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Sudan azo dyes and Para Red degradation by prevalent bacteria of the human gastrointestinal tract[☆]

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Abstract

Sudan azo dyes have genotoxic effects and ingestion of food products contaminated with Sudan I, II, III, IV, and Para Red could lead to exposure in the human gastrointestinal tract. In this study, we examined thirty-five prevalent species of human intestinal bacteria to evaluate their capacity to degrade Sudan dyes and Para Red. Among these tested bacterial strains, 23, 13, 33, 30, and 29 out of 35 species tested were able to reduce Sudan I, II, III, IV, and Para Red, respectively, to some extent. *Bifidobacterium infantis*, *Clostridium indolis*, *Enterococcus faecalis*, *Lactobacillus rhamnosus*, and *Ruminococcus obeum* were able to reduce completely all four tested Sudan dyes and Para Red. *Escherichia coli* and *Peptostreptococcus magnus* were the only two strains that were not able to reduce any of the tested Sudan dyes and Para Red to any significant extent. Metabolites of the reduction of the tested Sudan dyes and Para Red by *E. faecalis* were isolated and identified by HPLC and LC/ESI-MS analyses and compared with authentic standards. Thus it appears that the ability to reduce Sudan dyes and Para Red except Sudan II is common among bacteria in the human colon.

Keywords

Sudan dyes; Azo dyes; Intestinal bacteria; Aromatic amines; Biodegradation

1. Introduction

Synthetic azo dyes may be converted to potentially toxic and carcinogenic aromatic amines [1,2]. Mammalian and microbial enzyme systems have been reported to degrade azo dyes [3,4]. In the mammalian liver, azo compounds are enzymatically cleaved by cytosolic and microsomal enzymes to the corresponding amines. Some aromatic amines can be metabolically activated to DNA-binding intermediates that are mutagenic and carcinogenic [5,6]. The human skin and gastrointestinal tract harbor a complex and diverse microbiota comprised of at least several thousand species [7,8]. The microbiota also play roles in the

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degradation of azo dyes, with azo reduction being the most important reaction related to toxicity and mutagenicity [2,4,9–13]. Sudan dyes I, II, III, IV, and Para Red (Fig. 1) are oil-soluble azo dyes (1-amino-2-naphthol-based azo dyes) that are widely used for making plastics, printing inks, waxes, leather, fabrics and floor polishes [14–16]. Sudan I is a monoazo dye and Sudan II is a dimethyl derivative of Sudan I. Sudan III and Sudan IV are diazo dyes. Para Red is an azo dye used in printing that is chemically similar to Sudan I (Fig. 1) [17]. Sudan I is a liver and urinary bladder carcinogen in mammals and considered a possible human mutagen [3,18,19]. It can produce benzenediazonium ion during cytochrome P450 catalyzed metabolism, which is considered the possible mechanism of Sudan I activation to an ultimate carcinogen [3,18]. Sudan II causes mutations in *Salmonella enterica* Typhimurium TA 1538 in the presence of a rat liver preparation [20]. Concerns about the safety of Sudan III used in cosmetics have been raised due to potential metabolic cleavage by skin bacteria to yield 4-aminoazobenzene and aniline [7,21,22]. Sudan IV requires reduction and microsomal activation to be mutagenic [23].

Human exposure to Sudan dyes and Para Red occurs through ingestion, inhalation, or skin contact [11,24,25]. These dyes constitute a potential risk for public health if they enter the food chain. Sudan dyes and Para Red are often illegally used to enhance and/or maintain the appearance of food products and they have also been found as food contaminants in hot chili, other spices, and baked foods [26–28]. The risks of human exposure to Sudan dyes are documented [3,18,19,29]. There is evidence that some Sudan dyes have genotoxic effects and that ingestion of food products contaminated with the dyes could lead to exposure in the human gastrointestinal tract [11,12,19,24].

Sudan dye contamination in food has led national and international agencies to conduct toxicological safety assessments [3,19]. Most studies have developed methods for detection and quantification of Sudan dyes and Para Red in contaminated food products [15,30,31]. In addition, several studies have focused on Sudan I-DNA adducts and metabolic activation in the liver of mammals [3,14,19] and on the genotoxic effects and oxidative DNA damage in liver cells induced by Sudan I [14]. The recent detection of Sudan dyes and Para Red in various food commodities requires toxicological evaluation by regulatory agencies to determine the impact of these dyes on human health [32–35]. Our previous study showed that Sudan dyes and Para Red could be reduced by a human intestinal microbial consortium to produce aniline, 2,4-dimethylaniline, *o*-toluidine, and *p*-nitroaniline [21]. These aromatic amines are toxic, water-soluble, and easily absorbed by the human intestine [36,37]. This study examines the capability of thirty-five prevalent species of human intestinal bacteria to degrade Sudan dyes and Para Red. This study provides evidence that human gastrointestinal tract bacteria play a major role in the degradation of Sudan dyes and Para Red to aromatic amines.

