molecular oral microbiology

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Erythritol alters microstructure and metabolomic profiles of biofilm composed of *Streptococcus gordonii* and *Porphyromonas gingivalis*

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Keywords: biofilm; capillary electrophoresis time-of-flight mass spectrometry; metabolomics; *Porphyromonas*; *Streptococcus*; sugar alcohols
Accepted 18 June 2013
DOI: 10.1111/omi.12037

SUMMARY

The effects of sugar alcohols such as erythritol, xylitol, and sorbitol on periodontopathic biofilm are poorly understood, though they have often been reported to be non-cariogenic sweeteners. In the present study, we evaluated the efficacy of sugar alcohols for inhibiting periodontopathic biofilm formation using a heterotypic biofilm model composed of an oral inhabitant *Streptococcus gordonii* and a periodontal pathogen *Porphyromonas gingivalis*. Confocal microscopic observations showed that the most effective reagent to reduce *P. gingivalis* accumulation onto an *S. gordonii* substratum was erythritol, as compared with xylitol and sorbitol. In addition, erythritol moderately suppressed *S. gordonii* monotypic biofilm formation. To examine the inhibitory effects of erythritol, we analyzed the metabolomic profiles of erythritol-treated *P. gingivalis* and *S. gordonii* cells. Metabolome analyses using capillary electrophoresis time-of-flight mass spectrometry revealed that a number of nucleic intermediates and constituents of the extracellular matrix, such as nucleotide sugars, were decreased by erythritol in a dose-dependent manner. Next, comparative analyses of metabolites of erythritol- and sorbitol-treated cells were performed using both organisms to determine the erythritol-specific effects. In *P. gingivalis*, all detected dipeptides, including Glu-Glu, Ser-Glu, Tyr-Glu, Ala-Ala and Thr-Asp, were significantly decreased by erythritol, whereas they tended to be increased by sorbitol. Meanwhile, sorbitol promoted trehalose 6-phosphate accumulation in *S. gordonii* cells. These results suggest that erythritol has inhibitory effects on dual species biofilm development via several pathways, including suppression of growth resulting from DNA and RNA depletion, attenuated extracellular matrix production, and alterations of dipeptide acquisition and amino acid metabolism.

INTRODUCTION

Sugar alcohols (also known as polyols) such as erythritol, xylitol, and sorbitol are hydrogenated forms of carbohydrates and commonly used in place of sucrose, often in combination with high-intensity artificial sweeteners to counter their low level of sweetness (Monedero *et al.*, 2010). All known sugar alcohols have been tested *in vitro* for fermentation by oral microorganisms, and can be classified as hypo- or non-acidogenic (Makinen, 2010). Based on animal

experiments and intra-oral cariogenicity test results, it is concluded that sugar alcohols have extremely low or no cariogenicity (van Loveren, 2004). Caries and periodontal diseases are major oral infectious disorders, so it is considered important to determine whether sugar alcohols can counteract periodontal diseases in addition to caries. However, few studies have examined their effects on periodontal diseases.

Erythritol (1,2,3,4-butanetetrol) is a tetraol ($C_4H_{10}O_4$) produced by the reduction of erythrose ($C_4H_8O_4$) (Moon *et al.*, 2010). In humans, erythritol is efficiently absorbed, not metabolized, and excreted by renal processes, so the absence of systemic metabolism of erythritol means that it has limited potential to induce changes in plasma glucose and insulin levels (Munro *et al.*, 1998; Yokozawa *et al.*, 2002). Notably, erythritol has a lower calorie level (0–0.2 kcal kg^{-1}) compared with other sugar alcohols, yielding approximately 2 kcal kg^{-1}. Additionally, it is most unlikely to cause gastrointestinal side effects, because its maximum no-effect dose for causing diarrhea in humans is estimated to be the highest among sugar alcohols (Oku & Nakamura, 2007). Consequently, erythritol is often used as a functional sugar replacement in special foods prepared for individuals with diabetes and obesity. Interestingly, erythritol reportedly inhibited the growth of several mutans streptococci organisms in the same manner as xylitol (Makinen *et al.*, 2005). Additionally, an *in vitro* study of candidal biofilm showed that erythritol significantly enhanced the fungicidal effect of benzethonium chloride, compared with xylitol and sorbitol (Ichikawa *et al.*, 2008).

Streptococcus gordonii is a member of oral streptococci, which are major components of the dental biofilm. Once *S. gordonii* has formed biofilm on tooth surfaces, it provides an attachment substrate for subsequent colonization by more pathogenic species such as *Porphyromonas gingivalis* (Kuboniwa & Lamont, 2010; Whitmore & Lamont, 2011). Indeed, introduction of *P. gingivalis* into the mouths of human volunteers results in nearly exclusive localization in areas of streptococcal-rich plaque (Slots & Gibbons, 1978). This allows *P. gingivalis* to become established on tooth surfaces where colonization of the subgingival area can occur by spreading proliferation or translocation of dislodged progeny (Haffajee *et al.*, 1998; Socransky *et al.*, 1998; Ximenez-Fyvie *et al.*, 2000a,b).

Metabolomics, a novel approach for revealing global and dynamic biological information, is useful to comprehensively analyze intracellular metabolites and the results reflect the phenotype more closely compared with other -omics technologies (e.g. proteomics, transcriptomics). In particular, metabolomic analysis based on capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) is thought to be one of the strongest tools for detecting a broad range of characteristics of detectable metabolites and can be used to examine thousands of metabolites in bacteria, including the majority of metabolic substrates in major metabolic pathways (Ishii *et al.*, 2007; Monton & Soga, 2007).

In this study, we examined the effects of sugar alcohols on *S. gordonii* single species biofilm and *S. gordonii–P. gingivalis* mixed species biofilm development *in vitro*. In addition, we investigated alterations of the internal metabolomic profiles caused by sugar alcohols in each organism.

METHODS

Bacterial strains and culture conditions

Porphyromonas gingivalis strain ATCC 33277 was anaerobically cultured (80% N_2, 10% H_2, 10% CO_2) at 37°C in trypticase soy broth (TSB) containing 1 mg ml^{-1} yeast extract, 5 μg ml^{-1} hemin, and 1 μg ml^{-1} menadione. *Streptococcus gordonii* strain DL1 was grown at 37°C in Todd–Hewitt broth. For *S. gordonii* biofilm development, a chemically defined medium for streptococci (Loo *et al.*, 2000) was modified by using 0.8% sucrose (mCDM-B), as previously described (Kuboniwa *et al.*, 2006). Briefly, mCDM-B consisting of 58 mM K_2HPO_4, 15 mM KH_2PO_4, 10 mM $(NH4)_2SO_4$, 35 mM NaCl, 0.8% sucrose, 0.2% casamino acids, and 100 μM $MnCl_2$ $4H_2O$ (pH 7.4) was supplemented with filter-sterilized vitamins (0.04 mM nicotinic acid, 0.1 mM pyridoxine HCl, 0.01 mM pantothenic acid, 1 μM riboflavin, 0.3 μM thiamin HCl, 0.05 μM D-biotin), amino acids (4 mM L-glutamic acid, 1 mM L-arginine HCl, 1.3 mM L-cysteine HCl, 0.1 mM L-tryptophan), and $MgSO_4$ $7H_2O$ (2 mM). Investigation of the growth of *P. gingivalis* in a limited energy source was carried out using modified chemically defined medium, which did not contain α-ketoglutarate (mCDM-G) (Grenier *et al.*, 2001) consisting of 10 mM NaH_2PO_4, 10 mM KCl, 2 mM citric

acid, 1.25 mM $MgCl_2$, 100 μM $FeCl_3$, 20 μM $CaCl_2$, 0.1 μM Na_2MoO_4, 25 μM $ZnCl_2$, 50 μM $MnCl_2$, 5 μM $CuCl_2$, 10 μM $CoCl_2$, 5 μM H_3BO_3, 7.67 μM hemin, 2.91 μM menadione, and an appropriate carbon and nitrogen source (pH 7.0). For gingipain activity and metabolomic analyses, *P. gingivalis* cells were grown in TSB medium diluted three times (dTSB) with phosphate-buffered saline (PBS, pH 7.4) (Kuboniwa *et al.*, 2009). For metabolomic assays of *S. gordonii*, mCDM-B modified by low sucrose (0.1%) (mCDM-M) was used as the culture medium. For the metabolomic assays, we used a Transwell system (Corning Inc., Corning, NY), in which *P. gingivalis* and *S. gordonii* cells were co-cultured in PBS in the presence or absence of a sugar alcohol to observe its inhibitory effects on both individual growth and mutual nutrition transfer.

