ALA *in vitro* and excited with violet light. Though visualization of red bacterial fluorescence has been previously observed in oral biofilms [41], the results presented here focus on the detection of bacterial species relevant to chronic wounds in polymicrobial biofilms. These results potentiate new avenues for future investigations, including those focused on characterizing bacterial fluorescence in *in vivo* biofilm models and potentially determining if distinct fluorescence signatures are observed between planktonic and biofilm bacterial cultures.

Limitations

The goal of our *in vitro* studies was to test the ability of bacterial and yeast species to produce porphyrins detectable by a fluorescence imaging device used clinically in wound care. Growth of bacteria and yeast on plates embedded with artificially high levels of ALA enabled this test on species of clinical interest in a mono- or bi-microbial setting. However, the *in vitro* setting does not accurately mimic the *in vivo* wound setting, where bacteria exist polymicrobially above, beneath and within various tissues types and fluids. These wound components also emit endogenous fluorescence and can, in some cases, attenuate or obscure bacterial fluorescence and/or absorb the excitation and fluorescence light due to optical absorption of light by blood. Host cells also can contain endogenous porphyrins [42] which can alter the availability of iron, thereby influencing the heme biosynthesis of bacteria. Importantly, clinical use of the ***i:X*** has demonstrated that skin tissue produces a green fluorescent signal primarily due to extracellular matrix components such as collagen, NADH and fibrin [15], but not an appreciable red fluorescence signal, thereby allowing the red bacterial fluorescence to be visualized.

Depth penetration of the violet light presents an additional limitation in the clinical situation, not accounted for in this assay. Violet light has been shown to penetrate human skin to a depth of 1 mm [43]; however the presence

of optical absorbers and scatterers such as hyperkeratotic cells, melanin and blood can influence the penetration of the excitation light. Thus, in the clinical setting where bacteria can exist deeper within the wound or beneath the skin, the excitation light may not cause these deeper bacteria to fluoresce. This is a limitation of the device that was not addressed in these experiments as all bacteria imaged were imaged as they grew on the culture plate surface.

Conclusion

In conclusion, these data demonstrate the ability of violet light fluorescence imaging to detect many clinically-relevant porphyrin-producing bacteria *in vitro.* Our findings confirm porphyrin production as the main biological source of the red fluorescence detected. Furthermore, we demonstrate capacity of violet light fluorescence imaging to detect red fluorescence emitted from bacteria in polymicrobial biofilms. These data support clinical studies that have validated the use of violet light induced fluorescence imaging with the MolecuLight ***i:X*** [13–16] and provide additional insight into which bacterial species are contributing to the bacterial fluorescence.

Futures perspective

Looking ahead, as more is learned about porphyrin biosynthesis and the associated fluorescence characteristics, we speculate that it may be possible to distinguish between bacterial species or between planktonic bacteria and biofilm. Subtle differences in fluorescent emission wavelengths, in fluorescence hue and/or relative differences in abundance of various porphyrins across genera or species [18], may be distinguishable through machine-learning techniques applied to fluorescence images. The potential for real-time identification of species present in a wound or real-time distinction of regions of a wound burdened by biofilm, which machine-learning may provide, could help to facilitate more species-targeted wound treatments. Information on bacterial loads through fluorescence image analysis could also result from this approach. The automated analysis of fluorescent hue and intensity changes with increasing bacterial loads presented herein represents an early first step on that path.

Summary points

- Fluorescence imaging has recently become possible at point-of-care to visualize bacterial fluorescence in wounds.
- By supplementing the growth medium with δ-aminolevulinic acid, red fluorescence can be observed from porphyrin producing bacterial species under violet light illumination.
- A total of 32 common bacterial wound pathogens were investigated for porphyrin specific fluorescence; 28 produced visible red fluorescence.
- Red fluorescence can still be detected in situations where nonporphyrin producing bacterial species are predominant.
- Polymicrobial biofilms of porphyrin producing bacteria produce red fluorescence under violet light illumination in the presence of δ-aminolevulinic acid.
- Most yeast species tested (3/4) do not produce red fluorescence in this assay.
- Fluorescence imaging can be used to detect the majority of clinically-relevant bacterial species in wounds.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/fmb-2019-0279

Author contributions

LM Jones, D Dunham, MY Rennie, RS DaCosta and AC Smith were responsible for study conception and design; authors LM Jones, D Dunham, MY Rennie, AJ Lopez, KC Keim, W Little, A Gomez, J Bourke and H Ng were responsible for acquisition of data; authors LM Jones, J Kirman, MY Rennie and AC Smith were responsible for data analysis and drafting and revision of the manuscript.

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