

Communication

## Linear 3-Hydroxybutyrate Tetramer (HB4) Produced by *Sphingomonas* sp. Is Characterized as a Growth Promoting Factor for Some Rhizomicrobial Composers

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*Sphingomonas* spp. of  $\alpha$ -proteobacteria often play a role in assisting the development of microfloral communities under adverse soil conditions. Using a *Frateuria* sp. as an indicator for bacterial growth assay, we investigated the bacterial growth-promoting factor in the culture fluids of *Sphingomonas* sp. EC-K085. This factor was successfully isolated and identified as linear (R,R,R,R)-3-hydroxybutyrate tetramer (HB4), having a hydroxy-end and a carboxy-end group. When 28  $\mu$ g of HB4 was charged on a paper disc, impregnated *Frateuria* sp. cells in modified Winogradsky agar medium exhibited a promoted cell growth to form a clear colony emerging zone after a 2-day incubation.

**Key words:** bacterial cell growth-promoting factor; *Frateuria* sp.; linear 3-hydroxybutyrate tetramer; rhizobacteria; *Sphingomonas* sp.

*Xyris complanata* is a representative acid-tolerant, perennial herbal plant (Hashidoko *et al.*, in submission). We have been interested in its adverse soil-adapting strategy and the ecological role of rhizospheric microorganisms inhabiting the rhizosphere and rhizoplane of *X. complanata* grown on acidic tropical peat soils. As a means to address this research goal, we have isolated more than 100 strains of bacteria from the rhizosphere and rhizoplane of *X. complanata* grown in several environmental conditions, under modified Winogradsky's (MW, adding 1% saccharose and 0.005% yeast extract) mineral solution-base solid plates that we often use for screening of oligotrophs and heterotrophs in the plant rhizosphere.<sup>1</sup> We found subsequently that acid-tolerant and oligotrophic *Sphingomonas* spp.<sup>2</sup> and acid-susceptible and auxotrophic *Frateuria* spp.<sup>3</sup> (Fig. 1) are characteristic rhizobacterial groups in the rhizosphere and the rhizoplane respectively.

Due to compartmentalization in their habitat, we

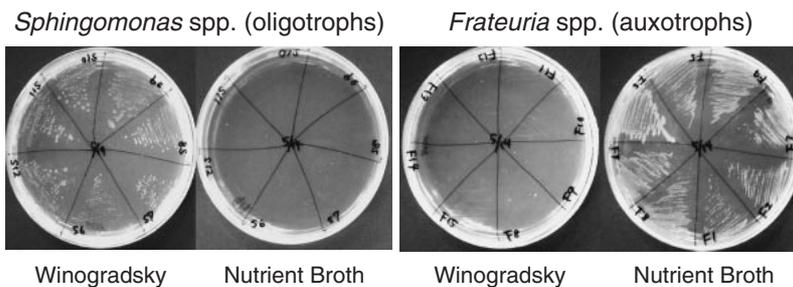
investigated the relationship between these *Sphingomonas* spp. and *Frateuria* spp. as to whether they affect each other in respect to their cell growth. Some *Sphingomonas* spp. displayed helper-like behaviors for a *Frateuria* sp. (Fig. 2A), similarly to observations in some *Sphingomonas* spp. towards other proteobacteria and/or fungi.<sup>4,5</sup> Generally, it is believed that bacteria are under severe competition in a nutrient-poor medium to acquire essential elements and energy sources to facilitate their survival. It is hence worth studying hypothetical biosignaling compounds produced by such *Sphingomonas* spp. that function to promote cell growth of auxotrophic *Frateuria* spp. in nutrient-poor media.

