Erythritol alters gene transcriptome signatures, cell growth, and biofilm formation in Staphylococcus pseudintermedius

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Abstract

· **Background:** Erythritol has recently been shown to have an inhibitory effect on the growth of *Staphylococcus pseudintermedius* isolated from dogs with superficial pyoderma. The present study aimed to define the changes in gene transcription signatures induced by erythritol in *S. pseudintermedius*. Changes in the gene transcription profiles of *S. pseudintermedius* by the presence of erythritol were analyzed by RNA sequencing and quantitative PCR. Gene ontology analysis was performed to assign functional descriptions to the genes.

· **Results:** We revealed that erythritol induced up-regulation of three genes (*ptsG*, *ppdK,* and *ppdkR*) that are related to the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS). Glucose supplementation restored the up-regulation of the PTS-related genes in response to erythritol. In addition, erythritol down-regulated eleven genes that are located in a single *pur*-operon and inhibited biofilm formation.

· **Conclusions:** These findings indicated that erythritol antagonistically inhibits PTS-mediated glucose uptake, thereby exerting a growth inhibitory effect on *S. pseudintermedius*. Moreover, erythritol inhibits the 'de novo' IMP biosynthetic pathway that may contribute to biofilm synthesis in *S. pseudintermedius*.

Background

Staphylococcus pseudintermedius is a coagulase-positive Gram-positive coccus that colonizes 90% of healthy dogs. It is an opportunistic pathogen and the most common cause of pyoderma and otitis externa in dogs [1]. Notably, the emergence of methicillin-resistant *S. pseudintermedius* (MRSP) has become a worldwide problem. Moreover, it has been reported that *S. pseudintermedius* is the cause of serious bacterial infections in immunosuppressed humans [2].

Erythritol is a sugar alcohol with four carbon atoms and is approximately 75% as sweet as sucrose; and the EU Scientific Committee on Food concluded in 2003 that it was safe to use in foods [3]. Erythritol was found to be more effective than xylitol, a sugar alcohol with one more carbon than erythritol, in inhibiting the growth of microorganisms, such as the caries-causing bacterium *Streptococcus mutans* [4], the indigenous oral bacterium *Streptococcus gordonii* [5], the periodontal disease bacterium *Porphyromonas gingivalis* [5], the causative bacteria of axillary and foot odor *Corynebacterium minutissimum*, *Corynebacterium striatum*, and *Staphylococcus epidermidis* [6], as well as the acne-causing bacterium *Cutibacterium acnes* [7]. We recently revealed that erythritol has an inhibitory effect on the growth of *Staphylococcus aureus* and *S. pseudintermedius*, including methicillin-resistant *S. aureus* and MRSP [8].

The mechanisms of the biological effects of erythritol and xylitol have been studied in streptococci. A previous study proposed the following mechanisms for the growth inhibitory effects of xylitol against *S. mutans*: (*1*) direct inhibition of glycolytic enzymes by the intracellular accumulation of xylitol 5-phosphate derived from xylitol through *the* phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS), and (*2*) indirect inhibition by competition between glucose and xylitol for phosphate donors used in PTS [9]. Another metabolomics study indicated that erythritol may inhibit the growth of *S. gordonii* and *P. gingivalis* by affecting various metabolic pathways, including nucleic acid synthesis and glycolytic pathways [5].

From these reports, it was speculated that the mechanism of growth inhibition by erythritol against staphylococci may also involve inhibition of the glucose metabolism pathway; however, no reports have clarified this possibility. This study was conducted to investigate the growth inhibition mechanism of erythritol against staphylococci using transcriptome analysis.

Results

Comprehensive gene expression analysis of erythritol-treated *S. pseudintermedius*

We recently revealed that erythritol at concentrations above 5% (w/w) inhibited the growth of a standard strain of *S. pseudintermedius,* JCM 17571 (SP) [8]. In this study, comprehensive gene expression analysis using RNA sequencing (RNA-seq) was performed for SP cultured in the presence or absence of erythritol (n = 3). To avoid complete suppression of cellular metabolism, a weak inhibiting concentration (5% [w/w]) was selected. After filtering the raw reads, we found that there were 19,142,672, 20,940,942, and 22,024,908 clean reads of the transcriptome in the control samples, whereas 19,068,865, 19,060,494, and 19,393,064 clean

reads were obtained from the erythritol-treated samples. A complete list of all reads is shown in Additional file 1. Among these genes, a total of 625 differentially expressed genes were identified using the calculated gene expression levels ($|\text{Log}_2$ FC (fold change)$| \geq 1$, p-value ≤ 0.05), with 244 genes markedly up-regulated and 381 genes markedly down-regulated in SP following erythritol treatment. A PCA plot showed a clear split between the control and erythritol-treated samples, with 95% of the variance explained by PC1 (Fig. 1a). A volcano plot showed that the differential expressions of the top up-regulated and down-regulated genes were statistically significant (Fig. 1b).

