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Reduction of bilirubin ditaurate by the intestinal bacterium *Clostridium perfringens*

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Bilirubin is degraded in the human gut by microflora into urobilinoids. In our study we investigated whether the bilirubin-reducing strain of *Clostridium perfringens* can reduce bilirubin ditaurate (BDT), a bile pigment of some lower vertebrates, without hydrolysis of the taurine moiety. *C. perfringes* was incubated under anaerobic conditions with BDT; reduction products were quantified by spectrophotometry and separated by TLC. Based on Rf values of BDT reduction products and synthetic urobilinogen ditaurate, three novel taurine-conjugated urobilinoids were identified. It is likely that bilirubin-reducing enzyme(s) serve for the effective disposal of electrons produced by fermentolytic processes in these anaerobic bacteria.

Key words: *Clostridium perfringens*, bile pigments, bilirubin ditaurate, intestinal metabolism, urobilinoids

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INTRODUCTION

Unconjugated bilirubin (UCB), a yellow, poorly water-soluble pigment, is the main heme catabolic product in the intravascular compartment. The predominant source of UCB is the breakdown of heme, originating from senescent or hemolyzed red blood cells. After its biotransformation in the liver, secretion into the bile, and then in the intestinal lumen, bilirubin is rapidly reduced to urobilinoids by the intestinal microflora (Vítek & Ostrow, 2009). The term urobilinoids covers the group of reduction products of bilirubin, including urobilinogen and stercobilinogen, along with their oxidized derivatives urobilins and stercobilins (Moscowitz *et al*., 1970). In the absence of bilirubin-reducing microflora, such as in the early newborn period (Vítek *et al*., 2000), or in patients treated with systemic antibiotics (Vítek *et al*., 2005), UCB may undergo substantial enterohepatic and enterosystemic circulation. Severe unconjugated hyperbilirubinaemia in neonates is of concern because of the potential danger to their central nervous system.

UCB is reduced by multiple sequential reactions into a series of urobilinogens; in turn, these colorless chromogens may be oxidized to urobilins, their respective yellowish oxidation products. These substances are believed to be nontoxic due to their increased polarity. Despite the importance of this catabolic pathway, only a few bacterial strains involved in bilirubin reduction have so far been isolated: *Clostridium ramosum* (Gustafsson & Lanke, 1960), *C. perfringens*, *C. difficile* (Vítek *et al*., 2000), and *Bacteroides fragilis* (Fahmy *et al*., 1972). In our recent study (Vítek *et al*., 2006), we undertook a detailed analysis of the products of the bacterial reduction of bilirubin and its derivatives formed by a novel strain of *C. perfringens* isolated from neonatal feces (Vítek *et al*., 2000). The *C. perfringens* strain reduced a wide variety of bile pigments that differed substantially in both their polarity and structure. The end-catabolic bilirubin products resulting from bacterial reduction were identified as urobilinogen species. The reduction process catalyzed by the bacterial strain studied did not proceed to the production of stercobilinogen (Vítek *et al*., 2006).

The aim of the present study was to assess whether bilirubin ditaurate, a pigment that occurs naturally in the bile of certain lower vertebrates (such as the marine fish *Seriola quinqueradiata*) (Sakai *et al*., 1987), can be reduced by the aforementioned strain of *C. perfringens* isolated from human neonatal feces, and secondly, to characterize the reduction products formed.

MATERIAL AND METHODS

***C. perfringens* cultivation.** The strain of *C. perfringens* used in our studies was isolated from stool of a healthy neonate (Vítek *et al*., 2000). The strain was classified in the National Reference Laboratory for Anaerobic Bacteria (Ostrava, Czech Republic) as non-pathogenic, based on the absence of any toxin production. The strain was incubated with BDT and UCB (both from Frontier Scientific, Inc., Logan, UT, USA) in broth (2% Yeast Extract, Oxoid, GB; prepared in 100 mM phosphate buffer, pH=8). Based on their polarity, UCB was dissolved in dimethylsulfoxide (Sigma, St. Louis, MO, USA) and bilirubin ditaurate (BDT) in the broth; both pigments were added to the late exponential phase *C. perfringens* culture, at the final concentration of 50 μmol/L (final concentration of DMSO was <3%, this concentration was found in separate experiments not to influence the growth of *C. perfringens*). The growth phase was monitored according to the optical density of the culture (spectrophotometry at 600 nm). The conversion rates of both bile pigments were analyzed under exactly the same conditions, and calculated from five independent measurements. Af-

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Abbreviations: BDT, bilirubin ditaurate; UCB, unconjugated bilirubin

ter incubation for 24 h at 37°C in Anaerostat (Oxoid, GB), the medium was sampled for urobilinoid analyses.

