Barbaloin Stimulates Growth of Eubacterium sp. Strain BAR, a Barbaloin-Metabolizing Bacterium from Human Feces

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Eubacterium sp. strain BAR, isolated from human feces, transformed barbaloin to aloe-emodin anthranthrene in a basal medium lacking carbohydrate. Barbaloin remarkably stimulated the growth of strain BAR in the basal medium, the stimulative extent of the growth depending on the amount of barbaloin added. The addition of D-glucose, D-galactose, maltose, cellobiose, sucrose or D-amygdalin to the basal medium containing barbaloin caused a decrease of the growth stimulated by barbaloin to the growth level with each sugar, resulting in a complete inhibition of the barbaloin transformation. On the other hand, the addition of D-fructose, which itself stimulated the growth of strain BAR, further increased the growth in the presence of barbaloin and little inhibited barbaloin transformation. Nojirimycin bisulfite, a specific inhibitor of glucosidases, potently inhibited the growth with barbaloin, but did not affect the growth with glucose or cellobiose. Also, nojirimycin bisulfite completely inhibited the transformation of barbaloin to aloe-emodin anthrone.

These results indicate that a unique enzyme capable of cleaving the C-glycosyl bond is induced in strain BAR by barbaloin and, consequently, strain BAR grows by utilizing as a nutrient the carbohydrate liberated from barbaloin. It is further suggested that the barbaloin-cleaving enzyme is inhibited by nojirimycin bisulfite and that the induction of the enzyme is repressed by D-glucose and D-galactose.

Keywords: barbaloin; aloe-emodin anthrone; Eubacterium sp. strain BAR; intestinal bacteria; metabolism

Introduction
Barbaloin is a popular laxative from aloe, but is less purgative than sennoisides, other popular laxatives from senna and rhubarb.1-3 Aloe-emodin anthrone, an aglycone of barbaloin, shows weaker laxative action than rhein anthrone, the principal purgative of sennosides.4,5 Therefore, aloe-emodin anthrone is considered to be the ultimate purgative of barbaloin. Intestinal flora play an important role in the transformation of sennoisides to rhein anthrone.6-15 We reported that barbaloin was also transformed to aloe-emodin anthrone by human intestinal flora.16 However, the intestinal flora of rats and mice and 23 defined strains of human intestinal bacteria did not reveal any barbaloin-metabolizing activity.16 The transformation of barbaloin to aloe-emodin anthrone seems to include a unique type of cleavage of the C-glycosyl bond. We isolated a new bacterium, called Eubacterium sp. strain BAR, capable of cleaving this bond of barbaloin from human feces.17

We describe here the stimulation of growth of strain BAR and the induction of a barbaloin-metabolizing enzyme by barbaloin.

Materials and Methods
Barbaloin is a popular laxative from aloe, but is less purgative than sennoisides, other popular laxatives from senna and rhubarb.1-3 Aloe-emodin anthrone, an aglycone of barbaloin, shows weaker laxative action than rhein anthrone, the principal purgative of sennosides.4,5 Therefore, aloe-emodin anthrone is considered to be the ultimate purgative of barbaloin. Intestinal flora play an important role in the transformation of sennoisides to rhein anthrone.6-15 We reported that barbaloin was also transformed to aloe-emodin anthrone by human intestinal flora.16 However, the intestinal flora of rats and mice and 23 defined strains of human intestinal bacteria did not reveal any barbaloin-metabolizing activity.16 The transformation of barbaloin to aloe-emodin anthrone seems to include a unique type of cleavage of the C-glycosyl bond. We isolated a new bacterium, called Eubacterium sp. strain BAR, capable of cleaving this bond of barbaloin from human feces.17

We describe here the stimulation of growth of strain BAR and the induction of a barbaloin-metabolizing enzyme by barbaloin.

