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Production of 4-hydroxybutyrate from succinate semialdehyde in butyrate biosynthesis in *Porphyromonas gingivalis*

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ABSTRACT

Background: Despite evidence demonstrating the importance of butyrate-producing bacteria in host health and disease, the characterization of enzymes responsible for butyrate production has not been fully elucidated in the periodontopathogen, *Porphyromonas gingivalis*.
Methods: LC-MS/MS and colorimetric analyses were employed to enzymatically characterize recombinant PGN_0724 in *P. gingivalis* as a succinate semialdehyde reductase. The concentration of short chain fatty acids in the culture supernatant of the wild-type bacteria and a mutant strain lacking the PGN_0724 gene were quantified using GC-MS.
Results: Incubation of recombinant PGN_0724 with succinate semialdehyde and NADH resulted in the production of 4-hydroxybutyrate as well as consumption of succinate semialdehyde. Double reciprocal plots showed that the reaction catalyzed by the PGN_0724 protein was associated with a ternary complex mechanism. The growth speed and final turbidity of the mutant strain were much lower than those of the wild-type cells. The capacity of the mutant strain to produce butyrate, isobutyrate, and isovalerate was 30%, 15%, and 45%, respectively, of that of the wild-type strain, while the mutant strain produced approximately 3.9-fold more propionate than the wild type.
Conclusions: The pathway responsible for butyrate production is important for the growth of *P. gingivalis* and appears to be associated with production of the other short chain fatty acids.
General significance: The aim of this study was to delineate the mechanisms involved in the production of 4-hydroxybutyrate, which is an intermediate in the biosynthetic pathway for production of butyrate, which is a virulence factor in *P. gingivalis*.

1. Introduction

Porphyromonas gingivalis, a Gram-negative, asaccharolytic, obligate anaerobic bacterium, is considered a major pathogen in the development of periodontal diseases [1,2]. This bacterium is also suggested to be involved in aspiration pneumonia in the elderly and responsible for the development of certain systemic diseases, including diabetes [3], atherosclerosis [4], and rheumatoid arthritis [5].

P. gingivalis possesses several virulence factors, including fimbriae, proteases, hemagglutinins, lipopolysaccharides, capsule polysaccharides, major outer membrane proteins, and cytotoxic metabolic end products [2,6]. Butyrate, which is the most toxic metabolic end product found in the oral cavity [7], induces apoptosis in gingival fibroblasts and in T and B cells [8–10], and produces reactive oxygen species affecting cell cycle progression in human gingival fibroblasts [11]. It is interesting that the concentration of butyrate in the periodontal pockets correlates significantly with clinical measures of inflammation and disease severity [12,13]. In the gastrointestinal tract, however, butyrate produced by bacteria is thought to play beneficial roles [14–16]. For example, butyrate induces proliferation of epithelial cells and regulatory T cells in normal intestinal tissue, reduces cell proliferation, increases apoptosis, and stimulates the differentiation of colon cancer cells, which may reduce the incidence and progression of colon cancer [17,18]. Butyrate may also improve the barrier function of the gastrointestinal epithelia and thus prevent diarrheal disorders induced by barrier failure [19].

The mechanism of butyrate production has been studied mainly in Gram-positive bacteria in the human intestine [20], where the Embden-Meyerhof-Parnas pathway is the main route for the catabolism of glucose via coupling of two molecules of acetyl-CoA [21]. In the other known butyrate-producing pathways, the lysine, glutarate, and 4-aminobutyrate pathways, amino acids serve as major substrates [22]. *P. gingivalis* mainly utilizes peptides as sources of energy and cell

Abbreviations: SCFA, short chain fatty acid; GST, glutathione S-transferase

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materials [23,24], especially glutamine/glutamate and asparagine/aspartate-containing peptides [25]. Glutamate and aspartate derived from these peptides are thought to be deaminated to succinyl-CoA [25,26], which is subsequently converted into butyryl-CoA by a sequential reaction (Fig. 1). Butyryl-CoA is then converted into acetyl-CoA with the formation of butyrate. Acetyl-CoA is further degraded to acetate with the production of ATP. The genes associated with the pathway in *P. gingivalis* were annotated from the database analysis [27], although the function of the genes was not experimentally elucidated.

In the current study, we identified the gene (PGN_0724) encoding succinate semialdehyde reductase (E.C. 1.1.1.61), also named 4-hydroxybutyrate dehydrogenase or γ-hydroxybutyrate dehydrogenase, which is involved in the intermediate step to produce 4-hydroxybutyrate from succinate semialdehyde. The recombinant protein was purified and enzymatically characterized. Deletion of PGN_0724 resulted in considerable effects on the capacity of *P. gingivalis* to produce SCFAs, including butyrate, propionate, isobutyrate, and isovalerate.