*Sphingomonas* sp. EC-K085 was cultured in modified Winogradsky's medium supplemented with 0.1% KNO<sub>3</sub> (w/v) at 25 °C at 80 rpm (rotary-shaking) for 7 d, and metabolites in the culture fluid (14,500 ml), from which the cultured bacterial cells had been removed by centrifugation (10,000  $\times$  g for 15 min), were subsequently trapped with a C<sub>18</sub>-coated silica gel (Cosmosil 75C<sub>18</sub>-PREP, Nacalai Tesque, Kyoto, Japan). The column was eluted with MeOH, and eventually 640 mg of crude extracts were obtained from 14.5-liter of the culture fluid. The crude extracts were re-dissolved in 36.25 ml of MeOH (400 fold of the culture fluid) and subjected to a preliminary growth-promotion assay. Onto a paper disc, 50  $\mu$ l of the extract solution (equivalent to 20 ml of the culture fluid) was charged, and the resulting disc was dried in vacuum to remove the solvent, and placed on a test bacterium-impregnated agar medium. The plate was incubated at 28 °C in the dark for 2 d. Growth-promoting activity toward the impregnated *Frateuria* sp. EC-K130 was observed in the crude extract, as shown in Fig. 2B.

To chase the active principles, a large portion of MeOH eluates (620 mg) was concentrated and re-dissolved in a small volume (about 1 ml) of MeOH.

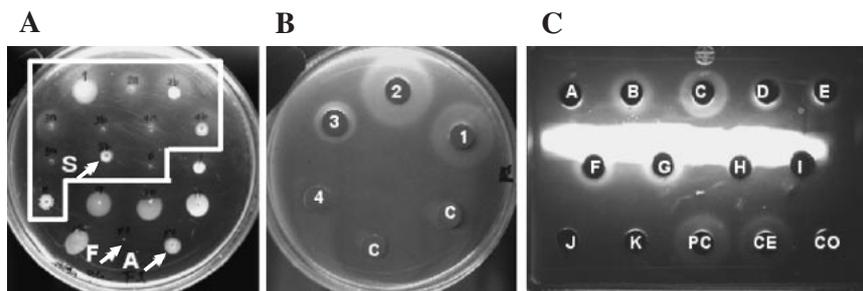
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Abbreviations: HB4, 3-hydroxybutyrate tetramer; PHB, poly-3-hydroxybutyrate; MW, modified Winogradsky



**Fig. 1.** Responses of *Spingomonas* spp. and *Frateuria* spp. to Nutrient-Poor or Nutrient-Rich Medium.

*Spingomonas* spp. and *Frateuria* spp., both isolated from several sites of *Xyris complanata*, were tested their growth performance on modified Winogradsky's medium (for oligotrophs) and nutrient broth medium (for auxotrophs). The agar plates (1.5% agar), on which these bacterial strains had previously been inoculated, were incubated at 28 °C for 2 d in the dark.



**Fig. 2.** Cell Growth-Promoting Effect of *Spingomonas* sp. EC-K085 on *Frateuria* sp. EC-K130 Impregnated in MW Agar Medium.

All of the plates, having been impregnated with the *Frateuria* sp. EC-K130 as described in "Materials and Methods," were incubated at 28 °C in the dark for 2 d. A, Not only *Spingomonas* sp. EC-K085 (arrow, S) but also a further 15 spingomonads isolated from *X. complanata* rhizosphere or the peat soil there were tested. Several isolates showed clear growth stimulation activities toward *Frateuria* sp. EC-K130 around the colonies of active spingomonads (framed with a white line). Although some areas of growth promotion are ambiguous in the photo, the activity of these positive strains was obvious in megascopic observation. *Frateuria* sp. EC-K130 (arrow, F) and an isolate of *Azospirillum* sp. (arrow, A) were also inoculated as reference bacteria. Other bacteria without any arrow are inactive spingomonads isolated from the same plant source. B, A cell growth-promoting principle was extracted with a Sep-Pak C18 cartridge (3 ml of reverse-phase, Varian) in which 20 ml of the bacterial culture fluid (0.1% KNO<sub>3</sub>-supplemented MW liquid medium, incubated for 7 d) had been passed. All of the MeOH eluates from the cartridge were concentrated and charged on a paper disc. The eluates also showed clear growth-stimulation activity toward impregnated EC-K130. Nos. 1–3 are culture extracts of *Spingomonas* spp., strains EC-K088, EC-K085, and EC-K129 respectively, while no. 4 is a culture fluid extract of *Frateuria* sp. EC-K130 prepared by the same process. C is a control (duplicated MeOH only). C, Among the fractions (A to K) obtained by the C<sub>18</sub> reversed phase silica gel column chromatography as described, the most significant activity was apparent in fraction C. PC and CE are positive controls (a volume-equivalent mixture of all fractions) and crude extract before column chromatography respectively, while CO is a control (MeOH only).