Sp. strain BAR was cultured anaerobically at 37°C in PYF broth containing barbaloin with or without various carbohydrates. An aliquot (1 ml) of the culture was taken out at suitable intervals. One half was vigorously mixed with 0.5 ml of butanol and an aliquot (10 μl) of the butanol layer was analyzed for barbaloin and aloe-emodin anthrone by thin-layer chromatography (TLC) as described below. The other half was mixed with 0.2 ml of 1% N,N-dimethyl-p-nitrosoaniline in pyridine, and then mixed thoroughly with 0.5 ml of butanol. An aliquot (10 μl) of the butanol layer was analyzed for anil (adduct of aloe-emodin anthrone and N,N-dimethyl-p-nitrosoaniline) by TLC as described below.

Thin-layer Chromatography: TLC for barbaloin and aloe-emodin anthrone was performed on silica gel plates (silica gel 60-F 254; layer thickness, 0.25 mm; Merck Co., Inc., Darmstadt, FRG) with the solvent system of chloroform-methanol-water (18:6:1). TLC for anil was performed on polyamide plates (Macherey-Nagel polyamide-6; layer thickness, 0.1 mm) with the solvent system of methanol-water (5:1). The amounts of barbaloin, aloe-emodin anthrone and anil were determined using calibration lines prepared with authentic samples as described previously.17

Protein: Protein was determined by the method of Lowry et al.18 Before the determination of bacterial proteins, Eubacterium sp. strain BAR cultured in broth containing barbaloin was harvested and then washed with ethanol to remove barbaloin and aloe-emodin anthrone.

Reagents: Barbaloin was isolated from the powder of aloin, which was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan, and purified by silica gel column chromatography, followed by repeated crystallization from ethanol. Nojirimycin bisulfite was synthesized as described.19 D-Glucopyranoside was purchased from Sigma Chemical Co., St. Louis, MO., U.S.A. GAM was a product of Nissui Seiyaku Co., Tokyo, Japan. All other reagents were of the best available commercial quality.

Results
Stimulation of Growth of Eubacterium sp. Strain BAR by Barbaloin: Eubacterium sp. strain BAR scarcely grew in PYF broth containing no carbohydrate, but its growth was stimulated remarkably by the addition of barbaloin, which was mostly transformed to aloe-emodin anthrone, as shown in Fig. 1. The transformation of barbaloin to aloe-emodin anthrone was in parallel to the growth. Moreover, the bacterial growth depended on the amount of barbaloin...
added to the medium (Fig. 2). These results suggest that *Eubacterium* sp. strain BAR utilizes barbaloïn as a nutrient to grow.

**Effects of Other Sugars on Barbaloïn Metabolism**  
*Eubacterium* sp. strain BAR in GAM broth, which contains D-glucose, grew well, but did not metabolize barbaloïn at all as reported earlier.\(^1\) Sugars such as D-glucose, D-galactose, D-fructose, maltose, cellobiose and sucrose stimulated the growth of strain BAR in PYF broth. D-Amygdalin also stimulated the growth, and barbaloïn was the best stimulator of them all.

Addition of D-glucose, D-galactose, maltose, cellobiose, sucrose and D-amylgadin into PYF broth containing barbaloïn inhibited the transformation of barbaloïn to aloe-emodin anthranthrone, and also decreased the growth of strain BAR to the growth level found with the respective sugar. Figure 3 shows the effect of D-glucose as an example. D-Fructose, however, stimulated the growth in the presence of barbaloïn but little inhibited the laxative’s transformation (Fig. 4). Moreover, sugars such as D-xylene and D-fructose, which themselves hardly stimulated the growth of strain BAR in PYF broth, did not inhibit the barbaloïn transformation (data not shown). These results suggest that barbaloïn-metabolizing enzymes in strain BAR are induced by barbaloïn and that their induction is repressed with D-glucose or D-galactose, but not with D-fructose.

**Inhibition of Barbaloïn-Stimulation of Growth and Barbaloïn Metabolism by Nojirimycin**  
Nojirimycin bisulfite, a specific inhibitor of glucosidases,\(^2\) in a final concentration of more than \(5 \times 10^{-4} \text{ M}\) completely inhibited the growth of strain BAR stimulated by barbaloïn as shown in Fig. 5; the growth in broth containing D-glucose
or D-cellobiose was not affected by nojirimycin bisulfite at all, however, indicating that the inhibitor had no antibacterial action. $I_{50}$ value of nojirimycin bisulfite against the growth was $8 \times 10^{-6}$ M. The growth stimulated by barbaloain was not inhibited by D-glucono-1,4-lactone, a specific inhibitor of $\beta$-D-glucuronidases (data not shown).