The top 15 up-regulated and down-regulated genes in response to erythritol

The predicted functional descriptions and GO biological process classes for the top 15 up-regulated and top 15 down-regulated genes in response to erythritol are shown in Table 2. Among the top 15 up-regulated genes with a predicted GO biological process class, genes related to two biological functional groups were present. Three genes (Up-Regulated Genes group 1, *URGs1*), BJK46_009280 (*ptsG*), BJK46_000300 (*ppdK*), and BJK46_000295 (*ppdkR*), were predicted to encode proteins related to PTS. The *ptsG* gene encodes the glucose-specific EIICBA component of PTS (PtsG), and the *ppdK* and *ppdkR* genes encode pyruvate phosphate dikinase (PPDK) and PPDK regulatory protein (PPDKR), respectively, which are involved in the regeneration of PEP that is required to drive PTS. Moreover, the other four genes (*URGs2*), BJK46_008080 (*vraT*), BJK46_008075 (*vraS*), BJK46_008070 (*vraR*), and BJK46_008015 (*sgtB*) were predicted to belong to the same group. The *vraTSR* genes belonging to a single gene cluster are predicted to comprise a three-component regulatory system necessary to promote resistance to cell wall agents, and *sgtB* is predicted to encode a glycosyltransferase associated with peptidoglycan biosynthesis. The GO classes of the biological process could not be predicted in the remaining 8 up-regulated genes.

Of the top 15 down-regulated genes, 11 genes (Down-Regulated Genes, *DRGs*), BJK46_008815 (*purD*), BJK46_008820 (*purH*), BJK46_008825 (*purN*), BJK46_008830 (*purM*), BJK46_008835 (*purF*), BJK46_008840 (*purL*), BJK46_008845 (*purQ*), BJK46_008850 (*purS*), BJK46_008855 (*purC*), BJK46_008860 (*purK*), and BJK46_008865 (*purE*), were located on a single *pur*-operon and assigned GO biological process classes predicted to be associated with 'de novo' IMP biosynthetic process. The GO biological process classes could not be predicted in the remaining 4 down-regulated genes.

The expression of the genes BJK46_008775 (encoding a PEP-protein phosphotransferase; PTS system enzyme I, E1), BJK46_002265 (encoding a histidine-containing phosphocarrier protein (HPr)), and BJK46_011055 (encoding a *pur*-operon repressor (PurR)) were not strongly induced or repressed by erythritol, exhibiting $\log_2$FC values of 0.550, 1.22, and 0.206, respectively, as shown in Additional file 1.

Glucose supplementation restored erythritol-induced up-regulation of PTS- and cell wall-related gene transcription and growth inhibition of SP

A real-time quantitative PCR (qPCR) analysis further confirmed the up-regulation of genes belonging to *URGs1* and *URGs2* in the presence of erythritol (Fig. 2). Because PTS contributes to glucose uptake, we thought that the up-regulation of PTS-related gene transcription was caused by glucose starvation in SP. Therefore, we compared the expression of PTS-related genes in SP between glucose-free and glucose-supplemented (1% [w/w]) conditions. We found that up-regulation of transcription of genes belonging to *URGs1* in response to erythritol was restored under glucose-supplemented conditions, suggesting that transcription of PTS-related genes was up-regulated in response to glucose starvation. We also found that up-regulation of transcription of genes belonging to *URGs2* in response to erythritol was restored under glucose-supplemented conditions. Moreover, supplementation of 0.1% glucose partially restored growth inhibition by erythritol (Fig. 3). Even when 1% glucose was added, the suppression of growth inhibition remained at the same level as when 0.1% glucose was added.

Erythritol inhibited biofilm formation in SP

Down-regulation of genes belonging to *DRGs* was also confirmed by qPCR analysis (Fig. 2). Previous reports suggested that down-regulation of *pur*-operon genes is associated with inhibition of biofilm formation in staphylococci [10]. Therefore, we hypothesized that erythritol inhibits biofilm formation in SP. Erythritol significantly inhibited the *growth of SP (Fig. 4a) and the amount of biofilm formation (Fig. 4b) in a dose-dependent manner. In the condition with 5% erythritol, the biofilm inhibition effect (41.8% of OD_{570} vs. control) is notably greater than the growth inhibition effect (74.9% of OD_{660} vs. control).*

Discussion

We performed a transcriptome analysis of SP with and without erythritol exposure to validate the mechanism of growth inhibition by erythritol. Among the top 15 up-regulated and top 15 down-regulated genes, we focused on the genes whose GO biological process classes were predicted for their gene products. A proposed summary of the cellular responses to the addition of erythritol is shown in Fig. 5.

The gene products of *URGs1* (*ptsG, ppdkR, ppdK*) are suggested to be involved in glucose transport. Microorganisms contain multiple PTS gene clusters with specificity for different sugars such as glucose, fructose, cellobiose, and xylose. The PTS is usually composed of one membrane-spanning protein and some soluble proteins. EI and HPr proteins are the general cytoplasmic PTS components, and in most organisms are involved in the uptake of all PTS carbohydrates. In contrast, the EIIA, EIIB, and EIIC (membrane-spanning) proteins are usually specific to one substrate or a small group of closely related carbohydrates [11]. These EII proteins are often fused to each other, and the *ptsG* gene of SP encodes the fused EIICBA protein. Bacterial PTS transports sugars up a concentration gradient with phosphorylation, and the phosphate donor is the energy-rich PEP. Pyruvate converted from PEP via PTS transportation is converted/reused by PPDK to PEP [12]. PPDK regulatory protein (PPDKR) activates PPDK via a Pi-dependent, PPi-forming phosphorolytic reaction [13]. The significant induction of *URGs1* expression by the addition of erythritol to SP cultures suggests that this may be a cellular response to erythritol-induced glucose starvation. Namely, erythritol may antagonistically inhibit PTS-mediated glucose uptake by binding to the substrate-binding site of PtsG, thereby inhibiting the growth of SP. The cellular response to this may be the induction of PtsG and its drivers, PPDK and PPDKR. This is supported by the fact that no significant induction of *URGs1* was observed under glucose-added conditions (Fig. 2). Since the growth inhibition was suppressed by adding 1/100th of the amount of glucose relative to erythritol, the affinity of erythritol to PtsG may be considerably lower than that of glucose. In the future, it will be necessary to verify whether erythritol binds to PtsG and competes with glucose uptake. In addition, PEP is well supplied by the glycolytic pathway in the presence of glucose, which may also be involved in suppressing the induction of PPDK and PPDKR by glucose.

