Metabolism of azo dyes by human skin microbiota

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Reduction of Methyl Red (MR) and Orange II (Or II) by 26 human skin bacterial species was monitored by a rapid spectrophotometric assay. The analysis indicated that skin bacteria, representing the genera *Staphylococcus*, *Corynebacterium*, *Micrococcus*, *Dermacoccus* and *Kocuria*, were able to reduce MR by 74–100 % in 24 h, with only three species unable to reduce completely the dye in that time. Among the species tested, only *Corynebacterium xerosis* was unable to reduce Or II to any degree by 24 h, and only *Staphylococcus delphini*, *Staphylococcus sciuri* subsp. *sciuri* and *Pseudomonas aeruginosa* were able to reduce completely this dye within 24 h. MR reduction started with early-exponential growth in *Staphylococcus aureus* and *Staphylococcus epidermidis*, and around late-exponential/early-stationary growth in *P. aeruginosa*. Reduction of Or II, Ponceau S and Ponceau BS started during late-exponential/early-stationary growth for all three species. Using liquid chromatography/electrospray ionization mass spectrometry analyses, MR metabolites produced by *Staph. aureus*, *Staph. epidermidis* and *P. aeruginosa* were identified as \( \text{N,N-dimethyl-p-phenylenediamine and 2-aminobenzoic acid.} \)

Searches of available genomic and proteomic data revealed that at least four of the staphylococci in this study, *Staphylococcus haemolyticus*, *Staph. epidermidis*, *Staphylococcus cohnii* and *Staphylococcus saprophyticus*, have hypothetical genes with 77, 76, 75 and 74 % sequence identity to azo1 encoding an azoreductase from *Staph. aureus* and hypothetical proteins with 82, 80, 72 and 74 % identity to Azo1, respectively. In addition, *Staphylococcus capitis* has a protein with 79 % identity to Azo1. Western analysis detected proteins similar to Azo1 in all the staphylococci tested, except *Staph. delphini*, *Staph. sciuri* subsp. *sciuri* and *Staphylococcus auricularis*. The data presented in this report will be useful in the risk assessment process for evaluation of public exposure to products containing these dyes.

INTRODUCTION

Azo dyes are characterized by one or more azo bonds (R–N=N–R) that allow visible light to be absorbed by the dyes. These dyes are used in a wide variety of consumer products including textile dyes, tattoo inks and cosmetics (Moller & Wallin, 2000). These products directly contact human skin for several hours on a daily basis (Golka et al., 2004). In 2007, approximately 95 million US women used cosmetics. Estimates indicate that over 45 million US citizens currently have some type of tattooing (tattoo or permanent make-up).

Azo dyes may be biotransformed into colourless aromatic amines that are more easily absorbed by the skin. Some of these metabolites may be less toxic than the original dye (Collier et al., 1993), while others, such as arylamines and free radicals, are potentially carcinogenic (Chung, 1983; Mason et al., 1977; Nakayama et al., 1983). Thousands of azo dyes are available and more than 500 contain potentially carcinogenic aromatic amines (Platzek et al., 1999). A cancer risk assessment for the National Institute of Public Health and the Environment of The Netherlands found carcinogenic aromatic amines in commonly used products coloured by azo dyes including textile toys, bed sheets and watch straps (Zeilmaker et al., 2000).

While azo dyes are generally considered to be persistent pollutants because they are typically recalcitrant to aerobic biotransformation (Chen, 2006; Stolz, 2001), they may be metabolized by azoreductases from intestinal microorganisms and from mammalian liver cells (Levine,
Azo dye metabolism has been described in anaerobic and aerobic bacteria (Levine, 1991; Xu et al., 2007).

Two types of azoreductases have been identified in bacteria: (1) monomeric flavin-free enzymes containing a putative NAD(P)H binding motif and (2) polymeric flavin-dependent enzymes (Chen, 2006). Although anaerobic azoreductases have been characterized from both aerobic and anaerobic dye reduction (Stolz, 2001), aerobic azoreductases have been characterized from many aerobic and anaerobic members of the human microbiome (Chen et al., 2004, 2005, 2008; Liu et al., 2007). A tetrameric, flavin-dependent, NADPH-dependent azoreductase (Azol) from Staphylococcus aureus (ATCC 25923) metabolizes Methyl Red (MR) to the colourless products 2-aminobenzoic acid and N,N-dimethyl-p-phenylenediamine, and also metabolizes the azo dyes Orange II (Or II), Amaranth, Ponceau BS and Ponceau S (Chen et al., 2005).