Dual species biofilm formation assays using dot blotting

Dot blotting assays were performed as previously described (Kuboniwa *et al.*, 2006). Briefly, *S. gordonii* biofilms deposited on nitrocellulose membranes using a Bio-Dot microfiltration apparatus [1×10^8 colony-forming units (CFU) dot^{-1}] were reacted with biotinylated *P. gingivalis* cells (4×10^8 CFU ml^{-1}) in the presence of 0, 0.8, 5, 10 or 20% meso-erythritol (Mitsubishi-Kagaku Foods Co., Tokyo, Japan) or D-sorbitol. After co-incubation, *P. gingivalis* accumulation was detected using alkaline phosphatase-conjugated streptavidin and an alkaline phosphatase-specific substrate.

Observation of biofilm microstructures

Streptococcus gordonii cells were stained with 15 μg ml^{-1} hexidium iodide, then suspended in mCDM-B or mCDM-B containing 10% erythritol, xylitol, or sorbitol. Bacterial suspensions (2×10^7 CFU) were anaerobically cultured for 16 h using a CultureWell® chambered coverglass system (Grace Bio-Labs, Bend, OR) to allow for biofilm development.

Heterotypic biofilm microstructures were observed as previously described (Kuboniwa *et al.*, 2006). Hexidium iodide-stained *S. gordonii* (2×10^7 CFU) cells were suspended in mCDM-B and anaerobically cultured for 16 h using a CultureWell® system. *P. gingivalis* cells were labeled with 6 μg ml^{-1} 5-(and-6)-carboxyfluorescein succinimidyl ester, then suspended in PBS or PBS containing 10% erythritol, xylitol, or sorbitol. Bacterial suspensions (8×10^6 CFU) were anaerobically reacted with surface-attached *S. gordonii* biofilms for 24 h, respectively.

Analysis of biofilm formation was performed using confocal laser scanning microscopy (Radiance 2100; Bio-Rad, Hercules, CA), as previously described (Nagata *et al.*, 2009).

Growth study

Streptococcus gordonii (1×10^8 CFU) cells were cultured in mCDM-M, mCDM-M supplemented with 10% erythritol, or mCDM-M modified by using 0.1% erythritol instead of 0.1% sucrose at 37°C. Optical density at 600 nm (OD_{600}) was determined at 1-h intervals for 8 h and then after 10, 12, and 24 h using a UV-visible recording spectrophotometer (UV-265FW; Shimadzu, Kyoto, Japan).

Porphyromonas gingivalis (1×10^8 CFU) cells were anaerobically cultured in mCDM-G supplemented with 0.5% erythritol, 3% bovine serum albumin (BSA), or 3% BSA including 10% erythritol at 37°C. OD_{600} was determined at 6-h intervals for 48 h.

Gingipain activity assay

Arg-gingipain (Rgp) activity assays were performed as previously described (Nakayama *et al.*, 1996). Briefly, *P. gingivalis* cells (5×10^5 CFU) were incubated in buffer containing 5 mM cysteine, 20 mM sodium phosphate buffer (pH 7.65), and 10 μM benzoyl-L-arginine-4-methylcoumaryl-7-amide at 37°C for 10 min. The proteolytic activities of Rgp were determined by the fluorescence intensity of released 7-amino-4-methylcoumarin detected at excitation of 355 nm and emission of 460 nm wavelengths using an $ARVO_{MX}$/Light (Perkin Elmer, Inc., Waltham, MA). Benzyloxycarbonyl-L-histidyl-L-glutamyl-L-lysine-4-methylcoumaryl-7-amide was applied as the substrate for Lys-gingipain (Kgp) (Abe *et al.*, 2000).

Intracellular metabolite extraction

Porphyromonas gingivalis cells were cultured anaerobically in dTSB containing 0, 0.8, or 10% erythritol at 37°C for 6 h. Bacterial cells were collected by centrifugation at 13,000 ***g*** for 2 min, then washed with

Milli-Q water followed by centrifugation. The bacterial pellets (1.4×10^{10}–1.8×10^{10} CFU) were immediately fixed by adding 5 μM internal standard-containing methanol (Ohashi *et al.*, 2008). *S. gordonii* cells (0.7×10^{10}–0.8×10^{10} CFU) were cultured in mCDM-M in the presence or absence of erythritol at 37°C for 6 h, and prepared in the same way as *P. gingivalis*.

CE-TOFMS conditions

CE-TOFMS was performed using an Agilent CE-TOF-MS System (Agilent Technologies, Santa Clara, CA) equipped with a fused silica capillary [50 μm (inner diameter) × 80 cm]. The conditions for measurement of cationic/anionic metabolites were as follows. Run buffer: a solution composed of Cation Buffer Solution [H3301-1001; Human Metabolome Technologies (HMT), Tsuruoka, Japan] and Anion Buffer Solution (H3302-1021), CE voltage: + 27 kV/+ 30 kV, MS ionization: ESI positive/ESI negative, MS capillary voltage: 4000 V/3500 V, MS scan range: m/z 50–1000, and sheath liquid: HMT Sheath Liquid (H3301-1020). Identification of metabolites and evaluation of the relative amounts were performed using MASTER HANDS (version 2.1.0.1 and 2.9.0.9; Keio University, Tokyo, Japan) with the HMT metabolite database. The relative amount of each metabolite was calculated with reference to the internal standard material (HMT).

Polysaccharide quantification

Porphyromonas gingivalis cells (5.0×10^9 CFU) were cultured anaerobically in PBS containing 0, 0.8, 5, 10 or 20% erythritol using glass bottom culture plates at 37°C for 16 h. After washing with sterilized Milli-Q water, bacterial cells were resuspended in Milli-Q water. The relative amounts of polysaccharides were estimated using the anthrone method (Hedge & Hofreiter, 1962). Briefly, 100 μl of 5% phenol solution and 500 μl of 95% sulfuric acid were added to 100 μl of each bacterial suspension, then color development was read in a spectrometric manner at 490 nm.

Metabolome analysis using Transwell system

A Transwell unit (Corning Inc., Corning, NY) consisting of upper and lower compartments with a 0.4-μm microporous membrane was used for co-cultures. The *P. gingivalis* cells (1.0×10^{10} CFU well^{-1}) were placed in the lower compartment and incubated with *S. gordonii* cells (1.0×10^{10} CFU well^{-1}) in the upper compartment in PBS in the presence or absence of 10% erythritol or 10% sorbitol at 37°C for 6 h under anaerobic conditions, as well as with those conditions in reverse. Metabolomics for intracellular metabolites extracted from each bacteria was performed using CE-TOFMS.

Statistical analysis

Except for metabolomic analysis, all data were evaluated using Tukey–Kramer's test with EXCEL STATISTICS 2006 software (SSRI, Tokyo, Japan). Multiple comparisons of metabolomic data were performed with Welch's *t*-test. Principal component analyses (PCA) of metabolomic data were carried out using SAMPLE STAT (version 3.14; HMT).

RESULTS

Development and microstructure of *S. gordonii*–*P. gingivalis* biofilms under polyol stresses

First, we examined the effects of erythritol using a heterotypic biofilm model with dot blotting. In clinical trials and *in situ* studies of sugar alcohols, their effects have generally been compared based on the given amount of daily consumption weight (6–10 g day^{-1}) or prescribed dosage, which is calculated according to the percentage by weight (w) in oral care products [e.g. 28–40% (w/w) in mouth rinses, 65–70% (w/w) in chewing gums] (van Loveren, 2004; Burt, 2006; Goncalves *et al.*, 2006). Therefore, we employed percentage by weight as the unit for evaluation in the present study.