The dissolved eluates were then diluted 100 fold with H<sub>2</sub>O in preparation for placing them onto 80 g of Cosmosil 75C<sub>18</sub>-PREP column. We obtained 11 fractions by eluting the column with the following MeOH–water solvent system: H<sub>2</sub>O (100 ml × 2, fractions A and B), 25% MeOH/H<sub>2</sub>O (100 ml × 3, C, D and E), 40% MeOH/H<sub>2</sub>O (100 ml × 2, F and G), 60% MeOH/H<sub>2</sub>O (100 ml × 2, H and I), 80% MeOH/H<sub>2</sub>O (200 ml, J) and 100% MeOH (200 ml, K). All of the fractions obtained were dried in vacuum, and the resulting eluates were then re-dissolved in a constant-volume of 1.40 ml of MeOH (each 10,000 fold of the culture fluid). Ten-times diluted extract solution (50 μl, equivalent to 50 ml) was each charged to a paper disc. The resulting discs were assayed by the same process as described for the crude extract.

Some fractions (B, C, D, E, F, and G) had growth-promoting activities toward EC-K130 (Fig. 2C). The

activity of fraction C (30.2 mg) was much clearer than those of other fractions exhibiting an ambiguous growth zone, so that a portion of fraction C (6.4 mg, obtainable from 3,000 ml of culture fluid) was further fractionated by preparative TLC (in MeOH–H<sub>2</sub>O 2:1) on reverse-phase silica gel plate (RP-18 F<sub>254S</sub> 15389, Merck, Darmstadt, Germany) in guidance with detection under UV 365 nm and 254 nm lights. Only one quenching spot at R<sub>f</sub> 0.56–0.62 (fraction C-1) showed activity.

The active fraction C-1 thus obtained was further subjected to a purification process using reversed phase HPLC in MeOH–H<sub>2</sub>O 2:1, setting an UV detector at 284 nm due to maximal UV absorption of fraction C-1. Accordingly, active substance possessing no conjugation system in the molecule was eluted (*t<sub>R</sub>* 4.0–5.9 min) in front of the main UV peak (*t<sub>R</sub>* 7.0 min), which was identified as 4-hydroxybenzaldehyde (spectral data not shown). The purified chemical substance