2. Materials and methods

2.1. Chemicals

Sudan I, II, III, IV, aniline, 2,4-xylidine (2,4-dimethylaniline), *o*-toluidine (2-methylaniline), and 4-nitroaniline were purchased from Sigma Chemical Co. Para Red was purchased from ABCR GmbH, Karlsruhe, Germany. Acetonitrile was purchased from J.T. Baker. Dimethyl

sulfoxide (DMSO) and ethyl acetate were purchased from Fisher Scientific Co. Stock solutions of Sudan dyes I, II, III, IV, and Para Red were made by dissolving each dye in DMSO (1 mg/ml).

2.2. Bacteria and culture conditions

The anaerobic bacterial species used in this study (Table 1) were obtained from the American Type Culture Collection (ATCC). All strains were preserved at -80°C in 10 to 15% glycerol stocks and revived as needed. The strains, except for *Lactobacillus* species, which were routinely cultured on deMann-Rogosa-Sharpe (MRS) broth or agar (Becton Dickinson & Company) [38], were routinely cultured on Brain Heart Infusion (BHI) broth supplemented with vitamin K and hemin or on PRAS brucella blood agar plates supplemented with vitamin K and hemin (Remel) at 37°C under an atmosphere of 91% nitrogen, 4% hydrogen and 5% carbon dioxide.

The bacterial strains were grown anaerobically at 37°C by using BHI broth or MRS supplemented with various Sudan dyes and Para Red. A loopful for each strain was cultured in static conditions at 37°C for 24 h in an Erlenmeyer flask containing 10 ml medium for use as a seed culture. The bacterial seed cultures of 1.5 ml were inoculated into flasks containing 100 ml BHI broth. Dye stock solutions were added to the medium at final concentrations of 10 $\mu\text{g/ml}$ (Sudan I, II, and Para Red) and 1.5 $\mu\text{g/ml}$ (Sudan III and IV), the cultures were incubated at 37°C in an anaerobic chamber for 2 days without agitation. Three control incubations consisted of sterile liquid medium, sterile liquid medium with bacteria, and one of sterile liquid medium with dyes.

2.3. Assay of the reduction of Sudan dyes and Para Red

After incubation for 2 days, 5 ml samples collected from the cultures were extracted with two equal volumes of ethyl acetate. The combined extracts were evaporated in a rotary evaporator at 40°C and the trace of solvent remaining was removed by evaporation at room temperature overnight. Each residue was dissolved in 0.2 ml acetonitrile and filtered through a 0.2 μm syringe filter. Forty μl of each sample was analyzed with a Hewlett–Packard 1050 Series high performance liquid chromatography (HPLC) equipped with a variable wavelength detector (detection wavelengths were 250 and 500 nm), an auto sampler, and a Luna C18 (2) column (150×3.0 mm, particle size, 5 μm , Phenomenex) with a guard column (40×3.0 mm, Phenomenex). The mobile phase was composed of a linear gradient of acetonitrile: water containing 0.1% formic acid from 30:70 to 95:5 for 40 min. The peak area was used with a standard curve to calculate the concentration of Sudan dyes and Para Red. Reduction of the dyes was determined by measuring the disappearance of the absorbance at 500 nm immediately after extraction with ethyl acetate as well.

2.4. Detection of metabolites from the reduction of Sudan dyes and Para Red

Identification of the metabolites was performed using a similar procedure as described previously [21]. Liquid chromatography/electrospray ionization mass spectrometric (LC/ESI-MS) data were acquired on a ThermoFinnigan Quantum Ultra mass spectrometer equipped with an Agilent 1100 Series HPLC system and a Prodigy ODS (3) 2.0×250 mm 5 μm 100 A HPLC column (Phenomenex). The mass spectrometer was operated in the

positive-ion electro-spray ionization (ESI) mode with an in-source collision-induced dissociation (CID) offset of 0 V. Other ESI conditions were spray voltage 4.0 kV, capillary temperature 350 °C, sheath gas 40 psi, ion sweep gas 0 and auxiliary gas 25. Standards and samples were extracted with ethyl acetate, which was dried and then redissolved in a starting buffer (acetonitrile: water containing 0.1% formic acid-5:95). Much of the dried sample was insoluble, so samples were taken without including the precipitate. The starting buffer was held 20 min, ramped in 1 min to acetonitrile: water containing 0.1% formic acid-95:5 at 21 min and held until 40 min. For the Para Red metabolite, a linear 21-min gradient of acetonitrile: water containing 0.1% formic acid from 5:95 to 95:5 was used.