Porphyromonas gingivalis biofilm formation on the *S. gordonii* substratum was prevented by addition of erythritol in a dose-dependent manner, with a plateau reached at concentrations > 10% (Fig. 1A). Sorbitol also showed a maximum inhibitory effect of 10% (data not shown), so we used 10% concentrations in the test solutions subjected to confocal microscopy analysis. Microscopic observations revealed that each polyol altered the morphology of *S. gordonii* and *P. gingivalis* microcolonies (Fig. 1B). Treatment with 10% erythritol resulted in sparsely formed microcolonies with markedly reduced numbers of *P. gingivalis* compared with

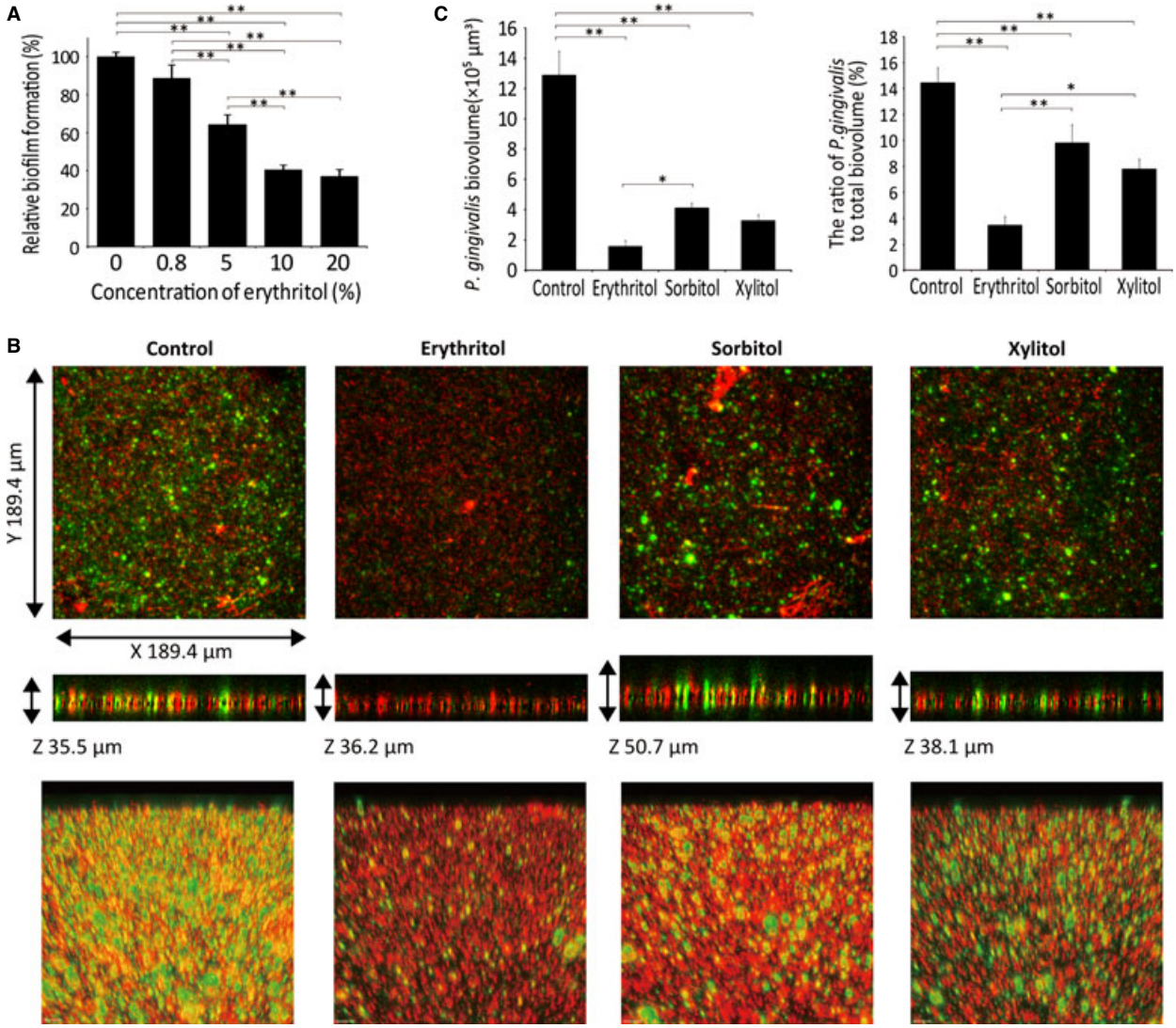

Figure 1 Effects of sugar alcohols on *Streptococcus gordonii–Porphyromonas gingivalis* biofilm formation. (A) Determination of relative amounts of *P. gingivalis* in *S. gordonii–P. gingivalis* mixed biofilms developed with various concentrations of erythritol using dot blotting assays. (B) Confocal laser scanning microscopy (CLSM) images of heterotypic biofilms consisting of *S. gordonii* (red) and *P. gingivalis* (green). The upper panels indicate *x-y* sections, the middle panels show representative *x-z* sections, and the lower panels are three-dimensional images constructed with the 'Iso Surface' function of IMARIS 5.0.1 software (Bitplane AG, Zurich, Switzerland). (C) Biovolume analysis of *P. gingivalis* in two mixed species mixed biofilms. Seven fields per sample were randomly recorded with CLSM and *P. gingivalis* biovolume was quantified using IMARIS software. $*P < 0.05$, $**P < 0.01$.

the control. Use of sorbitol also resulted in lower density biofilms than those of the control, though they had a thicker layer and taller microcolonies compared with the others. Xylitol treatment yielded a sparse distribution of microcolonies similar to erythritol, though the size and number of *P. gingivalis* microcolonies were larger than those formed with erythritol. On the other hand, the biovolume of *P. gingivalis* in heterotypic biofilms was significantly decreased in the presence of all of the tested polyol agents (Fig. 1C). Among these polyols, erythritol caused the most significant reduction in *P. gingivalis* biovolume. Moreover, treatment with erythritol resulted in a significant decrease in the ratio of *P. gingivalis* to *S. gordonii–P. gingivalis* total biovolume, compared with the others. Together, these results suggest that erythritol inhibits biofilm formation by *P. gingivalis* more effectively than sorbitol and xylitol.

Development and microstructure of *S. gordonii* biofilms under polyol stresses

To assess the extent to which the tested polyols contribute to *S. gordonii* monospecies biofilm formation, observation by confocal laser scanning microscopy was performed. Treatment with 10% erythritol resulted in sparsely formed microcolonies of *S. gordonii* with lower height compared with the control (Fig. 2A). In contrast, use of sorbitol resulted in taller biofilms compared with treatment with the others, which showed a rippling top layer, while xylitol-treated *S. gordonii* developed carpet-like dense and short biofilms without prominent microcolonies. Among the tested polyols, only erythritol caused a significant reduction in *S. gordonii* biovolume in comparison with the control (Fig. 2B), though the suppression rate was not as great as when added to cultures of *P. gingivalis* (Figs 1C and 2B).

Effects of erythritol on growth and gingipain activities of *P. gingivalis*

Porphyromonas gingivalis growth was not supported in the nutrition-limited chemically defined medium