At least several hundred species of bacteria are residents of the human skin (Gao et al., 1998; Kloos & Musselwhite, 1975). A number of species that were originally classified as micrococcii have been reclassified into the Kocuria, Kytopoccus and Dermacoccus genera (Stackebrandt et al., 1995). Any number of human skin microbiota species potentially express azoreductase(s) and may contribute to the metabolism of azo dyes with which they come into contact (Chen et al., 2005; Platzek et al., 1999).

The current report examines the reduction of azo dyes by specific skin bacteria from nine different genera, including Staphylococcus, Dermacoccus, Kocuria, Micrococcus, Kytopoccus and Corynebacterium. This study also detects proteins with some similarity to Azol that are expressed by many of the species examined.

### METHODS

**Bacterial strains.** Bacterial strains NCH 281–302 (Table 1) were obtained from Taxonometrics. Staphylococcus epidermidis (NTH 118) and Staph. aureus (NTH 125) were kindly provided by Dr Mark Hart (US Food and Drug Administration). The remaining strains were from the NCTR collection. All strains were maintained aerobically in brain heart infusion (BHI) medium at 37 °C for 16–18 h, except for proper growth, so reduction experiments were conducted under these conditions.

### Table 1. Azo dye reduction by skin bacteria

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Name</th>
<th>MR reduction</th>
<th>Or II reduction</th>
<th>Western*</th>
<th>Isolation source (ATCC no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCH 281</td>
<td>Dermacoccus nishinomiyaiensis</td>
<td>74 % by 24 h</td>
<td>15 % by 24 h</td>
<td>Multi</td>
<td>Water (29093)</td>
</tr>
<tr>
<td>NCH 282</td>
<td>Kocuria kristinae</td>
<td>100 % by 7 h</td>
<td>25 % by 24 h</td>
<td>+</td>
<td>Human skin (27570)</td>
</tr>
<tr>
<td>NCH 283</td>
<td>Micrococcus luteus</td>
<td>100 % by 14.5 h</td>
<td>35 % by 24 h</td>
<td>–</td>
<td>ATCC 4698</td>
</tr>
<tr>
<td>NCH 284</td>
<td>Micrococcus lylae</td>
<td>100 % by 12.5 h</td>
<td>35 % by 24 h</td>
<td>Multi</td>
<td>Human skin (27566)</td>
</tr>
<tr>
<td>NCH 285</td>
<td>Staphylococcus auricularis</td>
<td>100 % by 9 h</td>
<td>33 % by 24 h</td>
<td>–</td>
<td>Human external auditory canal (33753)</td>
</tr>
<tr>
<td>NCH 286</td>
<td>Staphylococcus capitis subsp. capitis</td>
<td>96.4 % by 7 h†</td>
<td>21 % by 24 h</td>
<td>+</td>
<td>Human skin (27840)</td>
</tr>
<tr>
<td>NCH 287</td>
<td>Staphylococcus caprae</td>
<td>100 % by 1.25 h</td>
<td>58 % by 24 h</td>
<td>+</td>
<td>Human skin (35538)</td>
</tr>
<tr>
<td>NCH 289</td>
<td>Staphylococcus chromogenes</td>
<td>100 % by 1.25 h</td>
<td>97 % by 24 h</td>
<td>+</td>
<td>Pig skin (43764)</td>
</tr>
<tr>
<td>NCH 290</td>
<td>Staphylococcus cohnii subsp. cohnii</td>
<td>100 % by 9 h</td>
<td>19 % by 24 h</td>
<td>+</td>
<td>Human skin (29974)</td>
</tr>
<tr>
<td>NCH 291</td>
<td>Staphylococcus delphini</td>
<td>100 % by 0.5 h</td>
<td>100 % by 21 h</td>
<td>–</td>
<td>Dolphin purulent matter (49171)</td>
</tr>
<tr>
<td>NCH 292</td>
<td>Staphylococcus hominis subsp. hominis</td>
<td>100 % by 8 h</td>
<td>41 % by 24 h</td>
<td>+</td>
<td>Human skin (27844)</td>
</tr>
<tr>
<td>NCH 294</td>
<td>Staphylococcus lugdunensis</td>
<td>100 % by 4.25 h</td>
<td>58 % by 24 h</td>
<td>+</td>
<td>Axillary lymph node (43809)</td>
</tr>
<tr>
<td>NCH 295</td>
<td>Staphylococcus saprophyticus</td>
<td>100 % by 5 h</td>
<td>34 % by 24 h</td>
<td>+</td>
<td>Human (15305)</td>
</tr>
<tr>
<td>NCH 297</td>
<td>Staphylococcus sciuri subsp. sciuri</td>
<td>100 % by 1 h</td>
<td>100 % by 23 h</td>
<td>–</td>
<td>Eastern grey squirrel skin (29062)</td>
</tr>
<tr>
<td>NCH 298</td>
<td>Staphylococcus simulans</td>
<td>100 % by 1.25 h</td>
<td>86 % by 24 h</td>
<td>+</td>
<td>Human skin (27848)</td>
</tr>
<tr>
<td>NCH 299</td>
<td>Staphylococcus warneri</td>
<td>100 % by 4.5 h</td>
<td>43 % by 24 h</td>
<td>+</td>
<td>Human skin (27836)</td>
</tr>
<tr>
<td>NCH 300</td>
<td>Staphylococcus xylosus</td>
<td>100 % by 3.5 h</td>
<td>68 % by 24 h</td>
<td>+</td>
<td>Human skin (29971)</td>
</tr>
<tr>
<td>NCH 301</td>
<td>Kytopoccus sedentarius‡</td>
<td>58 % by 48 h</td>
<td>12 % by 48 h</td>
<td>–</td>
<td>Bull (14392)</td>
</tr>
<tr>
<td>NCH 302</td>
<td>Staphylococcus haemolyticus</td>
<td>100 % by 5.5 h</td>
<td>40 % by 24 h</td>
<td>+</td>
<td>Human skin (29970)</td>
</tr>
<tr>
<td>NCH 303</td>
<td>Corynebacterium xerosis</td>
<td>100 % by 13.5 h</td>
<td>0 % by 24 h</td>
<td>Multi</td>
<td>Ear discharge of a child (373)</td>
</tr>
<tr>
<td>NCH 304</td>
<td>Pseudomonas aeruginosa</td>
<td>100 % by 12 h</td>
<td>100 % by 14.5 h</td>
<td>Multi</td>
<td>Blood culture (27853)</td>
</tr>
<tr>
<td>NCH 305</td>
<td>Pseudomonas putida</td>
<td>100 % by 12 h</td>
<td>62 % by 24 h</td>
<td>Multi</td>
<td>ATCC 23974</td>
</tr>
<tr>
<td>NCH 307</td>
<td>Serratia liquefaciens</td>
<td>100 % by 4.25 h</td>
<td>22 % by 24 h</td>
<td>–</td>
<td>Milk, Cork, Ireland (27592)</td>
</tr>
<tr>
<td>NCH 309</td>
<td>Streptococcus pyogenes</td>
<td>100 % by 11 h</td>
<td>16 % by 24 h</td>
<td>–</td>
<td>Pharynx of a child (19615)</td>
</tr>
<tr>
<td>NTH 118</td>
<td>Staphylococcus epidermidis</td>
<td>100 % by 4.5 h</td>
<td>67 % by 24 h</td>
<td>+</td>
<td>ATCC 12228</td>
</tr>
<tr>
<td>NTH 125</td>
<td>Staphylococcus aureus</td>
<td>100 % by 1.5 h</td>
<td>88 % by 24 h</td>
<td>+</td>
<td>ATCC 25923</td>
</tr>
</tbody>
</table>