Moreover, nojirimycin bisulfite completely inhibited the transformation of barbaloain to aloe-edomin anthrone in strain BAR as shown in Fig. 6. Thus, when nojirimycin bisulfite was added to the medium during the log phase of growth, both the growth of strain BAR and the barbaloain transformation were stopped. These results indicate that a barbaloain-metabolizing enzyme(s) capable of cleaving C-glycosyl bond is potently inhibited by nojirimycin and, consequently, the growth of strain BAR is interrupted. This enzyme cleaving the C-glycosyl bond may be a novel type of $\beta$-D-glucosidase.

**Discussion**

Barbaloain was reported to have appreciable purgative action in humans, though weaker than sennoside. Human intestinal flora transformed barbaloain to aloe-edomin anthrone, and *Eubacterium* sp. strain BAR having transforming capacity was isolated from human feces. Aloe-edomin anthrone, which shows weak purgative action, seems to be an ultimate purgative of barbaloain, in relation to the metabolic activation of sennoside. This study indicated that barbaloain induced the metabolizing enzyme system and stimulated the growth of strain BAR. In general, rapidly growing bacteria seem predominant in the human intestine and strain BAR seems to be inferior in number because it grows very slowly (1—2 d, Fig. 1). However, when barbaloain is administered orally to humans as an aloe extract (an oriental medicine), the growth of barbaloain-metabolizing bacteria alone such as strain BAR may be stimulated. If human beings ingest barbaloain every day, barbaloain-transforming ability in a lower part of intestine will increase, because sugars such as D-glucose do not remain, being absorbed and metabolized in an upper part of intestine, and the bowels are gradually loosened due to the formation of aloe-edomin anthrone.

Many bacteria having potent $\beta$-D-glucosidase activities did not metabolize barbaloain at all, and only *Eubacterium* sp. strain BAR cleaved the laxative C-glycosyl bond. The barbaloain-cleaving enzyme in strain BAR was inhibited by nojirimycin bisulfite, a specific inhibitor of glucosidases (Fig. 6), and the induction of the enzyme was repressed by D-glucose. Accordingly, the enzyme cleaving C-glycosyl bond may be a novel type of $\beta$-D-glucosidase (Chart 1), but an attempt to identify the structure of liberated sugar from

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**Chart 1. Two Possible Processes Cleaving C-Glycosyl Bond of Barbaloain**

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\text{HOH}_2C\text{OH} + \text{HOH}_2C\text{OH} + \text{CH}_3\text{OH} \rightarrow \text{hydrolysis} \\
\text{HOH}_2C\text{OH} + \text{HOH}_2C\text{OH} + \text{CH}_3\text{OH} \rightarrow \text{reduction}
\]

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**Fig. 5. Inhibition of Barbaloain-Stimulation of Growth by Nojirimycin**

Strain BAR was cultured in the presence of various concentrations of nojirimycin bisulfite in PYF broth containing 0.5% barbaloain (○), 0.5% glucose (△) or 0.5% cellobiose (□). The growth after cultivation for 2 d was measured by measuring turbidity at 540 nm. The growth without nojirimycin bisulfite was indicated as 100%.

**Fig. 6. Correlation of Barbaloain Cleavage and Growth**

Strain BAR was cultured in PYF broth containing 0.5 mg/ml barbaloain. After cultivation for 11 h and 17 h (arrows), $2 \times 10^{-5}$ mM nojirimycin bisulfite was added to the medium. Amount of anil (A) and turbidity at 540 nm (B) were measured as described in Materials and Methods.
barbaloin was unsuccessful in this study. The possibility exists that the C(10)–C(1') bond of barbaloin may be cleaved in a reductive manner similar to the cleavage of sennidine to rhein anthrone (Chart 1).

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References
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