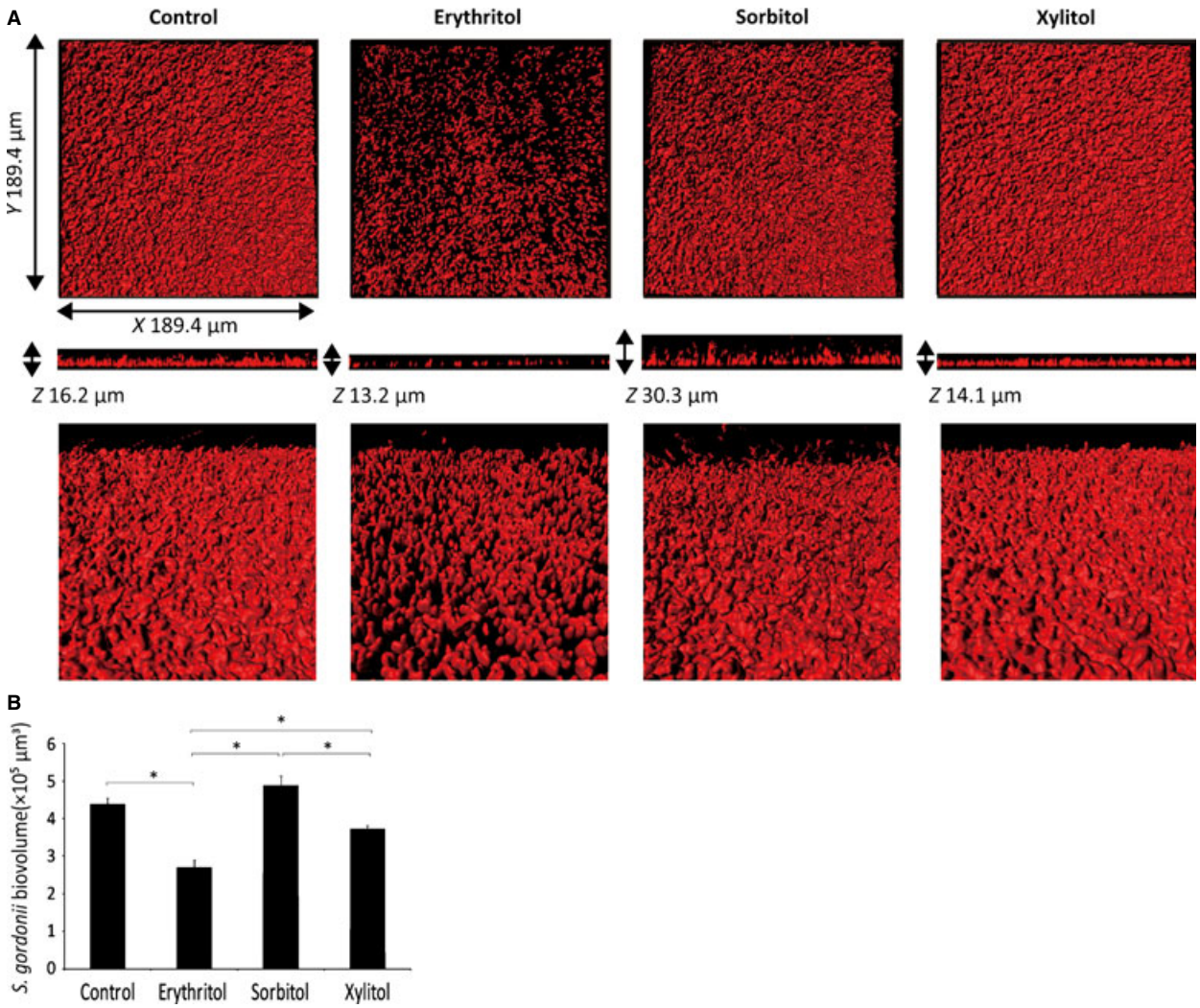

Figure 2 Effects of sugar alcohols on *Streptococcus gordonii* biofilm formation. (A) Confocal laser scanning microscopy (CLSM) images of homotypic biofilm formation by *S. gordonii* with various polyols. Upper panels show representative *x-y* sections and middle panels show representative *x-z* sections, while lower panels present three-dimensional images constructed with the 'Iso Surface' function of IMARIS 5.0.1 software (Bitplane AG). (B) Biovolume analysis of *S. gordonii* in homotypic biofilms. Seven fields per sample were randomly recorded with CLSM, then the biovolume was quantified using IMARIS software (Bitplane AG). $*P < 0.01$.

mCDM-G without a protein source (Fig. 3A). Similarly, *P. gingivalis* did not grow in the presence of 0.5% erythritol, indicating that erythritol is not a bacterial energy source. In contrast, the bacteria clearly grew in mCDM-G containing 3% BSA, whereas 10% erythritol suppressed their growth. The growth of *P. gingivalis* in mCDM-G containing protein has been shown to depend entirely on the availability of peptides generated by gingipains, which comprise Rgp and Kgp, and known to be the most powerful endopeptidases, because they acquire nutrition by degrading surrounding proteins (Grenier *et al.*, 2003). As we found that erythritol markedly suppressed the expression of Rgp protease protein in 0.8% erythritol-treated *P. gingivalis* cells in a quantitative proteomics study (data not shown), we further investigated the effects of erythritol on gingipain activities. Erythritol significantly inhibited the activity of cell-associated Rgp (Fig. 3B), though the level of inhibition was unlikely to fully account for the inhibition of growth. Therefore, we also performed metabolomic analysis to examine the inhibitory mechanisms of erythritol.

Effects of erythritol on growth of *S. gordonii*

Streptococcus gordonii growth was not supported when the cells were cultured in mCDM-M modified by using 0.1% erythritol instead of 0.1% sucrose (Fig. 3C), indicating that erythritol is not used as an energy source. In contrast, the bacteria quickly grew in mCDM-M until reaching a plateau at 4 h, whereas 10% erythritol retarded their growth, which reached a plateau after 3 h (Fig. 3C). Once the growth had plateaued, the OD_{600} values of *S. gordonii* cells cultured in mCDM-M with or without 10% erythritol showed a decreasing tendency along with the start of biofilm formation.

Effects of erythritol on metabolomic profiles of *P. gingivalis*

Hierarchical clustering analysis visualized by production of a heat map cluster illustrated that the metabolomic profile of *P. gingivalis* was altered in the presence of erythritol (see Table S1). In particular, crucial changes were induced with regard to nucleotide biosynthesis, amino sugar and nucleotide sugar biosynthesis, and the glycolytic pathways. Of the 22 metabolites detected in the purine metabolic pathway, 15 were decreased by erythritol (Fig. 4A). In particular, eight of those [adenylosuccinic acid, xanthine, guanosine, adenosine, adenine, deoxyadenosine 5′-triphosphate, adenosine 5′-monophosphate, inosine] were significantly reduced with 10% erythritol, whereas inosine 5′-monophosphate was not detected. In addition, 13 of 16 metabolites in the pyrimidine metabolic pathway were decreased in the presence of erythritol, of which 10 [uridine 5′-triphosphate, uridine 5′-diphosphate, uridine 5′-monophosphate, deoxythymidine 5′-triphosphate, deoxythymidine 5′-diphosphate, deoxythymidine 5′-monophosphate, cytidine 5′-monophosphate, uracil, uridine, thymidine] were significantly reduced in a dose-dependent manner (Fig. 4B). Furthermore, all identified nucleotide sugars and amino sugars were significantly decreased in the presence of erythritol (Fig. 4C). Erythritol at 10% also significantly reduced all metabolites in the pentose phosphate pathway, including ribulose 5-phosphate, ribose 5-phosphate and sedoheptulose 7-phosphate (Fig. 4D). Some metabolites in the preparatory phase of glycolysis, from glucose 6-phosphate (G6P) to glyceraldehyde 3-phosphate (G3P), were also decreased in the presence of 10% erythritol, whereas all metabolites detected in this phase tended to be increased with 0.8% erythritol (Fig. 4D, see Table S2).

Effects of erythritol on metabolomic profiles of *S. gordonii*

Heat map cluster findings showed that a large number of metabolites of *S. gordonii* changed their relative amounts following exposure to erythritol in a dose-dependent manner, though the clustering pattern and content construction of each cluster were different from those of *P. gingivalis* (see Table S3). As for the nucleotide biosynthesis pathways, the metabolomic profile of *S. gordonii* in the presence of erythritol was similar to that observed in *P. gingivalis.* Several metabolites in the nucleotide metabolic pathways, especially the pyrimidine metabolic pathway, showed a tendency to decrease in the presence of erythritol (Fig. 5A,B). Six metabolites in the purine metabolic pathway [deoxyadenosine 5′-diphosphate, deoxyadenosine 5′-triphosphate, guanosine 5′-diphosphate, guanosine 5′-triphosphate, adenosine 5′-diphosphate, adenosine 5′-triphosphate] and nine in the pyrimidine biosynthesis pathway [deoxycytidine 5′-diphosphate,