2.5. Binding of Sudan II to bacteria

Escherichia coli and *Enterococcus faecalis* cultures, grown on BHI broth supplemented with a final concentration of 10 µg/ml Sudan II at 37 °C for 4, 8, 24, and 48 h, were sampled. The samples were centrifuged at $3500 \times g$ for 10 min. The supernatants were removed with disposable glass pipettes and the bacterial pellets were photographed.

2.6. Disruption of bacteria and enzyme assay

E. coli and *E. faecalis* cells were suspended in 1 ml of 25 mM sodium phosphate buffer, pH 7.1, and disrupted mechanically with sea-sand using a Vortex Gene 2 (Scientific Industries) for 30 min twice at 4 °C, respectively. The mixtures were then centrifuged at $14,000 \times g$ for 10 min at 4 °C. The supernatant fraction was collected and used as cell-free extract. The cell pellet was washed three times with above phosphate buffer and the washed pellet was suspended in 1 ml of the buffer.

Azoreductase activity assay was conducted using a similar procedure as described previously [10]. A typical reaction mixture (2.0 ml) contained 25 mM potassium phosphate buffer (pH 7.1), 25 µM Methyl Red, and 0.1 mM NADH. The reaction was initiated by addition of the enzyme. Initial velocity was determined by monitoring the change in the amount of substrate in the first 2 min in a glass cuvette of 1.0-cm light path at 430 nm.

3. Results and discussion

3.1. Degradation of Sudan dyes and Para Red by thirty-five prevalent intestinal bacteria

Previously, we demonstrated that a microbial consortium in human fecal suspension was able to reduce Sudan dyes and Para Red [21]. As a part of a series of studies concerning Sudan dye contamination in food safety issue, we conducted experiments to evaluate the ability of individual human intestinal bacteria to degrade Sudan dyes and Para Red to potentially genotoxic aromatic amines. Thirty-five species of human intestinal bacteria (seven *Bacteroides* spp., five *Bifidobacterium* spp., seven *Clostridium* spp., three *Eubacterium* spp., two *Enterococcus* spp., two *Fusobacterium* spp., five *Lactobacilli* spp., two *Ruminococcus* spp., *Peptostreptococcus magnus*, and *E. coli*) were examined for their capacity to degrade Sudan azo dyes (Table 1). Among the tested bacterial strains, 23, 13, 33, 30 and 29 out of 35 species tested were able to completely or partially reduce Sudan I, II, III, IV, and Para Red, respectively. *Bifidobacterium infantis*, *Clostridium indolis*, *E. faecalis*, *Lactobacillus rhamnosus*, and *Ruminococcus obeum* were able to completely degrade

(100%) all five tested dyes. *Eubacterium tenue* was able to completely reduce Sudan III but not the other azo dyes. *E. coli* and *P. magnus* were not capable of degrading any of the tested dyes to any significant extent. An example of the decolorization capabilities of the seven *Bacteroides* spp. is shown in Fig. 2.

Sudan II was the most difficult dye to be reduced by the tested bacteria. This differs from results obtained from the reduction profile by the intestinal microbial consortium [21]. The diazo dyes, Sudan III and Sudan IV, were reduced much more slowly than the monoazo dyes, Sudan I, II and Para Red by the intestinal microbial consortium indicating that other bacterial species in the human intestinal microbiota or that synergism among the bacteria may play important role in reduction of monoazo dyes [21]. It is possible that the side groups of Sudan dyes may affect the reduction rate of the dyes by the tested bacteria as well [39].