The gene products of *URGs2* (*vraTSR* and *sgtB*) were suggested to be involved in cell wall drug resistance. It has been reported that *S. aureus* responds to diverse classes of cell wall-inhibitory antibiotics, like methicillin, using the two-component regulatory system VraSR to up- or down-regulate a set of genes that presumably facilitates resistance to these antibiotics [14], and VraT has been reported to be a positive modulator of VraSR [15]. SgtB is known as the core cell wall stress stimulon together with PBP2 and MurZ, and is also regulated by VraSR [16]. In Gram-positive bacteria, it has been reported that SgtB might participate in enhancing peptidoglycan biosynthesis and catalyzing the incorporation of UDP-*N* acetylglucosamine into peptidoglycan for cell wall elongation, thereby reducing sensitivity to antibiotics that inhibit cell wall synthesis [17]. Interestingly, no significant induction of *URGs2* was observed under glucose-added conditions (Fig. 2). The specific molecular signal responsible for *vraSTR* induction remains unknown, but some signaling from the PTS or glycolysis pathway might induce the expression of *vraTSR*. The above discussion suggests that erythritol may respond to the VraTSR three-component system and promote cell wall synthesis by inducing SgtB, which may contribute to the suppression of some erythritol stress.

Gene products of *DRGs* (*pur*-operon genes) were predicted to be involved in the 'de novo' IMP biosynthetic process, which leads to the purine biosynthesis pathway. It has been reported that the *pur*-operon repressor PurR of *Bacillus subtilis*, which is a structural homologue of PurR of staphylococci, regulates the transcription of all *pur*-operon genes encoding enzymes for synthesis of IMP from the starting material phosphoribosyl pyrophosphate (PRPP) [18]. Another study showed that PRPP appears to be the inducer of genes regulated by PurR, as it is the only molecule among many nucleobases, nucleosides, and nucleotides known to affect PurR-DNA binding *in vitro* [19]. The addition of erythritol to SP cultures significantly down-regulated *DRGs* under glucose-free conditions. This result may suggest that PRPP is not sufficiently synthesized in the presence of erythritol due to its inhibition of PTS and is not present in sufficient amounts to bind to PurR in order to release the expression of the *pur*-operon genes. In the glucose-fed condition, PRPP metabolized from glucose may be sufficiently present to interact with PurR, and the down-regulation of the *pur*-operon genes may thus be suppressed.

It has been reported that the 'de novo' IMP biosynthetic process is crucial for *S. aureus* growth in minimum media but not in rich media [20], likely due to the complementary action of the purine salvage pathway in rich media [10]. This suggests that inhibition of the 'de novo' IMP biosynthetic process by erythritol is not directly responsible for the inhibition of SP growth in the 'rich' 802 medium in this study.

Increased expression of the *pur*-operon genes, which were down-regulated by erythritol, has been reported to be found during biofilm formation in Gram-positive bacteria such as *S. aureus* and *Enterococcus faecalis*, and deletion of these genes significantly impaired biofilm formation in *S. aureus* [10]. Furthermore, erythritol has been shown to be more effective than xylitol in inhibiting not only streptococcal growth but also biofilm formation [4]. These previous results suggest that erythritol might inhibit biofilm formation in SP by reducing the expression of the *pur*-operon genes. In fact, erythritol showed an inhibitory effect on biofilm formation of SP that exceeded its inhibitory effect on growth (Fig. 4). The relationship between biofilm formation and the 'de novo' IMP biosynthetic process has not been fully elucidated; however, it has been suggested that during biofilm formation the requirement for high amounts of purine synthesized through 'de novo' IMP biosynthesis might be widespread among bacteria [10].

Biofilms are complex matrices produced by microorganisms, in which cells are bound to each other and linked to biotic or abiotic surfaces [21]. Biofilm formation of staphylococci strains is well known as a factor that increases the severity of diseases and antimicrobial resistance [22]. In the clinical setting, adhesion to surfaces such as catheters and subsequent biofilm formation can lead to a number of persistent infections [23]. The inhibition of growth and biofilm formation of staphylococci strains by erythritol may contribute to new treatments and assist in controlling the spread of infection.

Conclusion

The present study aimed to define the changes in gene transcription signatures induced by erythritol in *S. pseudintermedius*. Changes in the gene transcription profiles of *S. pseudintermedius* by the presence of erythritol were analyzed by RNA-seq and qPCR. We revealed that erythritol induced up-regulation of three genes (*ptsG*, *ppdK*, and *ppdkR*) that are related to the PTS. Glucose supplementation restored the up-regulation of the PTS-related genes in response to erythritol. These findings indicated that erythritol antagonistically inhibits PTS-mediated glucose uptake, thereby exerting a growth inhibitory effect on *S. pseudintermedius*. In addition, erythritol down-regulated eleven genes that are located in a single *pur*-operon and inhibited biofilm formation. Further data on the actual involvement of erythritol in these cellular responses are to be obtained in the future.