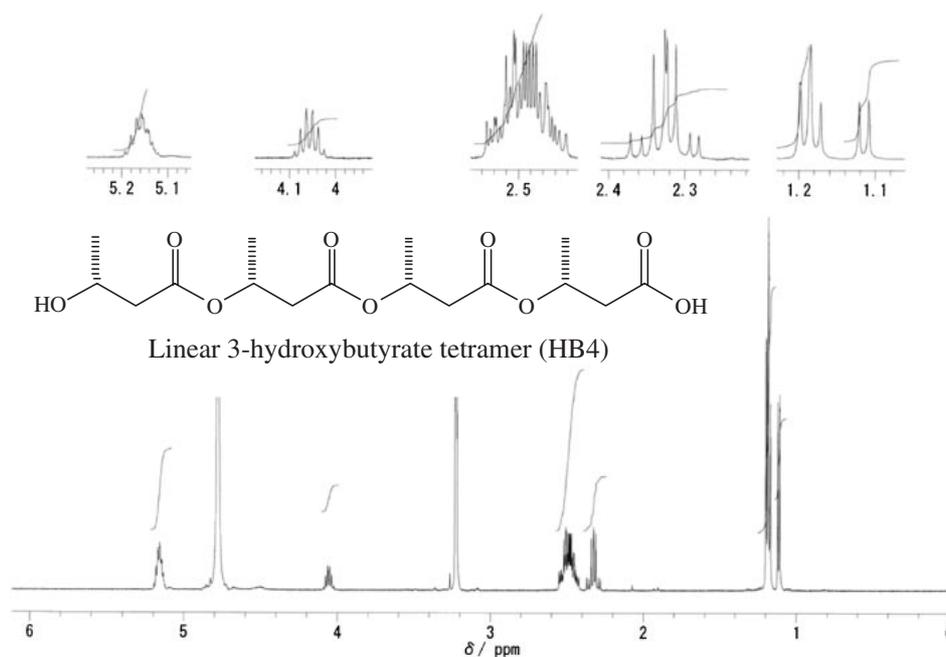


Fig. 3.  $^1\text{H-NMR}$  Spectrum ( $\text{CD}_3\text{OD}$ ) and Chemical Structure of Active Principle (HB4) Purified from Fraction C-1.

(1.7 mg) showed a growth-promoting activity nearly equivalent to the crude fraction C.

The purified active principle gave a parent ion at  $m/z$  363 ( $\text{M}^+ + \text{H}$ , 100%) in FD-MS. In EI-MS, its fragmentation indicated a repeated fission losing 86 mass units. By negative FAB-HR-MS analysis, the molecular formula ( $[\text{M} - \text{H}]^-$ ) was confirmed to be  $\text{C}_{16}\text{H}_{25}\text{O}_9$  (found 361.1505, calcd. 361.1499).  $^1\text{H-NMR}$  spectrum together with  $^1\text{H-}^1\text{H-COSY}$ ,  $^{13}\text{C-NMR}$ , DEPT, HMQC, and HMBC in  $\text{CD}_3\text{OD}$  showed the presence of four methyl ( $\delta_{\text{H}}$  1.11, 1.18, 1.19, and 1.19), four methine ( $\delta_{\text{H}}$  4.05 and at  $\delta_{\text{H}}$  5.12–5.19 for 3H), and four pairs of methylene ( $\delta_{\text{H}}$  2.31 and 2.35, and three sets at  $\delta_{\text{H}}$  2.41–2.56 for 6H) proton signals. The methyl signals (all doublets,  $J = 6\text{ Hz}$ ) were coupled with the oxygenated methine signals, and the set of methine signals was further coupled with the methylene signals. A set of proton-coupling sequences in those showing upfield-shifts at  $\delta_{\text{H}}$  1.11 ( $\text{CH}_3$ ), 4.05 (CH), and  $\delta_{\text{H}}$  2.31 and 2.35 ( $-\text{CH}_2$ ) was reasonably assignable to the hydroxy-end 3-hydroxybutyrate moiety of this compound (Fig. 3). Accordingly, the active principle was found to be a linear-type 3-hydroxybutyrate tetramer (HB4), of which  $^1\text{H-}$  and  $^{13}\text{C-NMR}$  chemical shifts were found to be in good accordance with those of authentic ( $R,R,R,R$ )-HB4, as previously reported by Bachmann and Seebach.<sup>6)</sup>

Poly-3-hydroxybutyrate (PHB, also a synonym of  $\beta$ -hydroxybutyrate polymer or 3-hydroxybutyrate polymer) is one of the most representative bacterial polymeric substances for several free-living nitrogen-fixing bacteria. Poly-3-hydroxybutyrate depolymerase (PHBDP), which catalyzes endo-type hydrolysis of PHB to produce several linear-type 3-hydroxybutyrate oligomers, including HB4, is also produced by many soil