Determination and isolation of urobilinoids. The rate of bile pigment conversion was calculated as the proportion of the urobilinoids produced to the initial bile pigment concentration. Concentrations of the UCB and BDT reduction products were determined spectrophotometrically (UV/Vis Spectrophotometer Lambda 20, Perkin-Elmer, USA) as the oxidation products of zinc complexes of urobilinoids, as previously described (Kotal & Fevery 1991).

The pigments were extracted from the culture medium using an SPE column (Strata C8 500 mg/6 ml, Phenomenex, Torrance, CA, USA). The columns were washed with 1 volume of methanol and distilled water. The urobilinoids were eluted with a minimal volume of MeOH:H_2O (1:1, by vol.). The elution mixture was evaporated; the extracted pigments were dissolved in methanol and separated by TLC.

To check whether the BDT-reducing enzyme(s) were secreted into the medium, BDT was incubated with a cell-free supernatant and a protein extract after a French press disintegration of bacterial cells. A *C. perfringens* culture grown overnight (500 ml) was centrifuged (15 000 × *g*, 10 min, 4°C) and the medium was filter-sterilized (Milipore Millex HV, PVDF 0.45 μm). The cells were re-suspended in a phosphate buffer (0.025 M, pH=8) containing 5 mM $MgCl_2$, 2 mM EDTA and 10% glycerol, and then disintegrated using a French press (6 cycles, 1500 psi, SLM-Aminco, USA). The cell debris was removed by centrifugation (15 000 × *g*, 10 min, 4°C) and the supernatant containing the protein extract was filter-sterilized as above. The cell-free supernatant and the protein extract were incubated separately with BDT, as described above; the production of urobilinoids was again determined.

Thin layer chromatography. Isolated BDT and UCB reduction products were separated by TLC, using HPTLC aluminum plates coated with silica gel (RP-18 W/UV_{254}, Macherey-Nagel, Germany) (solvent system: H_2O:MeOH:CH_3COOH (250:250:1, by vol.)) and examined under both visible and UV light (CAMAG TLC Scanner II, CAMAG, Muttenz, Switzerland). Urobilin (Frontier Scientific, Inc., Logan, UT, USA) and urobilinogen ditaurate were used as standards. Urobilinogen ditaurate was prepared by amalgam reduction of bilirubin ditaurate, as previously described (Watson, 1953); with its structure confirmed by mass spectrometry (Esquire 3000, Bruker Daltonics, Bremen, Germany).

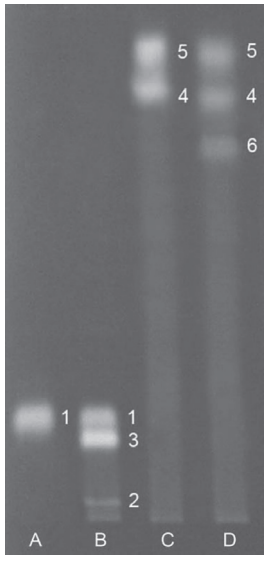

Figure 1. Products of UCB and BDT reduction by *C. perfringens*. The initial pigment concentration in the broth was 50 μmol/L. TLC system specification: HPTLC silica gel plate (RP-18 W/UV_{254}, Macherey-Nagel), solvent system: H_2O:MeOH:CH_3COOH (250:250:1, by vol.). **(Track A)** Urobilin (standard); **(Track B)** Reduction products of UCB; **(Track C)** Urobilinogen ditaurate (standard); **(Track D)** Reduction products of BDT. **(1)** Urobilin; **(2)** Mesobiliviolin; **(3)** Urobilinogen; **(4)** Urobilinogen ditaurate; **(5)** Urobilin ditaurate; **(6)** Mesobiliviolin ditaurate.

RESULTS AND DISCUSSION

The bilirubin-reducing strain of *C. perfringens* isolated from neonatal feces was able to reduce BDT efficiently. The conversion rates were 9±3 and 30±5% in 24 h for BDT and UCB, respectively; this is consistent with our previous findings (Vítek *et al.*, 2006). No reduction of BDT could be detected in cell-free post-culture medium; in contrast, a French press disintegration of the bacterial cells resulted in the release of enzyme(s) capable of reducing BDT, indicating that the BDT reductase is not secreted by the bacteria to the medium.

