Case Report

Exogenous coproporphyrin III production by *Corynebacterium aurimucosum* and *Microbacterium oxydans* in erythrasma lesionsAyako Yasuma,¹ Toyoko Ochiai,¹ Motoki Azuma,² Hiroyuki Nishiyama,³ Ken Kikuchi,⁴ Masao Kondo⁵ and Hiroshi Handa²

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Erythrasma is a superficial skin disease caused by Gram-positive *Corynebacterium* species. Coral-red fluorescence under Wood's light, strongly suggestive of erythrasma, can be attributed to the presence of porphyrins. Fractionated porphyrin analysis in erythrasma lesions is yet to be reported. We attempted to investigate erythrasma lesions by isolating the responsible bacteria and determining their exogenous porphyrin production by HPLC analysis. We observed a 78-year-old woman with erythrasma who had a well-demarcated slightly scaling patch on her left foot, between the fourth and fifth toes. Two kinds of colonies on 5 % sheep blood agar were obtained from this lesion. Analysis of the 16S rRNA sequence revealed the colonies to be *Corynebacterium aurimucosum* and *Microbacterium oxydans*. HPLC analysis demonstrated that coproporphyrin III (Copro III) levels were clearly elevated, although the amounts of protoporphyrin were diminished. These results indicate that the fluorescent substance was Copro III. This study supports the view that excess Copro III synthesis by *C. aurimucosum* and *M. oxydans* leads to accumulation of porphyrin in cutaneous tissue, which emits a coral-red fluorescence when exposed to Wood's light.

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Introduction

Erythrasma is a skin disease characterized by pink patches that sometimes become brown scales. The disease tends to occur at intertriginous sites, and exhibits a coral-red fluorescence under Wood's light (Hay & Adriaans, 1998). Sarkany *et al.* (1961) demonstrated the presence of Gram-positive rods in the scales of erythrasma and named the causative organism *Corynebacterium minutissimum* (Sarkany *et al.*, 1962). This bacterium is thought to produce excess coproporphyrins (Hay & Adriaans, 1998). Currently, we know very little about the epidemiology of erythrasma and the nature of porphyrin production by these bacteria. We have described a case of erythrasma from which *Corynebacterium aurimucosum* and *Microbacterium oxydans* were isolated. The aim of this study was to examine the amount of extracellular and intracellular porphyrin produced by these bacteria and to clarify the nature of the

porphyrin responsible for coral-red fluorescence. Photoinactivation of bacteria by endogenous porphyrins is of growing interest in the field of photodynamic therapy. If the existence of intracellular porphyrin produced by these bacteria is identified, an appropriate method of photodynamic therapy for erythrasma could be developed. To our knowledge, this is the first study to employ HPLC to investigate porphyrins of bacteria detected from erythrasma lesions.

Case report

A 78-year-old woman with a 1 month history of an asymptomatic scaling rash between the fourth and fifth toes on her left foot was seen in March 2007. Physical examination revealed a well-demarcated, reddish brown, slightly scaly patch with interdigital peeling. Samples dissolved in potassium hydroxide showed no dermatophytes in the affected areas. The patch exhibited coral-red

Abbreviations: Copro III, coproporphyrin III; Uro, uroporphyrin.

fluorescence under Wood's light (Fig. 1). Laboratory test results were within the normal range with the exception of slight hyperglycaemia. The clinical diagnosis was erythrasma. The patient was treated with a topical application of gentamicin ointment in the affected area. The coral-red fluorescence was no longer evident after 4 weeks of treatment.

Smear samples were taken from the scales present in the interdigital sites before treatment and cultured. The cultures were incubated at 35 °C on 5% sheep blood agar for 18 h and Gram stains were performed. Two different types of small, smooth colonies grew from the smear samples. One colony was white while the other was slightly yellow and both colonies fluoresced coral-red under Wood's light. Gram staining revealed similar Gram-positive rods from the colonies, 2–3 µm in length. The bacterial cells were arranged in a V formation or in palisades, characteristic of coryneform bacteria.

Identification tests for *Corynebacterium* were performed on the colonies using the commercial RapID CB PLUS System (Remel) according to the manufacturer's protocols (Funke *et al.*, 1998). As the results did not correspond to those for any corynebacteria, DNA from the two colonies was subjected to sequencing analysis targeting the 16S rRNA genes. Extraction of DNA was performed using achromopeptidase as previously described (Kikuchi *et al.*, 2003). The primers used for PCR amplification were Univ1 (5'-GAG AGT TTG ATC CTG GCT CAG GA-3') and Univ2 (5'-AAG GAG GTG ATC CAG CCG CA-3') and were synthesized by Operon Biotechnologies (Edwards *et al.*, 1989). The PCR was carried out in a 50 µl volume containing 2.5 U *Taq* polymerase (Perkin-Elmer Cetus), 10 × PCR buffer (TaKaRa), 0.2 mM dNTPs and 25 pmol of each primer. The thermal profile involved an initial denaturation step at 95 °C for 10 min, and then 30 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 90 s; the

reaction was terminated by a final extension step at 72 °C for 5 min.