3.2. Binding of dye to cells

As mentioned above, *E. coli* was not able to degrade any tested dye. On the other hand, *E. faecalis* was able to reduce all the tested dyes. To investigate the relationship between binding and degradation of Sudan dyes in bacteria, we chose these two bacterial species for a binding experiment. We observed that Sudan II quickly bound to the bacteria when the dye was added to the cultures (Fig. 3). With the decrease of Sudan II concentration in the *E. faecalis* culture, the amount of the dye bound to the bacteria was constant until the dye disappeared in the supernatant of the culture. It indicated that reduction of the dye may be an intracellular and/or membrane associated processes rather than an extracellular one. Azo and hydroxyl groups of Sudan dyes are capable of forming hydrogen bonds with the polar head groups of membrane phospholipids [40]. Although Sudan II can bind to *E. coli*, no apparent decolorization was observed. We found that 55 and 45% of azoreductase activity of *E. faecalis* were associated with the washed pellet (membrane-bound) and supernatant fraction (intracellular), respectively. However, no detectable enzyme activity was found in washed pellet and almost 100% of enzyme activity was found in supernatant fraction of *E. coli*. This suggests that *E. coli* lack enzymes in its membrane responsible for reduction of the dye and bound Sudan II are not efficiently transported into the cells [40]. Previously it has been demonstrated that Sudan dyes in the medium can penetrate into *E. coli* cells but most of the dyes remain in the membrane, resulting from the Langmuir adsorption of the dyes to membrane phospholipids via non-covalent interactions [40]. The results also suggested that membrane-bound azoreductases are crucial for degradation of water insoluble pigments such as Sudan dyes.

3.3. Identification of metabolites

Ethyl acetate extracts of broths in which *E. coli* and *E. faecalis* were incubated with Sudan I, II, III, IV, or Para Red at 37 °C for 2 days were dried. The residues were redissolved in the starting buffer and soluble metabolites were analyzed by LC/ESI-MS. Protonated molecules and adducts with acetonitrile seen in the fullscan spectra and retention times for metabolites were compared to those for authentic compounds for identification. These peaks were missing or greatly reduced in the controls that were analyzed. The metabolites produced from Sudan I and III by *E. faecalis* were identified as aniline, based on an identical retention

time of 4.05 min and ions at m/z 94 [MH^+] and 135 [MH^+ + acetonitrile] (Fig. 4A and C). The metabolite produced from Sudan II by *E. faecalis* was identified as 2,4-dimethylaniline, based on an identical retention time of 16.55 min and ions at m/z 122 [MH^+] and 163 [MH^+ + acetonitrile] (Fig. 4B). The metabolite produced from Sudan IV by *E. faecalis* was identified as *o*-toluidine, based on an identical retention time of 7.42 min and ions at m/z 108 [MH^+] and 149 [MH^+ + acetonitrile] (Fig. 4D). The metabolite produced from Para Red by *E. faecalis* was identified as 4-nitroaniline, based on an identical retention time of 36.00 min and ions at m/z 139 [MH^+] and 180 [MH^+ + acetonitrile] (Fig. 4E). 1-Amino-2-naphthol from all tested Sudan dyes and Para Red, 1, 4-phenylenediamine from Sudan III, and 2,5-diaminotoluene from Sudan IV (Fig. 1) could not be detected in the extracted samples as demonstrated before [21]. No metabolites of Sudan dyes and Para Red produced by *E. coli* were detected by LC/ESI-MS, indicating that the dyes were not degraded by the bacterium.

Aniline, a metabolite of Sudan I and III, is considered hazardous because of its toxicity and carcinogenicity [36]. 2,4-Dimethylaniline, a metabolite of Sudan II, is able to damage DNA in the liver cells of mice in the comet assay under alkaline conditions [6,41]. *o*-Toluidine (2-methylaniline), a metabolite of Sudan IV, is degraded *in vivo* into a number of compounds, some of which are active genotoxins [42]. 4-Nitroaniline, a metabolite of Para Red, is highly toxic by inhalation, ingestion and if absorbed through skin [43].

4. Conclusions

Most of the tested prevalent intestinal bacterial species are able to reduce the tested Sudan dyes and Para Red, except Sudan II, to form potentially toxic aromatic amines. The degradation of the tested dyes by the bacteria depends upon the characteristics of each individual species regardless of its genus. This initial investigation provides data examining the role of intestinal microbiota in the degradation of Sudan azo dyes. This information will be useful in the risk assessment process when evaluating public exposure to these azo dyes.

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Abbreviations

DMSO	dimethyl sulfoxide
BHI	Brain Heart Infusion
MRS	deMann-Rogosa-Sharpe
HPLC	high performance liquid chromatography

LC/ESI-MS liquid chromatography/electrospray ionization mass spectrometry

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Fig. 1.
Chemical structures of Sudan azo dyes and Para Red.