*Multi, multiple proteins were detected by the polyclonal anti-Azo1 serum.
†By 24 h, only 97.2 % had been reduced.
‡Kytopoccus sedentarius required 48 h at 26 °C for proper growth, so reduction experiments were conducted under these conditions.

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Kytococcus sedentarius (NCH 301), which was maintained at 26°C for at least 42 h.

**Azo dye reduction assay.** Spectrophotometric monitoring of the reduction of 200 μM MR in BHI by *Staph. epidermidis* (NTH 118) at 430 nm for 3 h indicated no difference in the reduction pattern between samples with and without cells present.

To establish optimal dye concentrations, BHI supplemented with 25–200 μM MR was added (180 or 190 μl per well) to a 96-well plate (Becton Dickinson), with BHI (blank) or overnight *Staph. epidermidis* culture bringing volumes to 200 μl (each in triplicate). MR reduction was monitored in a SpectraMax Plus 384 plate reader (Molecular Devices) at 37°C for 3 h, with 10 s of shaking before readings. The mean optical density (OD) of cells + BHI was taken from the mean OD of cells + MR. This method was used for Or II (482 nm), Ponceau S (520 nm) and Ponceau BS (502 nm).

The optimum OD of the initial inoculum was determined using initial OD_{340} of 0.005–1.0 with *Staph. aureus*, *Staph. epidermidis* and *Pseudomonas aeruginosa*. An OD of 0.1 allowed growth and ensured no significant lag in dye reduction. An initial inoculum OD of 0.05 was used in some experiments to observe dye reduction closely during early growth.