Methods

Bacterial strain and preparation of media

SP was obtained from the Japan Collection of Microorganisms (JCM). For cultivation, 802 medium (1% hipolypepton [Fujifilm Wako, Osaka, Japan], 0.2% yeast extract [Fujifilm Wako], 0.1% $MgSO_4$ $7H_2O$ [Fujifilm Wako], pH 7.0) with or without erythritol (B Food Science Co., Ltd., Tokyo, Japan) and/or glucose (Fujifilm Wako) were used.

RNA-seq

A single colony of SP was inoculated into 3 mL of 802 medium, and cultured at 30°C for 15 hours with shaking at 210 rpm using a BR-23FP MR shaker (Taitec, Aichi, Japan) until OD_{660} = 3.4. For RNA preparation of bacteria cultured in 802 medium with or without 5% (w/w) erythritol, 60 or 30 µL of the above seed culture was inoculated into 3 mL of 802 medium, respectively, and incubated at 30°C until OD_{660} = 0.8–1.0. The RNA extraction and RNA-seq operations were performed according to the procedures described in a previous report *[7]*. The sample size was n = 3 for each condition. The Principal Component Analysis (PCA) plot and volcano plot were created to visualize the RNA-seq results using the Nucleic Acid SeQuence Analysis Resource (NASQAR; https://nasqar.abudhabi.nyu.edu/#). The top 15 up-regulated and down-regulated genes in response to erythritol obtained by RNA-seq analysis were annotated using Protein ANNotation with Z-scoRE (PANNZER2; http://ekhidna2.biocenter.helsinki.fi/sanspanz/) to predict functional descriptions and Gene Ontology (GO) classes of the biological process.

Analysis of qPCR

Analysis of qPCR was performed as follows. Total RNA of the cells cultured in 802 medium was extracted as above. Total RNA of the cells cultured in 802 medium containing 1% (w/w) glucose was prepared in the same manner as that described above (n = 3). Complementary DNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Ottweiler, Germany) with random primers. The primer sets used for qPCR had 100% identity with the gene sequences of SP (GenBank: MLGE00000000.2) and were selected from the primer sets selected using Primer-BLAST (Table 1). A qPCR reaction was performed using these primer sets and TB Green® Fast qPCR Mix (Takara Bio, Shiga, Japan) with 45 cycles comprised of conditions of

95℃ for 5 s and 55℃ for 60 s on a Thermal Cycler Dice® Real-Time System III (Takara Bio). Relative gene expression levels were assessed using the *recA* gene as a reference gene [24].

Growth test of SP

SP was inoculated into 802 medium containing 0.1% (w/w) glucose and cultured at 37℃ for 2 hours to serve as an inoculum (OD_{660} = 1.47). After dispensing 0.6 mL of 802 medium with 0% erythritol and 0% glucose, 0% erythritol and 0.1% glucose, 10% erythritol and 1% glucose, 10% erythritol and 0.1% glucose, and 10% erythritol or 0% glucose into 96 Deep Well Plates (AxyGen Scientific, CA, USA), each well was inoculated with 20 µL of the SP broth and cultured at 37℃ using the MBR-034P shaker (Taitec) with shaking at 1,000 rpm. After 1.5, 3, 4.5, 6, and 7.5 hours of incubation, twenty microliters of these cultures were suspended in 180 µL of water in the 96-well flat-bottomed plate 4845-96F (Watson Bio Lab, CA, USA), and turbidity (OD_{660}) was measured using the microplate reader SpectraMax M2 (Molecular Devices, CA, USA) (n = 4).

Biofilm adhesion assay

Biofilm adhesion was examined using a crystal violet staining assay based on a previously reported method [25]. Briefly, SP was inoculated into 2X 802 medium and cultured at 37℃ with shaking at 210 rpm until OD_{660} = 1.0. A 100-µL aliquot of this culture was added to 100 µL of erythritol solution containing 0, 5, 10, and 15% erythritol (w/w) dispensed into 96-well cell culture plates (197-96CPS; Watson Bio Lab). In the case of glucose-containing cultures, glucose was added at a concentration of 1.0% (w/w). After incubation at 37°C for 24 hours without shaking to allow biofilm formation, OD_{660} was measured and used as an indicator of the cell population or bacterial growth. The contents of the wells were then discarded and each well was washed with water to remove non-adherent cells while carefully maintaining the integrity of the formed biofilms. Biofilms were heat-fixed at 60°C for 60 min. Adherent cells were stained with 150 µL of 0.2% (w/v) crystal violet for 15 min at room temperature and then dried at 30°C. After resolubilization with 95% ethanol, OD_{570} was measured and used as an indicator of the amount of biofilm formation. In the statistical analyses, multiple comparisons were performed using Tukey's method with SPSS @ Statistics Version 26.0. The sample size was n = 8.

Abbreviations

SP
Staphylococcus pseudintermedius JCM 17571
MRSP
methicillin-resistant *Staphylococcus pseudintermedius*
PTS
phosphoenolpyruvate-dependent sugar phosphotransferase system
PEP
phosphoenolpyruvate
PRPP
phosphoribosyl pyrophosphate
qPCR
real-time quantitative PCR
FC
fold change
URGs
Up-Regulated Genes group
DRGs
Down-Regulated Genes
JCM
Japan Collection of Microorganisms

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

T. Fujii and T. Tochio are employed by B Food Science Co., Ltd. The remaining authors declare no conflicts of interest.

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Authors' contributions

T. Fujii, T. Tochio, and K. Nishifuji were involved in the study design and data interpretation. T. Fujii was involved in the experiments, data analysis. T. Fujii and K. Nishifuji were involved in writing. All authors critically revised the report, commented on drafts of the manuscript, and approved the final report.