Fig. 2. Reduction of Sudan dyes and Para Red by seven *Bacteroides* spp. in 2 days. C, control; 1, *B. vulgatus*; 2, *B. ovatus*; 3, *B. uniformis*; 4, *B. distasonis*; 5, *B. fragilis*; 6, *B. thetaiotaomicron*; and 7, *B. caccae*.

Fig. 3.

Interaction between bacteria and Sudan II. Sudan II was able to bind to the cell surfaces of both *E. coli* and *E. faecalis*. However, *E. faecalis* was capable of reducing Sudan II, but the dye could not be degraded by *E. coli*.

Fig. 4.

LC/ESI-MS chromatograms of samples from the incubation of Sudan azo dyes and Para Red with *E. faecalis*. Ions included m/z 94 for the metabolite of Sudan I (A), m/z 122 for the metabolite of Sudan II (B), m/z 94 for the metabolite of Sudan III (C), m/z 108 for the metabolite of Sudan IV (D), and m/z 139 for the metabolite of Para Red (E). Peaks also observed in the control for dye without *E. faecalis* are designated with an asterisk (*).

Table 1

Sudan azo dye reduction by thirty-five prevalent human intestinal bacterial species.

	Sudan I reduction (%)	Sudan II reduction (%)	Sudan III reduction (%)	Sudan IV reduction (%)	Para Red reduction (%)
<i>Bacteroides vulgatus</i> ATCC 8482	40 ^a	– ^b	100	100	60
<i>B. ovatus</i> ATCC 8483	100	60	60	100	100
<i>B. uniformis</i> ATCC	100	–	100	100	100
<i>B. distasonis</i> ATCC 8503	100	40	100	100	100
<i>B. fragilis</i> ATCC 23745	100	–	100	100	40
<i>B. thetaiotaomicron</i> 29148	100	40	100	100	100
<i>B. caccae</i> ATCC 43185	100	40	100	100	100
<i>Bifidobacterium longum</i> ATCC 15707	–	–	100	60	40
<i>B. adolescentis</i> ATCC 15703	–	–	100	40	–
<i>B. infantis</i> ATCC 15697	100	100	100	100	100
<i>B. catenulatum</i> ATCC 27539	–	–	40	–	–
<i>B. angulatum</i> ATCC 27535	–	–	40	40	40
<i>Clostridium perfringens</i> ATCC 13124	100	–	100	100	100
<i>C. butyricum</i> ATCC 19398	100	–	100	60	100
<i>C. ramosum</i> ATCC 25582	–	–	100	–	60
<i>C. difficile</i> ATCC 9689	100	–	100	60	100
<i>C. indolis</i> ATCC 25771	100	100	100	100	100
<i>C. leptum</i> ATCC 29065	–	–	40	60	100
<i>C. clostridioforme</i> ATCC 29084	100	60	100	100	100
<i>Eubacterium aerofaciens</i> ATCC 25986	100	–	100	100	100
<i>E. limosum</i> ATCC 8486	60	–	100	100	100
<i>E. tenue</i> ATCC 25553	–	–	100	–	–
<i>Enterococcus faecalis</i> ATCC 27274	100	100	100	100	100
<i>E. faecium</i> ATCC 19434	100	40	100	100	100
<i>Escherichia coli</i> ATCC 25922	–	–	–	–	–
<i>Fusobacterium russii</i> ATCC 25533	100	40	100	100	100
<i>F. nucleatum</i> ATCC 25586	100	–	100	100	100
<i>Lactobacillus bifidus</i> ATCC 11146	–	–	80	80	–

	Sudan I reduction (%)	Sudan II reduction (%)	Sudan III reduction (%)	Sudan IV reduction (%)	Para Red reduction (%)
<i>L. paracasei</i> ATCC 27092	100	20	100	100	100
<i>L. reuteri</i> ATCC 23272	–	–	100	100	60
<i>L. rhamnosus</i> ATCC 53103	100	100	100	100	100
<i>L. ruminis</i> ATCC 25644	–	–	80	40	40
<i>Peptostreptococcus magnus</i> ATCC 14955	–	–	–	–	–
<i>Ruminococcus obeum</i> ATCC 29174	100	100	100	100	100
<i>R. gnavus</i> ATCC 29149	100	–	100	100	100

^aData were presented in % rounded to –, 20, 40, 60, 80, and 100 by the means from triplicate incubations.

^bNo reduction detected.