Sequencing reactions were performed using a Big Dye terminator (version 1.1) Cycle Sequencing kit (Applied Biosystems) in conjunction with an ABI PRISM 3100 genetic analyser (Applied Biosystems) (Sasaki *et al.*, 2007). Multiple alignment was carried out by the CLUSTAL_X program (Thompson *et al.*, 1997). Construction of the unrooted phylogenetic tree was performed by the neighbour-joining method (Saitou & Nei, 1987).

We extracted haem precursors from the *C. aurimucosum* and *M. oxydans* strains and conducted HPLC analysis on these precursors (Fig. 2a, b). To detect haem precursors in the bacteria, colonies obtained from the sheep blood agars were grown for 96 h in thioglycollate medium (Becton Dickinson) at 37 °C (Thomson & Miller, 2003). The concentration and absolute number of cells in culture was measured using the McFarland method (McFarland, 1907). The culture was centrifuged at 3000 g for 10 min at 37 °C and the supernatant and precipitate were processed separately for HPLC analysis. As a control, a strain of *Staphylococcus aureus* that was isolated from a case of impetigo contagiosum and did not exhibit fluorescence under Wood's light was used in the present study. The precipitates were homogenized with 5 ml ethyl acetate:acetic acid (3:1, v/v). After centrifugation at 5000 g for 10 min, the supernatant was concentrated into 1 ml and 10 µl samples and subjected to chromatography on a C₁₈ reverse-phase HPLC column equipped with a fluorometric detector (Kondo & Kajiwara, 1987; Azuma *et al.*, 2008). The culture medium was lyophilized, extracted with 5 ml ethyl acetate:acetic acid (3:1, v/v) and subjected to chromatography (Nitzan *et al.*, 2004). In the culture medium, the amount of coproporphyrin III (Copro III) was significantly increased in *C. aurimucosum* and *M. oxydans* compared to *S. aureus* strains ($P < 0.05$, Student's *t*-test), while the levels of uroporphyrin (Uro) and other haem precursors were not increased (Fig. 2c, d). In the cell pellets, the amounts of Uro and Copro III were significantly increased in *C. aurimucosum* and *M. oxydans* compared with *S. aureus* ($P < 0.05$).

The Japanese Ministry of Health, Labour and Welfare provides guidelines on ethics regarding clinical research. These guidelines state that if the research was carried out as a part of a physical examination, and the field of research is directly related to micro-organisms detected in specimens of patients, and is not a phenomenon of the patient's health, then ethics approval is not required. Therefore, we consider that our study required no ethics approval or informed consent.

Discussion

In this study, we isolated *C. aurimucosum* and *M. oxydans* from erythrasma lesions, conducted 16S rRNA sequence analysis on the isolates, and identified Copro III in the

Fig. 1. Well-demarcated, reddish brown, slightly scaly patch seen on the left foot of the patient between the fourth and fifth toes. The patch fluoresced coral red under Wood's light.

Fig. 2. Analysis of haem biosynthesis in *C. aurimucosum*, *M. oxydans* and *S. aureus*. (a) Haem precursors in culture medium obtained from *C. aurimucosum*, *M. oxydans* and *S. aureus* were analysed by HPLC. Colonies were grown for 96 h at 37 °C. The culture medium was volatilized under negative pressure and then porphyrins were extracted with 5 ml ethyl acetate : acetic acid (3 : 1, v/v) and subjected to chromatography on a C₁₈ reverse-phase HPLC column equipped with a fluorometric detector. (b) Intracellular haem precursors from *C. aurimucosum*, *M. oxydans* and *S. aureus*. Bacteria were grown for 96 h at 37 °C and homogenized with 5 ml ethyl acetate : acetic acid (3 : 1, v/v). After centrifugation at 5000 g for 10 min, the supernatant was concentrated and subjected to chromatography. Quantification of each haem precursor obtained from culture medium (c) and from *C. aurimucosum*, *M. oxydans* and *S. aureus* cells (d). In the medium, the amount of Copro III was increased in *C. aurimucosum* and *M. oxydans* compared with *S. aureus* (c). In the cells, the amount of Uro and Copro III were significantly increased in *C. aurimucosum* and *M. oxydans* compared to *S. aureus* (d). The data were analysed with a fluorometric detector. Uro, Uroporphyrin; Copro, coproporphyrin; 7CP, heptaporphyrin; 6CP, hexaporphyrin; 5CP, pentaporphyrin; Proto, protoporphyrin. Quantification of haem precursors is presented as the mean $\pm$ SEM from six independent experiments. Student's *t*-test was performed to determine statistical significance among the experimental groups; **P* < 0.05 was considered statistically significant.

culture medium. To the best of our knowledge, there have been no previous reports in which *C. aurimucosum* and *M. oxydans* were isolated from lesions associated with erythrasma.