bacteria.<sup>7,8)</sup> Sang has reported that the absolute configuration of 3-hydroxybutyric acid and the 3-hydroxybutyrate unit of PHB produced by proteobacteria is generally in the  $R$ -configuration.<sup>9)</sup> Bachmann and Seebach have also reported optical rotation of synthesized ( $R,R,R,R$ )-HB4 as  $[\alpha]_{\text{D}} -16^\circ$  ( $c = 1.0$  in MeOH),<sup>6)</sup> and the HB4 we have isolated showed a levorotatory ( $[\alpha]_{\text{D}} -12^\circ$ ,  $c = 0.65$  in MeOH). Considering these features together, it is concluded that all of the four chiral carbons of HB4 produced by EC-K085 are in the  $R$ -configuration.

Since saccharose is contained in MW medium at 1% concentration, it is obvious that the effective role of HB4 is not a simple carbon source. Instead, it is most likely playing a role as a certain functional principle. Because PHB is known to be a chelating agent towards several metal cations by capturing them inside the helix of its chain,<sup>10,11)</sup> it may function as metal cation ionophores. In fact, in not a few cases, cell growth of a hardly culturable microorganism is regulated by only one limiting nutritional factor. Hoo *et al.* reported that  $\text{Mg}^{2+}$  was the limiting factor for the cell growth of *Sphingobium yanoikuyae* EC-S001, which is difficult to culture in commercial potato-dextrose broth (Difco, Boston, MA).<sup>12)</sup> In this case, the threshold level of  $\text{Mg}^{2+}$  for normal growth of strain EC-S001 is 0.10 mM, but the standard potato-dextrose broth medium contained only 0.05 mM of  $\text{Mg}^{2+}$ . Similarly to this example, HB4 may assist effective assimilation of certain cations or other nutrient serving as a limiting factor for the cell growth of auxotrophic *Frateruia* sp. in nutrient-poor media. It is necessary to confirm whether HB4 is applicable in artificial media to enhance the growth of ceratin hardly culturable bacteria.

At the present time, the physiology and ecological roles of PHB in polymer-producing bacteria is not well understood. Some studies using bacterial mutants which lacked the ability to produce PHB showed that PHB suppresses bacterial metabolisms and also extends bacterial life-spans in bulk soil.<sup>13)</sup> In our study, HB4 produced by *Sphingomonas* sp. EC-K085 was characterized as a candidate for an important chemical signal among rhizospheric microbial communities that functions to increase their microfloral diversity. As a related phenomenon, Tanaka *et al.* reported that a *Sphingomonas* sp. in activated sludge enabled an unculturable *Catellibacterium nectariphilum* to become culturable, contributing to microfloral development in the sludge.<sup>5)</sup> Despite efforts at isolation and characterization of the growth promoting chemical factor produced by the *Sphingomonas* sp., it is not yet characterized completely.

Some other acidic soil tolerant rhizobacteria (*e.g.*, *Burkholderia* spp.) also showed similar growth-promoting effects on impregnated slow-growing rhizobacteria (*e.g.*, another *Burkholderia* sp. and a *Pantoea* sp. isolated from *Melastoma malabathricum* and *Artocarpus champeden*). It is thus most likely that HB4 is the major growth-promoting principle, and chemical investigation of them may reveal a complicated regulation system of unculturable microfloral communities. Further ecochemical and biochemical studies of 3-hydroxybutyrate oligomers are currently in progress, and their details will be reported elsewhere as a full paper.

The materials and methods part in this communication is as follows.

**General:** EI-MS and FD-MS were measured by JEOL JMS-01SG-2 and JEOL JMS-DX300 (Tokyo) respectively. <sup>1</sup>H- and <sup>13</sup>C-NMR data were assigned by 2D-NMR experiments (HH-COSY, HMBC and HMQC) by using a Bruker AMX500 (<sup>1</sup>H: 500 MHz and <sup>13</sup>C: 125 MHz). The optical rotation value was measured in MeOH with a JASCO DIP-370 Digital Polarimeter, using a quartz cell ( $\phi$  3.5 mm  $\times$  10 mm thick). Final purification of the active principle was done by HPLC (a Hitachi L-6000 equipped with a Hitachi L-4200H UV-VIS detector) connected with a reverse phase column (YMC-Pac PolymerC18,  $\phi$  4.6 mm  $\times$  250 mm long). MeOH-H<sub>2</sub>O 2:1 was used as an isocratic solvent with a UV detector set at 284 nm to yield two chemical substances.