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tables

Table 1 Sequences of PCR primers used for qPCR.

Primer set	Sequence (5’–3’)	PCR product length (bp)
purN	ACTCGCGCATATCGAAGTCA	195
	TACACCTTCCTGACGCAACC	
ptsG	GCATTGGTGGCTCGTGATTC	192
	AAGGCAATTCGCTTGCAGTC	
ppdK	TCATTCCACCGTGTGTCGTT	153
	CGGCGACGGGTAAAATTGTG	
ppdkR	CTCACCGCAAGCCCTGAATA	157
	TGACTGTCGCATTGAGCTGT	
vraS	CAATACGTTACGACATGCTG	169
	ACCAATCTCTATTGCACGTT	
vraR	CGTTAGATGCCGGTGTCGAT	177
	TCTCGCGCTCAGTCAACAAT	
sgtB	CGCTCAATCCGTTTAGCGAC	150
	ACGTGCGATTTTTGCATCGG	
purN	ACTCGCGCATATCGAAGTCA	195
	TACACCTTCCTGACGCAACC	
purF	ATCCGAACGCCATTGGTCAT	176
	TTGGAAAATGCCGCCTGTTG	
recA	GGGCCGAGCTCTGAAATTCT	185
	CTCCTTCGCGTGAAATCCCT	

Table 2 Top 15 up-regulated genes (top table) and top 15 down-regulated genes (bottom table) by erythritol.

Log_2FC	*p*-value	Gene_id	Gene product	Gene name	Log_2FC		p-value		
					Estimated PPV (>0.5)	Description	Estimated PPV (>0.5)	GO-id	Description
7.01	0	BJK46_006305	hypothetical protein						
6.99	9.00E-300	BJK46_000955	hypothetical protein						
5.39	0	BJK46_002585	DUF1361 domain-containing protein		0.52	DUF1361 domain-containing protein (fragment)			
5.24	0	BJK46_008880	DUF5011 domain-containing protein		0.51	chitinase			
4.76	1.42E-302	BJK46_008085	hypothetical protein						
4.73	0	BJK46_005200	M50 family peptidase		0.67	M50 family metallopeptidase			
4.63	2.45E-302	BJK46_009280	PTS glucose transporter subunit IICBA	*ptsG*	0.72	PTS system glucose-specific EIICBA component	0.82	GO:1904659	glucose transmembrane transport
							0.72	GO:0009401	phosphoenolpyruvate-dependent sugar phosphotransferase system
							0.59	GO:0016310	phosphorylation
4.62	2.79E-249	BJK46_006810	LytR family transcriptional regulator						
4.5	6.13E-307	BJK46_008015	glycosyltransferase	*sgtB*	0.50	monofunctional glycosyltransferase	0.66	GO:0008360	regulation of cell shape
							0.66	GO:0009252	peptidoglycan biosynthetic process
							0.66	GO:0071555	"
4.47	3.41E-230	BJK46_000295	kinase/pyrophosphorylase	*ppdkR*		Putative pyruvate, phosphate dikinase regulatory protein	0.70	GO:0006470	protein dephosphorylation
							0.63	GO:0006468	protein phosphorylation
4.22	2.28E-202	BJK46_000300	pyruvate, phosphate dikinase	*ppdK*	0.63	Pyruvate, phosphate dikinase	0.67	GO:0006090	pyruvate metabolic process
							0.59	GO:0016310	phosphorylation
4.15	7.55E-274	BJK46_008070	DNA-binding response regulator	*vraR*			0.67	GO:2000112	regulation of cellular macromolecule biosynthetic process
							0.63	GO:0000160	phosphorelay signal transduction system
							0.58	GO:0006355	regulation of transcription, DNA-templated
4.14	2.29E-274	BJK46_008080	transporter	*vraT*	0.69	Transporter yvqF			
4.1	5.04E-252	BJK46_008075	sensor histidine kinase	*vraS*	0.89	Sensor protein VraS	0.68	GO:0018106	peptidyl-histidine phosphorylation
							0.63	GO:0000160	phosphorelay signal transduction system
4.1	5.40E-246	BJK46_000760	DUF1002 domain-containing protein		0.79	Extracellular protein			