Reduction assays were performed twice (each in triplicate) for each bacterial species with MR and with Or II. Results in Table 1 reflect the mean of at least four wells. Bacterial growth was monitored at 550 nm, or at 600 nm for Ponceau S (absorption maximum = 520 nm).

Bacterial species with inconsistent reduction patterns from the plate reader, *Staphylococcus capitis* subsp. *capitis* (NCH 286) and *Serratia liquefaciens* (NCH 307), were assayed manually in 100 ml. Aliquots were taken periodically from MR cultures and at 24 h from Or II cultures. Aliquots were centrifuged (10 000 g, 1 min) and supernatants were assayed.

**Analysis of MR metabolites.** Cultures of *Staph. aureus*, *Staph. epidermidis* and *P. aeruginosa* were cultivated overnight in BHI broth supplemented with 200 μM MR. The same volume of absolute ethanol was added to 10 ml of the cultures, the mixtures were vortexed, and then centrifuged at 12 000 g for 10 min. Supernatants were filtered using integral 0.2 μm PTFE filters in Whatman filtration vials. The samples and a mixture containing 10 μg ml⁻¹ each of authentic *N,N*-dimethyl-β-phenylenediamine and 2-aminobenzoic acid were similarly analysed by LC/ESI-MS. A TSQ Quantum Ultra mass spectrometer (ThermoFinnigan) coupled with an 1100 Agilent HPLC system was operated in the positive-ion mode at 4.0 kV, CID offset 14 V, sheath gas 20, sweep gas 4.0 and aux 1100. Between samples with and without cells present.

**RESULTS**

**Azo dye reduction by bacterial cultures.**

The disappearance of MR and Or II, indicative of azoreductase activity, was measured spectrophotometrically in the presence of various bacterial species (Table 1).

MR reduction times varied among the bacteria (Fig. 1a). Only three of the 26 species did not completely reduce MR by 14.5 h. *Staph. capitis* subsp. *capitis* (NCH 286) reduced MR by 96.4 % at 7 h, and by 97.2 % at 24 h. *Dermacoccus nishinomiyaensis* (NCH 281) reduced MR by 74 % at 24 h. *Kyt. sedentarius* (NCH 301) required 48 h at 26°C for growth, and reduced MR by 58 % under those conditions.

Or II reduction also varied among the tested species, with no distinct groupings based on reduction times (Fig. 1b). Half (13) of the bacterial species were only able to reduce between 15 and 43 % of Or II by 24 h (Table 1). *Kyt. sedentarius* had reduced only 12 % of Or II by 48 h. By 24 h, five species had reduced between 58 and 68 % and six species had reduced more than 85 % of Or II. Only three species, *Staphylococcus delphini* (NCH 291), *Staphylococcus
sciuri subsp. sciuri (NCH 297) and P. aeruginosa (NCH 304), completely reduced Or II within 24 h. Corynebacterium xerosis (NCH 303) was unable to reduce Or II at all.

**Bacterial growth during azo dye reduction**

To examine the growth phase during which azo dye reduction occurred, Staph. aureus (NTH 125), Staph. epidermidis (NTH 118) and P. aeruginosa were grown in the presence and absence of MR (Fig. 2), Or II, Ponceau S and Ponceau BS. Azo dye disappearance and bacterial growth were measured simultaneously.

Reduction of MR by Staph. aureus and Staph. epidermidis started in early-exponential phase and was complete by 5 h (Fig. 2). Staph. aureus and Staph. epidermidis Or II reduction began around late-exponential/early-stationary phase and was not complete by 24 h. The two species did not begin Ponceau S or Ponceau BS reduction until late-exponential/early-stationary phase. At 24 h, Staph. aureus had reduced Ponceau S and Ponceau BS by 37 and 70%, and Staph. epidermidis had reduced the dyes by 57 and 87%, respectively.

P. aeruginosa reduction of MR, Or II, Ponceau BS and Ponceau S started at approximately the same time during growth, late-exponential/early-stationary phase (Fig. 2). P. aeruginosa completely reduced these dyes by 15 h.

**Identification of MR metabolites by bacterial cultures**

MR metabolites, produced in cultures of Staph. aureus, Staph. epidermidis and P. aeruginosa, were directly analysed by LC/ESI-MS. The retention times and product ion spectra of the MR metabolites were compared to those of authentic standards for identification. The metabolite...
eluting at 3.62 min had a protonated molecule at $m/z$ 137 that fragmented at 20 eV to give product ions at $m/z$ 122, 121, 107, 93 and 80. It was identified as N,N-dimethyl-p-phenylenediamine, since it eluted at the same retention time as did the standard and had an identical product ion spectrum. The metabolite eluting at 25.11 min had a protonated molecule at $m/z$ 138 that fragmented at 20 eV to give product ions at $m/z$ 120, 92 and 65. It was identified as 2-aminobenzoic acid since it eluted at the same retention time as did the standard and had an identical product ion spectrum.