It is known that erythrasma is a superficial skin disease caused by *Corynebacterium* species. Sarkany *et al.* (1961) originally detected Gram-positive rods from lesions associated with erythrasma and named the bacterium *C. minutissimum* (Sarkany *et al.*, 1962). Somerville (1973) classified aerobic cutaneous diphtheroids into 15 groups from the results of nine tests. These groups encompass lipophilic characteristics, lipolytic ability, porphyrin production, nitrate reduction and ability to decompose glucose, maltose, sucrose, fructose and galactose. This classification differs from that described in *Bergey's Manual of Systematic Bacteriology*. Somerville (1973) stated that all groups of fluorescent diphtheroids were isolated from erythrasma lesions as the result of an overgrowth of diphtheroid members in the normal skin flora, and that *C. minutissimum* belonged to group 2. Takei (1996) succeeded in the isolation of 24 fluorescent diphtheroids from 43 lesions and identified them using API biochemical tests. Sixteen isolates were *C. minutissimum*, six were *Corynebacterium jeikeium*, one was coryneform group G-1 and the final isolate was coryneform group G-2. Takei (1996) also stated that *C. jeikeium* and *C. minutissimum* belonged to group 2 of Somerville's classification scheme.

The strain of *C. aurimucosum* isolated in this study was novel, as described by Yassin *et al.* (2002). Strains originally designated CDC coryneform group 4 bacteria represent a heterogeneous group and include pigmented variants of *C. aurimucosum*. *Corynebacterium nigricans* is related to *C. aurimucosum* at the species level as demonstrated by DNA–DNA hybridization analysis and sequencing of the 16S rRNA gene (Daneshvar *et al.*, 2004). Yassin *et al.* (2002) stated that *C. aurimucosum* is genealogically related to *C. minutissimum* (99.8% nucleotide identity for 16S rRNA) with a similar basic biochemical profile. The DNA–DNA hybridization experiments demonstrated that *C. aurimucosum* is distinct from *C. minutissimum* with only 42% nucleotide sequence identity (Yassin *et al.*, 2002).

M. oxydans is a coryneform bacterium that grows aerobically, is asporogeneous, irregularly shaped, not acid-fast, and can be visualized as Gram-positive rods. The majority of *Microbacteria* form colonies with a yellow pigment on nutrient agar. *M. oxydans* is one of the species of *Microbacteria* most frequently isolated from clinical specimens.

Only a small number of studies examining porphyrins from erythrasma lesions have been published. Sarkany *et al.* (1961) examined aqueous extracts of fluorescent substances from culture medium using a spectrophotometer and described that the substances had a maximum peak at 400–402 nm and a single sharp peak at 603 nm, although fractionated porphyrin analysis was not demonstrated. Hay

& Adriaans (1998) stated that the coral-red fluorescence under Wood's light in erythrasma lesions was due to Copro III, but did not provide any further details of their results.

In our study, HPLC analysis of haem precursors from the culture medium of *C. aurimucosum* and *M. oxydans* revealed that Copro III levels were clearly elevated (Fig. 2c) and the amounts of protoporphyrin were diminished. Analysis of intracellular porphyrin showed that the Uro and Copro III levels were very high, although the protoporphyrin levels were low (Fig. 2d). These results indicate that the fluorescent substance was actually Copro III. We could not clarify the complex mechanisms of excess Copro III production in the supernatants in this study. The conversion from Copro III to protoporphyrinogen IX is carried out by coproporphyrinogen oxidase (Hayashi, 1995). Almost nothing is known about the regulation of haem-biosynthetic enzyme activities in *Corynebacterium* bacteria. We postulate that the low levels of coproporphyrinogen oxidase activity in *C. aurimucosum* and *M. oxydans* likely lead to an accumulation of large quantities of Copro III.

Recently, photoinactivation of bacteria by endogenous porphyrins has garnered interest in photodynamic therapy (Maisch *et al.*, 2004; Nitzan *et al.*, 2004). In this study, analysis of intracellular porphyrin in *C. aurimucosum* and *M. oxydans* showed that the levels of Uro and Copro III were very high. Porphyrins have a common absorption maximum (Soret band) around 400 nm and another peak at 500–650 nm (Kondo, 1995). The photodestructive wavelength range of porphyrins is reckoned to be 407–420 nm (Ashkenazi *et al.*, 2003). The bacterium *Propionibacterium acnes* produces mainly Copro III, which accumulates in the cells. The effectiveness of photodynamic therapy for acne treatment has been demonstrated by Kawada *et al.* (2002), who reported that blue light (407–420 nm) was effective for acne patients in a clinical setting. Ashkenazi *et al.* (2003) stated that illumination of the endogenous coproporphyrin with blue light played a major role in *P. acnes* photoinactivation. We believe that photodynamic therapy, especially with light in the blue wavelengths, could be applied to treat erythrasma lesions.

In conclusion, the results of this study support the view that excess Copro III synthesis by *C. aurimucosum* and *M. oxydans* leads to accumulation of porphyrin in cutaneous tissue, which fluoresces a coral-red colour when exposed to Wood's light.

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