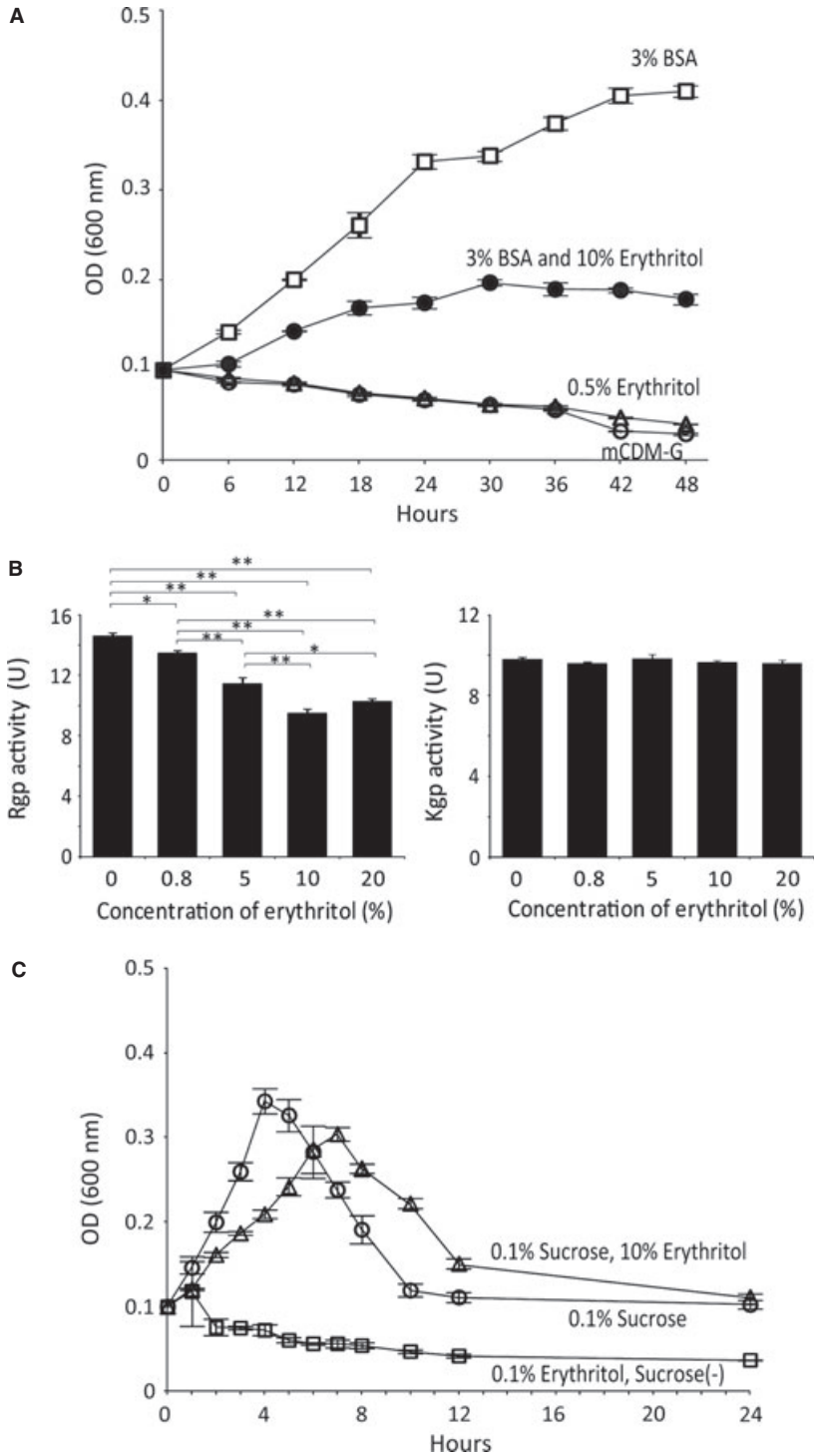

Figure 3 Effects of erythritol on bacterial growth and gingipain activity. (A) Effects of erythritol on growth of *Porphyromonas gingivalis* in mCDM with various reagents. (B) Effects of erythritol on bacterial cell-associated Rgp and Kgp activities of *P. gingivalis*. U = enzyme activity to degrade 20 nM substrate in 1 min. $*P < 0.05$, $**P < 0.01$. (C) Effects of erythritol on *Streptococcus gordonii* growth in mCDM with various reagents.

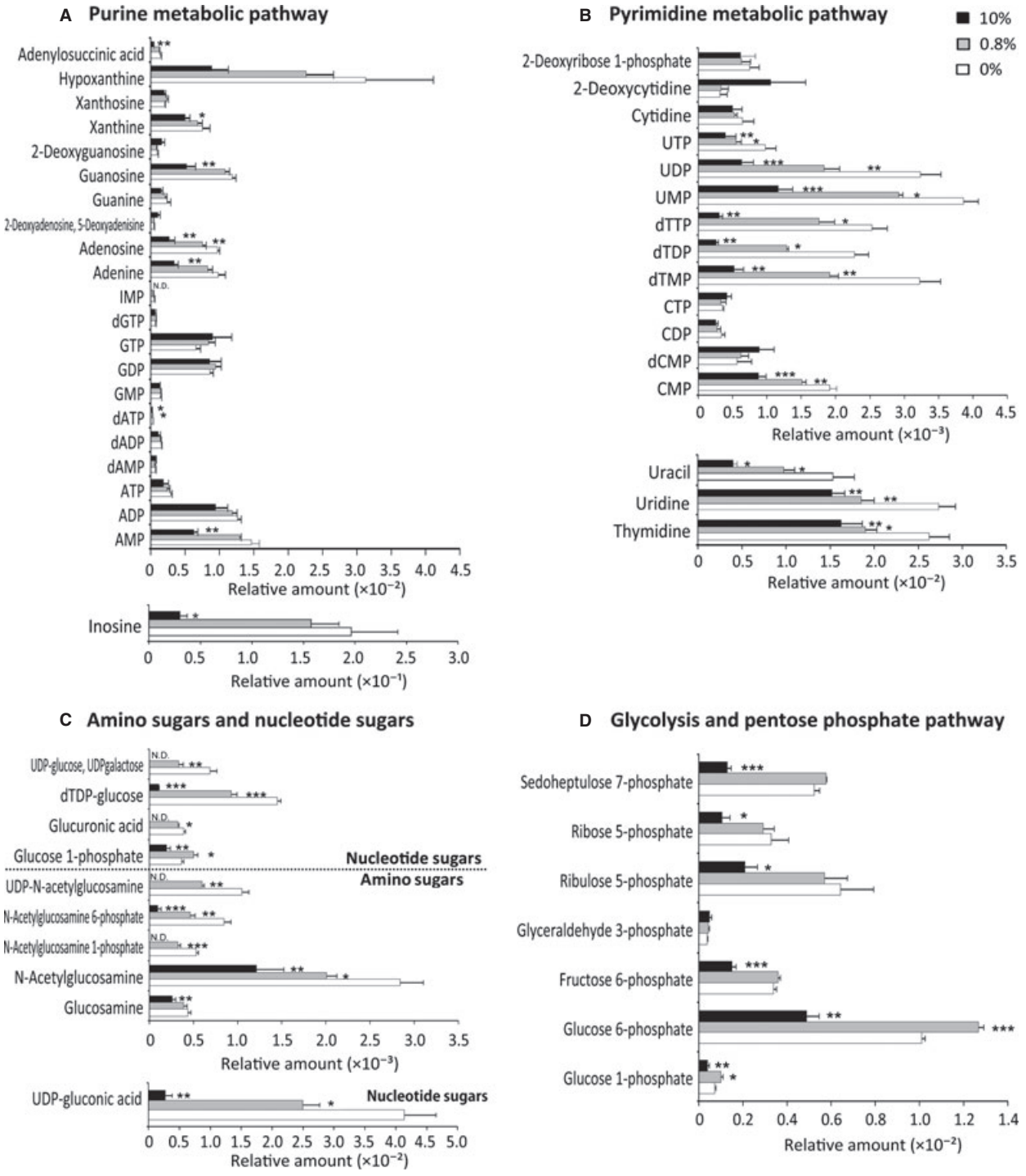

Figure 4 Effects of erythritol on *Porphyromonas gingivalis* metabolomic profile. (A) Purine metabolic pathway. (B) Pyrimidine metabolic pathway. (C) Amino and nucleotide sugars. (D) Glycolysis and pentose phosphate pathway. White, gray, and black bars represent 0, 0.8, and 10% erythritol, respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

deoxycytidine 5′-triphosphate, deoxythymidine 5′-diphosphate, deoxythymidine 5′-triphosphate, 2-deoxyribose 1-phosphate, cytidine 5′-diphosphate, cytidine 5′-triphosphate, uridine 5′-diphosphate, uridine 5′-triphosphate] were significantly reduced by 10% erythritol (Fig. 5A,B, see Table S4). Furthermore, nucleotide