Log_2FC	*p*-value	Gene_id	Gene product	*Gene name*	Description (shown by Pannzer2)		Biological process (shown by Pannzer2)		
					Estimated PPV (>0.5)	Description	Estimated PPV (>0.5)	GO-id	Description
-5.33	0	BJK46_009895	hypothetical protein		0.59	surface rod structure-forming protein G (fragment)			
-4.84	2.28E-219	BJK46_012045	hypothetical protein						
-4.8	0	BJK46_008825	phosphoribosylglycinamide formyltransferase	*purN*	0.56	phosphoribosylglycinamide formyltransferase			
-4.79	0	BJK46_008820	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	*purH*	0.54	bifunctional purine biosynthesis protein PurH			
-4.69	0	BJK46_008830	phosphoribosylformylglycinamidine cyclo-ligase	*purM*	0.67	phosphoribosylformylglycinamidine cyclo-ligase			
-4.67	0	BJK46_008835	Amidophosphoribosyltransferase	*purF*	0.63	amidophosphoribosyltransferase	0.75	GO:0009113	purine nucleobase biosynthetic process
							0.70	GO:0006189	de novo' IMP biosynthetic process
							0.70	GO:0009116	nucleoside metabolic process
							0.69	GO:0006541	glutamine metabolic process
-4.56	2.34E-321	BJK46_008815	phosphoribosylamine–glycine ligase	*purD*	0.65	phosphoribosylamine–glycine ligase	0.74	GO:0009113	purine nucleobase biosynthetic process
							0.69	GO:0006189	de novo' IMP biosynthetic process
-4.54	4.33E-245	BJK46_008840	phosphoribosylformylglycinamidine synthase subunit PurL	*purL*	0.59	phosphoribosylformylglycinamidine synthase subunit PurL	0.70	GO:0006189	de novo' IMP biosynthetic process
-4.47	3.47E-294	BJK46_008850	phosphoribosylformylglycinamidine synthase subunit PurS	*purS*	0.63	phosphoribosylformylglycinamidine synthase subunit PurS	0.71	GO:0006189	de novo' IMP biosynthetic process
-4.42	0	BJK46_008845	phosphoribosylformylglycinamidine synthase subunit PurQ	*purQ*	0.60	phosphoribosylformylglycinamidine synthase subunit PurQ	0.70	GO:0006189	de novo' IMP biosynthetic process
							0.69	GO:0006541	glutamine metabolic process
-4.3	0	BJK46_008855	phosphoribosylaminoimidazolesuccinocarboxamide synthase	*purC*	0.63	phosphoribosylaminoimidazole-succinocarboxamide synthase	0.71	GO:0009236	cobalamin biosynthetic process
							0.70	GO:0006189	de novo' IMP biosynthetic process
-3.86	1.28E-235	BJK46_008860	5-(carboxyamino)imidazole ribonucleotide synthase	*purK*	0.61	N5-carboxyaminoimidazole ribonucleotide synthase	0.70	GO:0006189	de novo' IMP biosynthetic process
-3.8	4.19E-66	BJK46_011905	peptidoglycan-binding protein						
-3.63	3.78E-181	BJK46_009875	ABC-type cobalamin Fe3+-siderophores transport system periplasmic component		0.73	hydroxamate siderophore binding lipoprotein			
-3.25	1.40E-126	BJK46_008865	5-(carboxyamino)imidazole ribonucleotide mutase	*purE*	0.57	N5-carboxyaminoimidazole ribonucleotide mutase	0.71	GO:0006189	de novo' IMP biosynthetic process

Figures

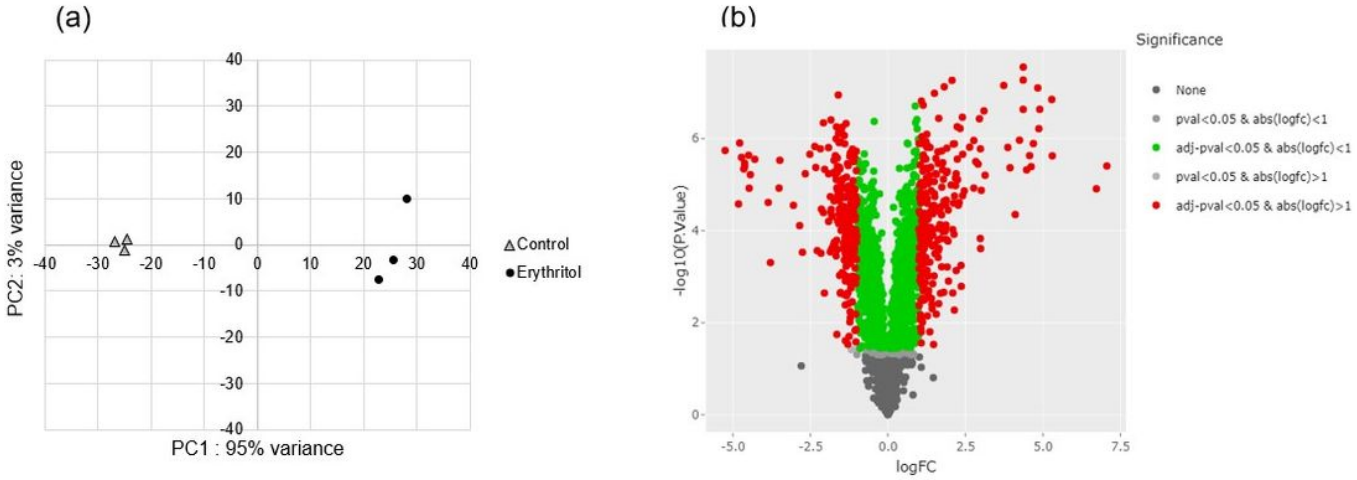

Figure 1

RNA-seq analysis and verification by NASQAR.

(a) PCA shows clustering of RNA-seq samples with (circles) or without (triangles) erythritol treatment. PC1, which explains 95% of the total variance, separates the treated samples from control samples. (b) Volcano plot showing RNA-seq samples. The 244 and 381 genes were significantly up- or down-regulated by erythritol, respectively ($|\text{Log}_2\ \text{FC}| \geq 1$, p value ≤ 0.05).