**Isolation and identification of bacteria.** *X. complanata*, used as a bacterial source, was a specimen forming a pure community in a topsoil-removed peatland near Palangkaraya, Central Kalimantan, Indonesia. The root of the plant was first washed well with clean water in a handy spray bottle, and the resulting roots were cut into several fragments (2–3 cm) and transferred into a 50 ml-Falcon tube, in which 30 ml of sterilized water was filled. The Falcon tube was then shaken for 5 s, and the root fragment was inoculated into N-free gellan gum medium to trap rhizoplane bacteria. The remaining root washings in the Falcon tube were directly used as an

inoculant to search for rhizospheric bacteria on MW agar plates. Bacterial identification was done based on 16S rRNA gene sequence determination and a homology search on GeneBank database. *Sphingomonas* sp. EC-K085 (accession no. AB264174) and *Frateuria* sp. EC-K130 (AB264175) were isolated from the rhizosphere and rhizoplane of the host plant respectively.

**Bioassay:** We performed a paper disc assay (8 mm diameter with 2 mm thickness, Advantec, Tokyo) in the search for growth-promoting factors in the culture fluid of *Sphingomonas* sp. MW solution adjusted to a final pH of 6.0 with a small volume of 4 M H<sub>2</sub>SO<sub>4</sub> was added to 0.5% agar and then autoclaved at 120 °C for 30 min. Cooling below 45 °C, a cell suspension of *Frateuria* sp. EC-K130 (OD 0.1 or more) precultured on an MW agar plate for 2–3 d (1 ml, 10<sup>7</sup> cells) was added and vortexed well to cast onto agar plates (9 cm diameter, 15 ml of gel volume).

**Physicochemical properties of linear (R,R,R,R)-3-hydroxybutyrate tetramer (HB4):** colorless syrup; FD-MS (*m/z*, rel. int. %): 363 (M<sup>+</sup> + H, 100); EI-MS (*m/z*, rel. int. %): 277 (15), 259 (13), 214 (17), 191 (25), 173 (63), 155 (95), 128 (27), 105 (20), 87 (61), 69 (100), and 43 (23); FAB-HR-MS (negative mode): C<sub>16</sub>H<sub>25</sub>O<sub>9</sub> (found 361.1505, calcd. 361.1499); [ $\alpha$ ]<sub>D</sub> –12° (*c* = 0.65 in MeOH); <sup>1</sup>H-NMR ( $\delta$ <sub>H</sub> in CD<sub>3</sub>OD, 500 MHz): 5.12–5.19 (3H, m), 4.05 (1H, m), 2.41–2.56 (6H, m), 2.35 (1H, dd, *J* = 15 and 7 Hz), 2.31 (1H, dd, *J* = 15 and 5 Hz), 1.19 (6H, d  $\times$  2, *J* = 6 Hz), 1.18 (3H, d, *J* = 7 Hz), and 1.11 (3H, d, *J* = 6 Hz); <sup>13</sup>C-NMR ( $\delta$ <sub>C</sub> in CDCl<sub>3</sub>, 125 MHz): 172.3 (CO), 169.5 (CO  $\times$  2), 169.4 (CO), 68.0 (CH), 68.0 (CH), 67.9 (CH), 64.7 (CH), 43.5 (CH<sub>2</sub>), 41.0 (CH<sub>2</sub>  $\times$  2), 40.7 (CH<sub>2</sub>), 22.4 (CH<sub>3</sub>), 20.1 (CH<sub>3</sub>), 20.0 (CH<sub>3</sub>), and 19.9 (CH<sub>3</sub>).

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