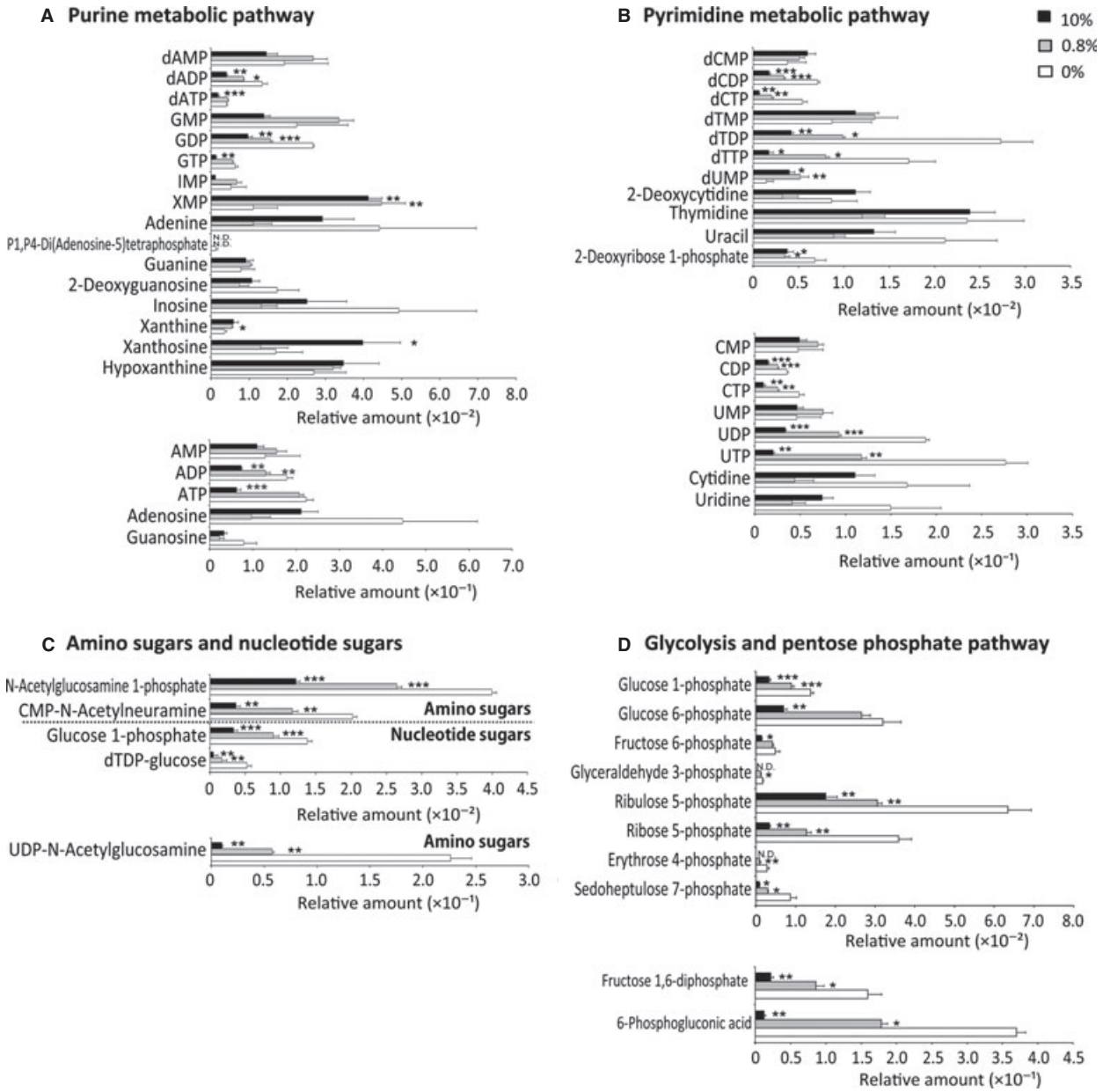

Figure 5 Effects of erythritol on *Streptococcus gordonii* metabolomic profile. (A) Purine metabolic pathway. (B) Pyrimidine metabolic pathway. (C) Amino and nucleotide sugars. (D) Glycolysis and pentose phosphate pathway. Open, gray, and solid bars represent 0, 0.8, and 10% erythritol, respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

and amino sugars showed significant dose-dependent decreases in the presence of erythritol (Fig. 5C). Meanwhile, most of the metabolites in both the preparatory phase of glycolysis and the pentose phosphate pathway were decreased in an erythritol dose-dependent manner. In contrast to *P. gingivalis*, all metabolites (G6P to G3P) in the preparatory phase of glycolysis were significantly reduced, with nearly all decreasing in an erythritol dose-dependent manner (Fig. 5D, Table S4).

Shifts of metabolites in central carbon metabolic pathways

Metabolome analysis of *P. gingivalis* revealed that most metabolites in the pentose phosphate pathway,

as well as the amino and nucleotide sugar metabolic pathways, were decreased in the presence of erythritol (Fig. 6). The glycolytic intermediates from glycerate 3-phospate to acetyl Coenzyme A showed a tendency to decrease in an erythritol dose-dependent manner, while the levels of several amino acids (Gly, Glu, Ala, Ser, Asp) were increased or maintained. Meanwhile, the overall metabolites of *S. gordonii* related to amino sugar and nucleotide sugar metabolism, as well as the pentose phosphate pathway, showed reduced abundance in the presence of erythritol (Fig. 7). In the glycolytic pathway, metabolites from glucose 1-phosphate (G1P) to G3P tended to decrease with lower concentrations of erythritol and were clearly reduced at a concentration of 10%. In addition, some amino acids (Gly, Glu, Ser, Asp) showed reduced abundance, which was different from the results of *P. gingivalis*.

Polysaccharide production

In *P. gingivalis* biofilm, the relative amount of polysaccharide was decreased in a dose-dependent manner with erythritol and reached a plateau at concentrations > 10% (Fig. 8A). Similarly, the amount of polysaccharide of *S. gordonii* was significantly reduced in the presence of erythritol (Fig. 8B).

Comparative analysis of metabolomes altered by erythritol and sorbitol

Principal component analyses was employed to examine the effects of erythritol in comparison to sorbitol on the *P. gingivalis* metabolomic profile. Our results clearly showed distinct effects of erythritol compared with sorbitol. Although polyol-treated cells were characterized by a plus score with the PC1 axis, in contrast to the minus score of the control, the distinct feature of erythritol was association of plus score with the PC2 axis (Fig. 9A). In *P. gingivalis* cells treated with 10% erythritol, several branched chain fatty acids in the biosynthesis/degradation pathway of Val, Leu, and Ile, such as 2-oxoisovaleric acid, 4- or 3-methyl-2-oxovaleric acid, and 2-oxobutyric acid, were significantly increased, whereas the levels of all detected dipeptides including Glu-Glu, Ser-Glu, Tyr-Glu, His-Glu, Ala-Ala, Thr-Asp, Gly-Asp, Gly-Leu, and Gly-Gly were significantly decreased (see Table S5). *P. gingivalis* cells treated with 10% sorbitol, which are plotted in the PC1 plus and PC2 minus areas (Fig. 9A), showed significantly higher concentrations of Arg, Gln, and lactic acid, as well as several fatty acids and dipeptides including Ala-Ala, Gly-Leu, β-Ala-Lys, Thr-Asp, His-Glu, and Tyr-Glu as compared with the control and 10% erythritol-treated cells (Table S5). A number of metabolites in the gluconeogenesis pathway, as well as nucleotide and amino sugars were reduced by both types of sugar alcohols in *P. gingivalis* (Table S5). On the other hand, PCA of the metabolome sets of *S. gordonii* illustrated that 10% sorbitol treatment caused the opposite alteration of metabolomic profiles compared with the control and 10% erythritol treatment in this organism, which was characterized as a plus score on the PC1 axis (Fig. 9B). In detail, sorbitol 6-phosphate was detected only in 10% sorbitol-treated *S. gordonii* cells and the amount of trehalose 6-phosphate was drastically increased under the same conditions (see Table S6). In addition, Ala-Ala and Gln were significantly increased in the 10% sorbitol group, as also observed in *P. gingivalis*. Consistent with the metabolomic profiles of 10% erythritol-treated *S. gordonii* cells in mCDM-M (Table S4), Ser, Gly, and Asp were significantly decreased by 10% erythritol in *S. gordonii* co-cultured with *P. gingivalis* in PBS (Table S6).

DISCUSSION

In the present study, erythritol showed significant inhibitory effects on *P. gingivalis*–*S. gordonii* heterotypic biofilm development and the bacterial growth of *P. gingivalis,* as well as moderate inhibitory effects on monotypic biofilm development by and growth speed of *S. gordonii* (Figs 1, 2, 3A,C). Erythritol transmuted the microstructures of biofilms formed by both species and decreased their biovolumes to a greater degree than the other sugar alcohols (Figs 1 and 2), whereas *P. gingivalis* cell surface-associated Rgp activity was also suppressed (Fig. 3B). These results suggest that erythritol has multiple suppressive effects on the *S. gordonii*–*P. gingivalis* heterotypic community.

We also performed metabolomic evaluations to elucidate the molecular basis of the effects of erythritol on *P. gingivalis*. In the presence of erythritol, a large number of metabolites showed changed concentrations, especially those in the purine and pyrimidine biosynthesis pathways, which were significantly