2. Materials and methods

2.1. Bacterial strains and culture conditions

P. gingivalis ATCC 33277 and its derivative were grown anaerobically at 37 °C in modified GAM broth (Nissui, Tokyo, Japan) or on Brucella HK agar (Kyokuto Pharmaceutical Industrial, Tokyo, Japan), supplemented with 5% rabbit blood and, when necessary, with 20 μg ml^{-1} erythromycin. *Escherichia coli* strains DH5α (Invitrogen, Carlsbad, CA) and BL21 (Novagen, Madison, WI), used for DNA manipulation and recombinant protein purification, respectively, were grown aerobically in 2 × YT broth (BD Japan, Tokyo, Japan) at 37 °C supplemented with 100 μg ml^{-1} ampicillin or 200 μg ml^{-1} erythromycin.

2.2. Purification of recombinant enzymes

Recombinant PGN_0724 from *P. gingivalis* was obtained using the expression vector pGEX-6P-1 (GE Healthcare Japan, Hino, Japan), as described previously [28]. The coding sequence, which was PCR amplified from the genomic DNA of *P. gingivalis* ATCC 33277 using the primers listed in Table 1, was ligated into the pGEX-6P-1 vector via the *Bam*HI and *Sal*I restriction sites, juxtaposing each gene downstream of the coding sequence for glutathione *S*-transferase (GST) and a PreScission protease cleavage site. The resulting plasmid was sequenced to verify the fidelity of the PCR amplification and then used to transform *E. coli* BL21 cells. A 50 μl aliquot of overnight culture was inoculated into 50 ml of fresh medium, and the cultures were grown at 37 °C to an optimal density at 600 nm (OD_{600}) of approximately 0.7. After induction of protein expression with 0.3 mM isopropyl-β-D-thiogalactopyranoside, the cells were incubated at 37 °C for 2 h, harvested by centrifugation at 4 °C, resuspended in 0.5 ml of PBS, and lysed by ultrasonication on ice. The lysate was centrifuged at 30,000 × *g* for 1 h, and the GST

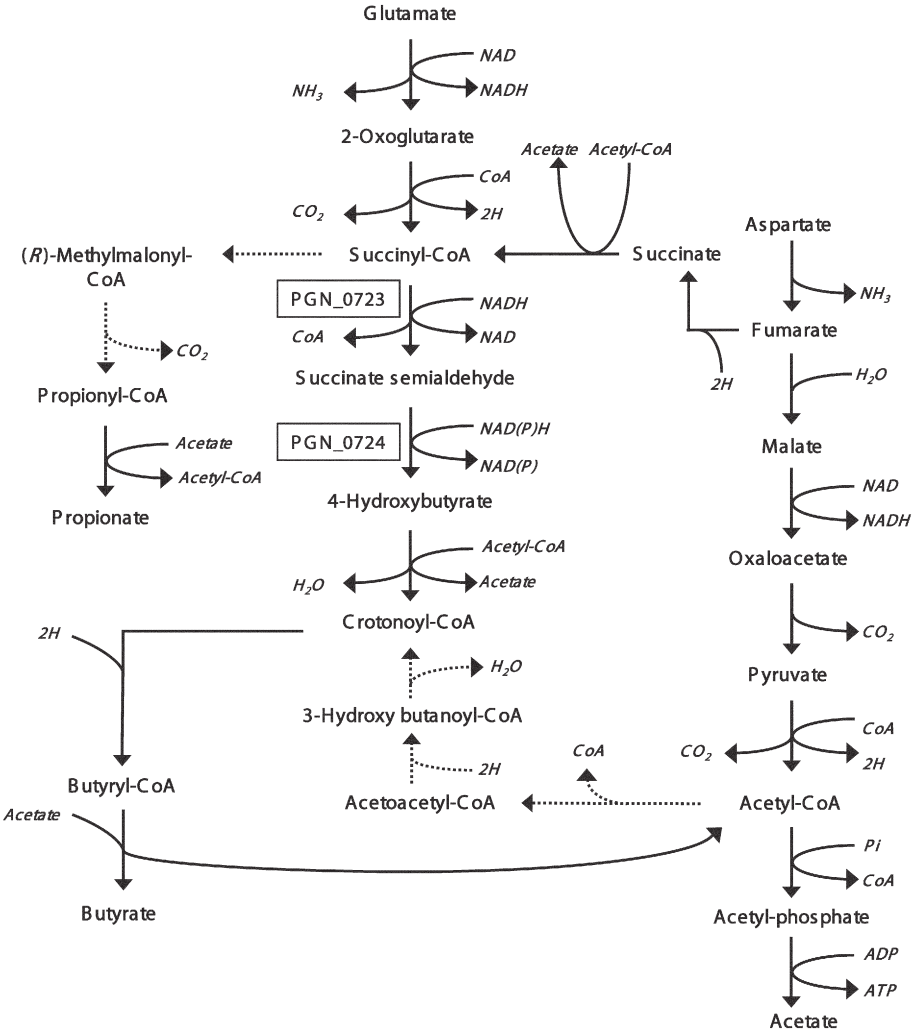

Fig. 1. Proposed metabolic pathways for use of glutamate and aspartate in *P. gingivalis* based on previously described pathways [22,25,26,44]. Broken lines indicate expected pathways that were not supported by the experimental evidence.