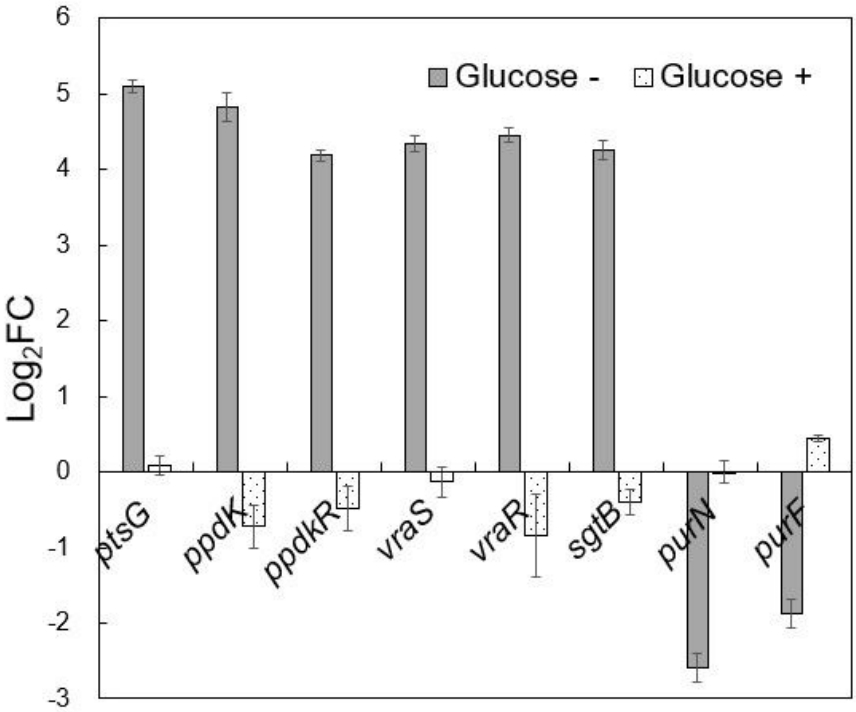

Figure 2

Differential gene expression analysis by qPCR.

The expression levels of several genes whose expression was regulated by erythritol were examined by qPCR in the presence and absence of 1% glucose.

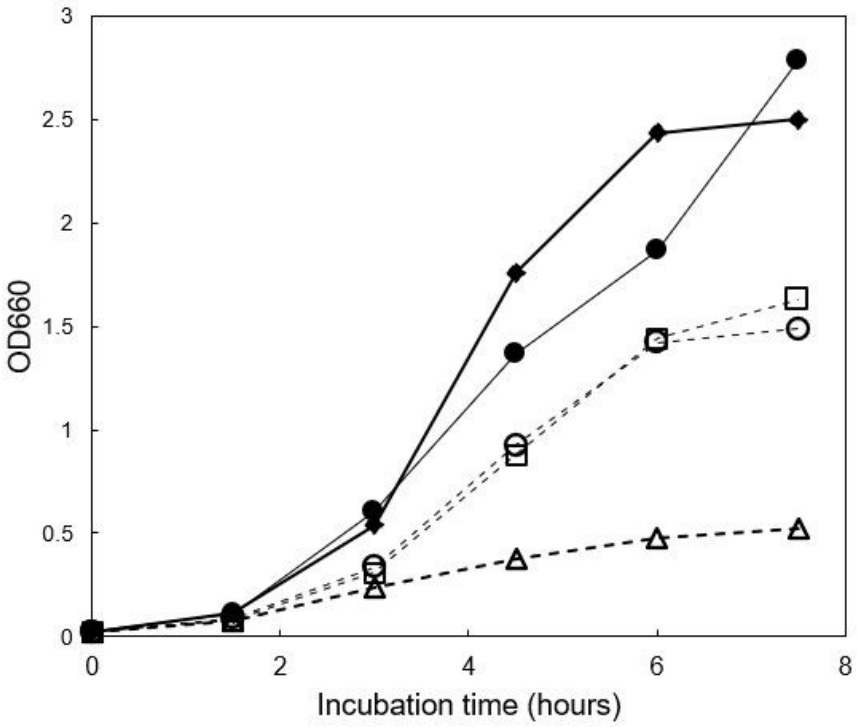

Figure 3

Glucose addition partially suppressed growth inhibition by erythritol (n = 3).

SP in 802 medium with 0% erythritol and 0% glucose (closed diamonds), 0% erythritol and 0.1% glucose (closed circles), 10% erythritol and 1% glucose (open squares), 10% erythritol and 0.1% glucose (open circles), and 10% erythritol and 0% glucose (open triangles).