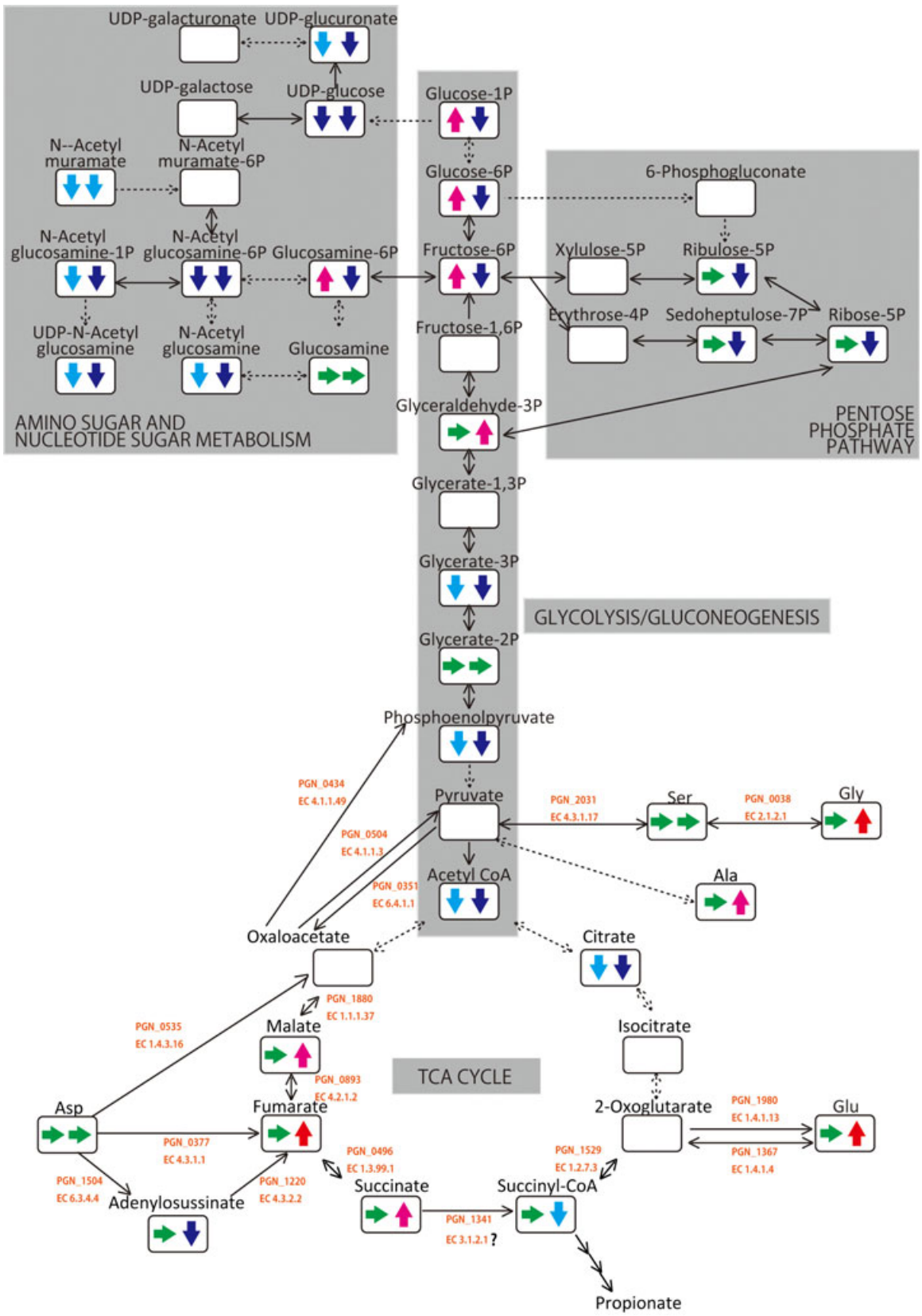

Figure 6 Visualized map of central carbon metabolism pathway in *Porphyromonas gingivalis* relative to data presented in Fig. 4. The left and right arrows for each metabolite represent 0.8 and 10% erythritol, respectively. Red upward arrows indicate remarkably increased abundance [comparative ratio of relative amounts of each erythritol group to controls (CR) $\geq$ 2.0]. Magenta upward arrows represent increased abundance (1.3 $\leq$ CR $<$ 2.0). Green rightward arrows show no change (0.7 $<$ CR $<$ 1.3). Cyan downward arrows indicate decreased abundance (0.5 $<$ CR $\leq$ 0.7). Blue downward arrows show remarkably decreased abundance (CR $\leq$ 0.5). Continuous lines with arrows indicate protein catalyzing each step in the pathway as annotated in the Kyoto Encyclopedia of Genes and Genomes (KEGG). Dashed lines with arrows show proteins not annotated by KEGG. Proteins catalyzing each step between amino acids (Gly, Ala, Asp, Glu) and phosphoenolpyruvate are shown by their *P. gingivalis* PGN designation and EC (enzyme commission) number.

decreased in a dose-dependent manner (Fig. 4A,B). Furthermore, erythritol significantly reduced the precursors for nucleotide synthesis in the pentose phosphate pathway, including ribulose 5-phosphate, ribose 5-phosphate and sedoheptulose 7-phosphate (Fig. 4D). In addition to these results, a previously performed proteomics analysis showed that thymidylate synthase (PGN_2062), which mediates the synthesis of deoxythymidine 5′-monophosphate, and transaldolase (PGN_0333), which synthesizes sedoheptulose 7-phosphate, were decreased in the presence of 0.8% erythritol (data not shown). A number of recent studies have reported that extracellular DNA, as a constituent of the polymeric matrix, is required for forming biofilm (Whitchurch *et al.*, 2002; Vilain *et al.*, 2009; Flemming & Wingender, 2010). However, we did not observe a significant decrease in eDNA following erythritol treatment (data not shown). Together, these results suggest that erythritol decreases DNA/RNA synthesis, resulting in retarded growth of *P. gingivalis*.

In addition to nucleic acids, various amino and nucleotide sugars were also decreased in the presence of erythritol (Fig. 4C). As the former are essential constituents of bacterial peptidoglycans, our findings suggest that erythritol inhibits bacterial replication. On the other hand, nucleotide sugars are known to be components of extracellular polymeric substrates, so reduction of nucleotide sugar production by erythritol probably retards extracellular matrix biosynthesis, resulting in a decrease in *P. gingivalis* biovolume. This supports the present finding of decreased amounts of polysaccharides in *P. gingivalis* in the presence of erythritol (Fig. 8).

Streptococcus gordonii metabolomic profiles in the presence of erythritol showed that it suppresses nucleotide biosynthesis, especially in the pyrimidine synthesis pathway, similarly to that seen with *P. gingivalis* (Fig. 5A,B). Moreover, extracellular polysaccharides, and the internal levels of amino and nucleotide sugars were also markedly decreased (Figs 5C and 8). Together, these results suggest that the inhibitory effect of erythritol on mixed species biofilm development is attributable to repression of both *P. gingivalis* and *S. gordonii* growth by inhibition of nucleic acid synthesis and decreased extracellular matrix production.

It has been speculated that *P. gingivalis* cannot use sugars because of a mutation in the hexokinase-encoding gene (PGN_0380) (Naito *et al.*, 2008). However, various metabolites in the glycolysis pathway were detected by our metabolomics approach, indicating that their production occurs via gluconeogenesis during the process of amino acid catabolism (Mazumdar *et al.*, 2009). The overall profile of central carbon metabolism including glycolysis/gluconeogenesis was clearly different between asaccharolytic *P. gingivalis* and saccharolytic *S. gordonii* following erythritol treatment, suggesting different effector pathways in these species (Figs 6 and 7).

In *P. gingivalis* cells co-cultured with *S. gordonii*, all detected dipeptides were significantly decreased by 10% erythritol treatment (Table S5), whereas they were increased by 10% sorbitol. Furthermore, the same tendency was observed with *P. gingivalis* cells treated with 0.8% erythritol (Table S2). On the other hand, there was no clear tendency with regard to the dipeptides in *S. gordonii* (Tables S4 and S6). It was previously reported that the primary carbon and nitrogen sources of *P. gingivalis* are dipeptides rather than free amino acids (Takahashi & Sato, 2002), so the significant inhibitory effect of erythritol on the growth of asaccharolytic *P. gingivalis* is presumed to be caused by a shortage of dipeptides. To acquire dipeptides from protein sources, *P. gingivalis* uses an elaborate proteolytic system composed of endopeptidases (Rgp, Kgp, periodontain, PrtT protease, Tpr protease) and oligopeptidase in a cascade-like manner (Guo *et al.*, 2010). Our results confirmed that erythritol has an ability to suppress the most aggressive endopeptidase Rgp, so this effect may cause a dipeptide shortage.