BDT was reduced by *C. perfringens* into several species of taurine-bound urobilinoids, demonstrating that taurine hydrolysis did not precede the enzymatic reduction (Fig. 1). After TLC separation, no unconjugated urobilinoids derived from BDT could be detected. Based on comparison of the bilirubin reduction products with synthetic urobilinogen ditaurate, three reduction products of BDT were identified: urobilinogen ditaurate, urobilin ditaurate, and (most likely) mesobiliviolin ditaurate (Fig. 1). A more polar taurine-bound urobilin derivative detected in both metabolized BDT and synthetic urobilinogen ditaurate tracks was most likely formed spontaneous oxidation of the urobilinogen ditaurate.

Another bilirubin conjugate, bisglucuronosyl bilirubin, was reduced by the same *C. perfringens* strain into unconjugated urobilinoids; the glucuronoside bond was hydrolyzed prior to the reduction of the tetrapyrroles (Vítek *et al.*, 2006). The amide bond of BDT is presumably resistant to hydrolysis by the enzymes of the intestinal microflora; surprisingly, however, BDT can still be reduced into taurine-bound urobilinoids. These results demonstrate a very broad substrate specificity of the enzyme(s) reducing bilirubin in the human gastrointestinal tract and may help to understand the function of bilirubin reductase. Such a broad substrate specificity of the bilirubin-reducing enzyme(s) presumably serve for the effective disposal of electrons produced by fermentolytic processes in these anaerobic bacteria in a manner similar to that described for microbial bile acid dehydroxylation (Ridlon *et al.*, 2006).

It is interesting to note that despite their ubiquitous occurrence in the human intestinal tract, as well as their high therapeutic potential, the bacterial enzymes responsible for bilirubin reduction have, to date, not been identified.

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REFERENCES

Fahmy K, Gray CH, Nicholson DC (1972) The reduction of bile pigments by faecal and intestinal bacteria. *Bioch Bioph Acta* **264**: 85–97.

Gustafsson BE, Lanke L (1960) Bilirubin and urobilins in germfree, exgermfree, and conventional rats. *J Exp Med* **112**: 975–981.

Kotal P, Fevery J (1991) Quantitation of urobilinogen in feces, urine, bile and serum by direct spectrophotometry of zinc complex. *Clin Chim Acta* **202**: 1–9.

Moscowitz A, Weimer M, Lightner A, Petryka ZJ, Davis E, Watson CJ (1970) The in vitro conversion of bile pigments to the urobilinoids by a rat clostridia species as compared with the human fecal flora; III. Natural d-Urobilin, synthetic i-urobilin, and synthetic i-urobilinogen. *Biochem Med* **4**: 149–164.

Ridlon JM, Kang DJ, Hylemon PB (2006) Bile salt biotransformations by human intestinal bacteria. *J Lip Res* **47**: 241–259.

Sakai T, Watanabe K, Kawatsu H (1987) Occurrence of ditaurobilirubin, bilirubin conjugated with two moles of taurine, in the gallbladder bile of yellowtail, Seriola quinqueradiata. *J Biochem* **102**: 793–796.

Vítek L, Ostrow JD (2009) Bilirubin chemistry and metabolism; harmful and protective aspects. *Curr Pharm Des* **15**: 2869–2883.

Vítek L, Kotal P, Jirsa M, Malina J, Černá M, Chmelař D, *et al.* (2000). Intestinal colonization leading to fecal urobilinoid excretion may play a role in the pathogenesis of neonatal jaundice. *J Ped Gastroenterol Nutr* **30**: 294–298.

Vítek L, Zelenka J, Zadinová M, Malina J (2005). The impact of intestinal microflora on serum bilirubin levels. *J Hepatol* **42**: 238–243.

Vítek L, Majer F, Muchová L, Zelenka J, Jirásková A, Branny P, *et al.* (2006). Identification of bilirubin reduction products formed by Clostridium perfringens isolated from human neonatal fecal flora. *J Chromat B* **833**: 149–157.

Watson CJ (1953). The direct preparation of crystalline urobilin from bilirubin. *J Biol Chem* **200**: 691–696.