**Western analysis using polyclonal anti-Azo1**

A polyclonal antibody to *Staph. aureus* Azo1 was used in Western analyses to detect similar proteins in skin bacteria cultured in the presence of MR. Among the 15 staphylococci examined, the antibody bound to a protein in each, except *Staphylococcus auricularis* (NCH 285), *Staph. sciuri* subsp. sciuri and *Staph. delphini* (Fig. 3).

Among the non-staphylococcal species, only *Kocuria kristinae* (NCH 282) produced a protein that was specifically detected by the *Staph. aureus* Azo1 antibody (Fig. 3). The antibody bound non-specifically to multiple proteins from *P. aeruginosa, Pseudomonas putida* (NCH 305), *Micrococcus lylae, D. nishinomiyaensis* and *C. xerosis*.

**DISCUSSION**

Azo dye reduction on the surface of skin could potentially lead to the formation of carcinogenic aromatic amines that are more readily absorbed by the skin than the original dyes (Platzer *et al.*, 1999). Investigation of the ability of human skin microbiota to reduce azo dyes used in cosmetics, tattoo inks and other products that routinely contact skin is essential for evaluation of potential health risks involved in using these products (Chen *et al.*, 2005).

The results of this study suggest that at least a portion of the human skin microbiota is capable of azo dye reduction.

All of the bacteria studied were able to reduce MR to some extent within 24 h. Fifteen staphylococcal species completely reduced MR by 9 h. *Staph. capitis* subsp. *capitis* was the only staphylococcus that did not completely reduce MR by 24 h. However, this species had achieved 96.4 % reduction.
by 7 h without significant further reduction. Among the other genera, only *Kyt. sedentarius*, which required a longer incubation time, and *D. nishinomiyaensis* had not completely reduced MR by 24 h.

Although Or II reduction was generally not as rapid or as complete as that of MR, only *C. xerosis* was unable to reduce Or II to any degree. Only *Staph. delphini*, *Staph. sciuri* subsp. *sciuri* and *P. aeruginosa* completely reduced Or II within 24 h.

Bacterial growth varied among the species, but was not affected by the dyes. The slowest growers, including *D. nishinomiyaensis*, *M. lylae*, *Kyt. sedentarius* and *C. xerosis*, were among the slowest to reduce MR. *Staph. delphini*, the first to finish MR reduction, was among the fastest growing species. Although adequate growth was likely required for dye reduction, growth did not appear to directly correlate with MR reduction among many of the species.

MR metabolites from *Staph. aureus*, *Staph. epidermidis* and *P. aeruginosa* cultures were identified as N,N-dimethyl-p-phenylenediamine and 2-aminobenzoic acid, indicating cleavage of the MRazo bond to form aromatic amines (Chen et al., 2005; Sugiuira et al., 1999).

BlastN and BlastP searches using azoI and Azo1 sequences, respectively, revealed that the staphylococcal species for which genomic and proteomic sequences were available, *Staph. haemolyticus*, *Staph. epidermidis*, *Staph. cohnii* and *Staph. saprophyticus*, have hypothetical genes with 77, 76, 200 and 75% identity to azoI, and hypothetical proteins with 82, 80, 72 and 74% identity to Azo1, respectively. In addition, *Staph. capitis* has a protein with 79% identity to Azo1.

Proteins similar to Azo1 were detected by Western blotting in most of the staphylococcal tested, confirming Blast data. Although the amount of protein used in the Western analyses was the same for each species, some protein bands were more intensely detected than others, likely due to the polyclonal nature of the Azo1 antisera.

Azoreduction is not performed by a single bacterial enzyme (Brige et al., 2008). Azo1-like proteins were not detected in *Staph. delphini* or *Staph. sciuri* subsp. *sciuri*, the first two species to completely reduce MR in assays. No obvious pattern of dye reduction among the species was associated with the presence of an Azo1-like protein. In addition, *Staph. aureus* and *Staph. epidermidis* began MR reduction much earlier during growth than for reduction of the other dyes, while *P. aeruginosa* reduced all dyes at approximately the same point in growth. Together, these observations suggest that additional bacterial enzymes are likely involved in azo dye reduction.

This study has provided data evaluating the role of skin microbiota in the metabolism of azo dyes. This information will be essential in the risk assessment process to evaluate public exposure to products containing azo dyes.

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**REFERENCES**