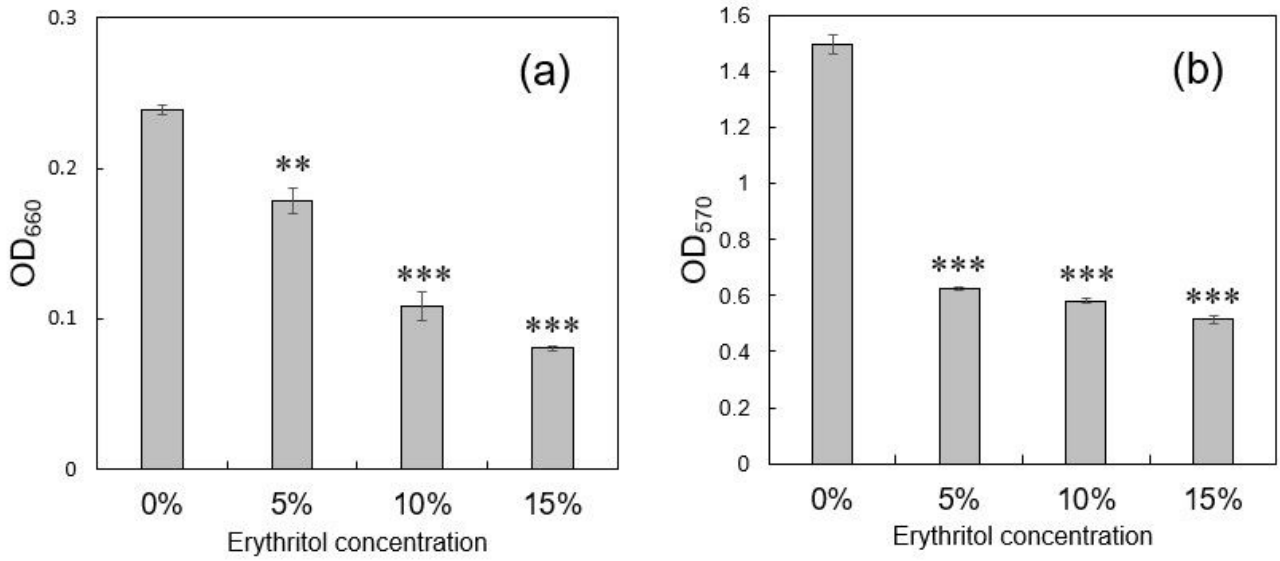

Figure 4

Inhibition of growth and biofilm formation of SP by erythritol.

Cell growth as indicated by OD_{660} (a) and biofilm formation as indicated by OD_{570} (b) of the SP culture medium without erythritol (0%) and with erythritol (5, 10, 15%) (n = 8) were measured. Double asterisk indicates $p < 0.01$ and triple asterisk indicates $p < 0.001$ between the control (0%) group and each erythritol-added group.

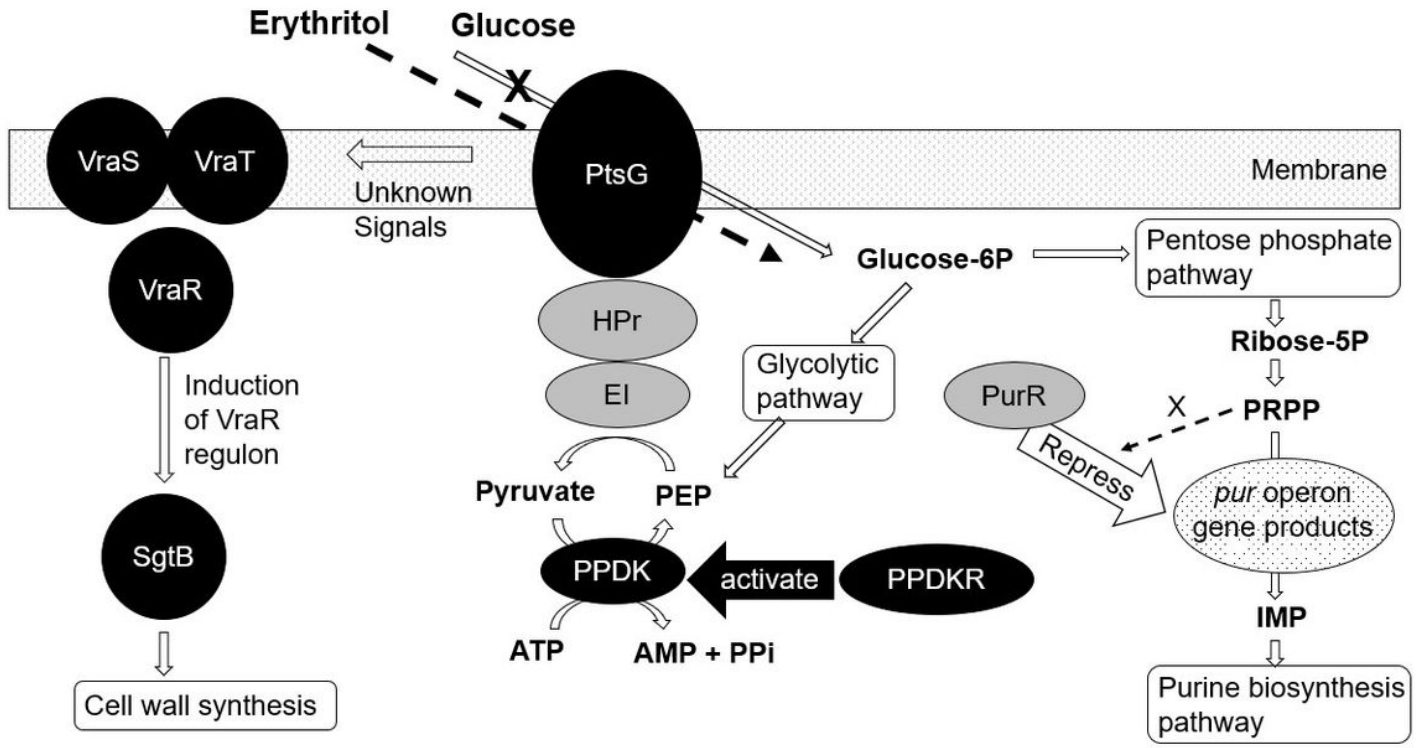

Figure 5

A proposed summary of the cellular responses to the addition of erythritol.

Gene products of *URGs1*, *URGs2* (black circles), and *DRGs* (gray circles) and their related gene products (dotted circles) are shown. Compound names are shown in bold. Erythritol may antagonistically inhibit PTS-mediated glucose uptake by binding to the substrate binding site of PtsG, thereby exerting a growth inhibitory effect on SP. The cellular response to this may be induction of PtsG and its drivers, PPDK and PPDKR. Erythritol may respond to the VraTSR three-component system and promote cell wall synthesis by inducing SgtB, which may contribute to the suppression of some erythritol stress. Erythritol-induced glucose starvation may limit PRPP synthesis, preventing the synthesis of sufficient amounts of PRPP for binding to the PurR repressor and releasing expression of the *pur*-operon genes.

Supplementary Files

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- Additionalfile1.docx