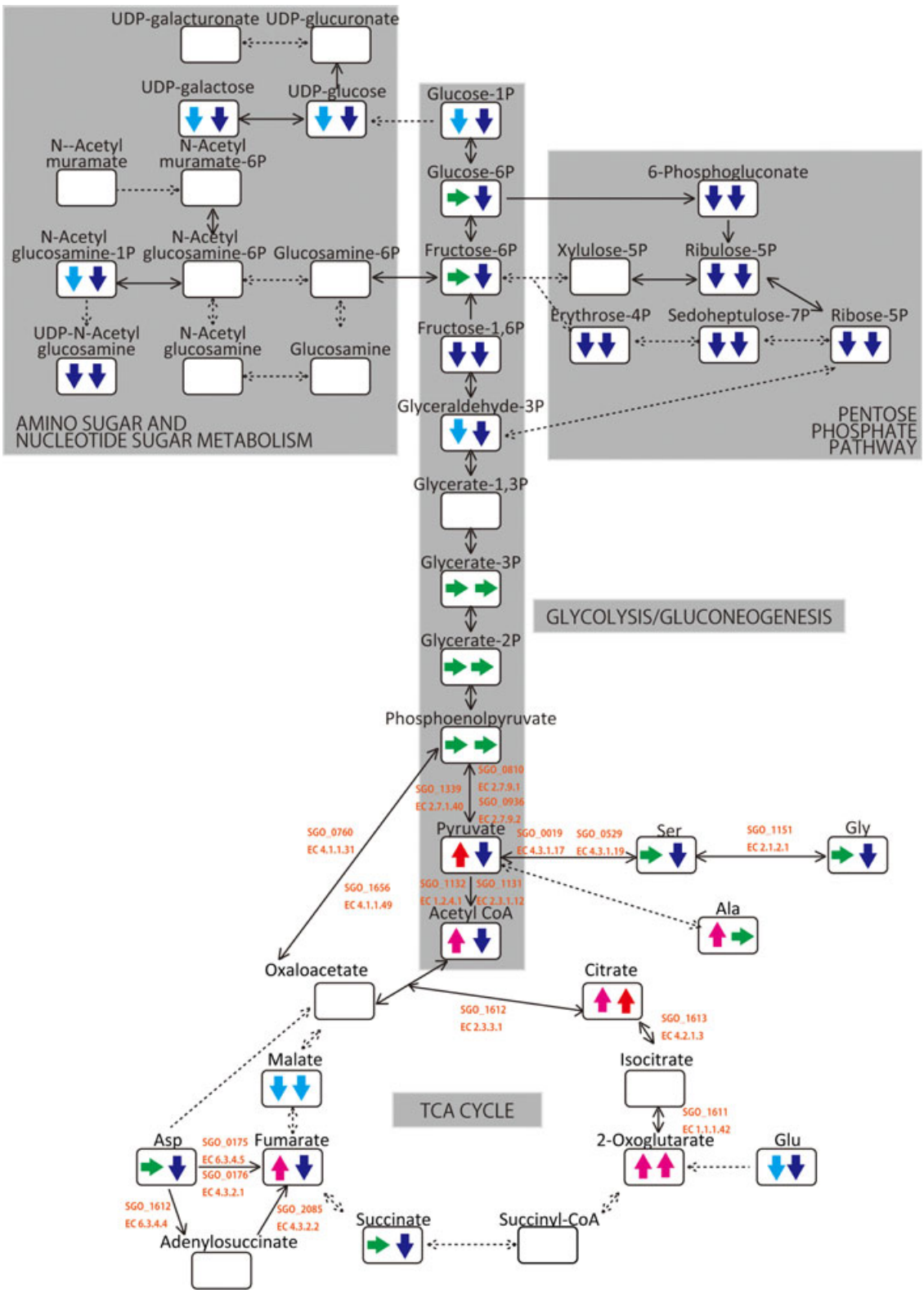

Figure 7 Visualized map of central carbon metabolism pathway in *Streptococcus gordonii* relative to data presented in Fig. 5. The arrows used follow the same conventions as in Fig. 6. Proteins catalyzing each step between amino acids (Gly, Ala, Asp, Glu) and phosphoenolpyruvate are shown by their *S. gordonii* SGO designation and EC (enzyme commission) number.

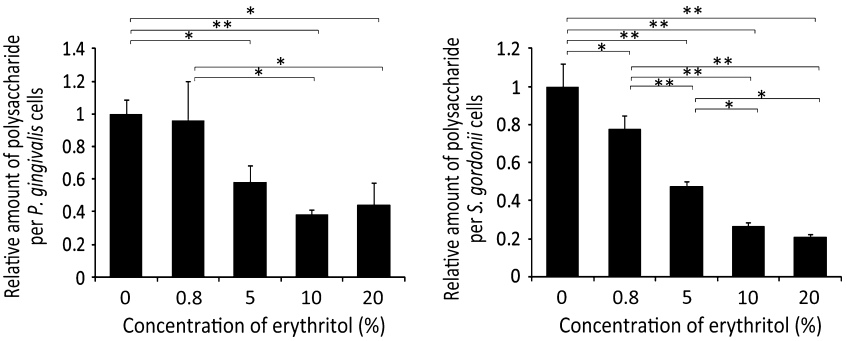

Figure 8 Effects of erythritol on polysaccharide production of each bacterium. $*P < 0.05$, $**P < 0.01$.

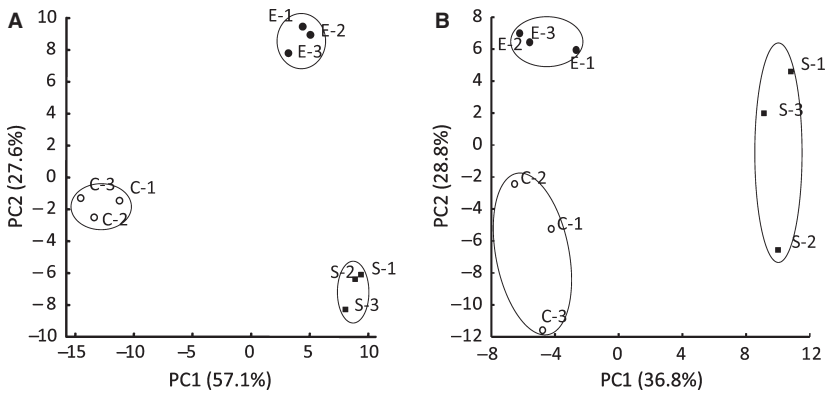

Figure 9 Score plots of principal component analyses (PCA). To examine specific effects of erythritol on *Porphyromonas gingivalis* and *Streptococcus gordonii* metabolomic profiles by comparing sorbitol, PCA was employed. (A) *P. gingivalis* metabolomic data. (B) *S. gordonii* metabolomic data. Open dots, solid dots, and solid squares show control (no sugar alcohols), 10% erythritol, and 10% sorbitol, respectively.

In a previous study, *Escherichia coli* growth and glucose use were stopped when the bacterial cells were exposed to 0.8 M sucrose, whereas upshocked cells remained viable and metabolically active (Roth *et al.*, 1985). Furthermore, osmotically stressed *Lactobacillus rhamnosus* cells had a slight loss of growth ability in cultures with a concentration of sucrose > 1.2 M, whereas intact membranes were evident as well as esterase activity as signs of viability (Sunny-Roberts & Knorr, 2008). In the present study, we used 10% (0.82 M) erythritol and 10% (0.55 M) sorbitol, so it was necessary to take the effect of osmotic pressure into consideration. To test whether erythritol has a specific inhibitory effect, we performed further metabolomic analyses of *P. gingivalis* and *S. gordonii* in the presence of 10% erythritol and 10% sorbitol. With both tested microbes, PCA (PC2 axis in Fig. 9A, PC1 axis in Fig. 9B) indicated that a different physiological response was induced by each sugar alcohol, even though there were some common characteristics due to osmotic effects (PC1 axis in Fig. 9A, PC2 axis in Fig. 9B).

Most bacteria extend their osmotolerance by accumulating organic osmolytes that restore cellular hydration more effectively and act as protein stabilizers. As with eukaryotes, these compounds include trehalose, proline, glycine, betaine, and ectoine (Wood, 2007). In the present study, *S. gordonii* showed massively pooled trehalose 6-phosphate after 10% sorbitol treatment (comparative ratio to control 7.9, $P = 0.008$), whereas 10% erythritol did not trigger that phenomenon (comparative ratio to control 0.7, $P = 0.022$). Hence, 10% erythritol might cause a milder osmotic stimulus than 10% sorbitol.

To suppress *Staphylococcus aureus* biofilm formation, a cream containing 5% xylitol is used for skin treatment (Katsuyama *et al.*, 2005). In addition to already developed oral care products, erythritol could be applied as a mixture with an appropriate cream or paste base into the subgingival region to maintain an

effective concentration. In dentistry, this sugar alcohol is employed as a sweetener for preventing caries, but evidence of its effects for preventing periodontal disease remains lacking. Nevertheless, the present findings show that erythritol can be used to prevent periodontal diseases.

ACKNOWLEDGEMENTS

This work was supported by grants-in-aid for Research Activity Start-up (23890112) and Scientific Research (C; 24593150) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the 'Challenge to Intractable Oral Diseases' Project of Osaka University Graduate School of Dentistry. The authors report no conflicts of interest related to this study

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Hierarchical clustering analysis of erythritol-treated *Porphyromonas gingivalis* metabolomes.

Table S2. Comparative analysis of erythritol-treated *Porphyromonas gingivalis* metabolomes.

Table S3. Hierarchical clustering analysis of erythritol-treated *Streptococcus gordonii* metabolomes.

Table S4. Comparative analysis of erythritol-treated *Streptococcus gordonii* metabolomes.

Table S5. Comparative analysis to compare between erythritol- and sorbitol-treated *Porphyromonas gingivalis* metabolomes.

Table S6. Comparative analysis to compare between erythritol- and sorbitol-treated *Streptococcus gordonii* metabolomes.