Aerobic conditions increase isoprenoid biosynthesis pathway gene expression levels for carotenoid production in Enterococcus gilvus

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One sentence summary: Our results indicate that Enterococcus gilvus can increase carotenoid production by up-regulation of the isoprenoid biosynthesis pathway. This article provides novel insights into the aerobic metabolism of lactic acid bacteria.

ABSTRACT

Some lactic acid bacteria that harbour carotenoid biosynthesis genes (crtNM) can produce carotenoids. Although aerobic conditions can increase carotenoid production and crtNM expression levels, their effects on the pathways that synthesize carotenoid precursors such as mevalonate and isoprene are not completely understood. In this study, we investigated whether aerobic conditions affected gene expression levels involved in the isoprenoid biosynthesis pathway that includes the mevalonate and isoprene biosynthesis pathways in Enterococcus gilvus using real-time quantitative reverse transcription PCR. NADH oxidase (nox) and superoxide dismutase (sod) gene expression levels were investigated as controls for aerobic conditions. The expression levels of nox and sod under aerobic conditions were 7.2- and 8.0-fold higher, respectively, than those under anaerobic conditions. Aerobic conditions concomitantly increased the expression levels of crtNM carotenoid biosynthesis genes. HMG-CoA synthase gene expression levels in the mevalonate pathway were only slightly increased under aerobic conditions, whereas the expression levels of HMG-CoA reductase and five other genes in the isoprene biosynthesis pathways were 1.2–2.3-fold higher than those under anaerobic conditions. These results demonstrated that aerobic conditions could increase the expression levels of genes involved in the isoprenoid biosynthesis pathway via mevalonate in E. gilvus.

Keywords: lactic acid bacteria; carotenoid; aerobic; isoprene; mevalonate; gene expression

INTRODUCTION

Reactive oxygen species that are generated from oxygen during oxidative stress can damage lactic acid bacteria (LAB) (Miyoshi et al. 2003). To protect against cell damage due to oxidative stress, LAB produce various antioxidants such as superoxide dismutase and thioredoxin reductase (Pedersen et al. 2008). For metabolic engineering, it is useful to investigate the stress tolerance mechanisms of LAB. Several studies have applied stress response mechanisms to provide LAB with stress tolerance (Desmond et al. 2004; Bruno-Bárcena et al. 2005). We recently demonstrated that a carotenoid, 4,4′-diaponeurosporene, could increase the oxidative stress resistance of Lactococcus lactis (Hagi et al. 2013). 4,4′-diaponeurosporene is considered to enhance oxidative stress tolerance because of its ability to act as antioxidant, which is derived from its conjugated double bonds (Young and Lowe 2001; Garrido-Fernández et al. 2010). Furthermore, based on the responses of carotenoids to oxidative stress,
aerobic conditions significantly increased carotenoid production and expression levels of carotenoid biosynthesis genes (crtNM) in Enterococcus gilvus (Hagi et al. 2014). These results suggested that carotenoids provide one mechanism for oxidative stress tolerance of LAB.

Oxidative stress can regulate various antioxidant and metabolic pathways in LAB, for which responses to oxygen have been reviewed (Condon 1987; Miyoshi et al. 2003). For L. lactis, Pedersen et al. (2008) reported that the expression levels of stress response genes, including alkyl hydroperoxide reductase (ahr), glutathione reductase (grh) and superoxide dismutase (sodA), were up-regulated under aerobic conditions. In comparison, glucose metabolism was altered under aerobic conditions (Nordkvist, Jensen and Villadsen 2003; Kang, Korber and Tanaka 2013). Under aerobic conditions, LAB consume oxygen and the activity of NADH oxidase, which causes changes in the redox balance, is increased. Consequently, glucose metabolism through pyruvate and acetyl-CoA is altered and lactic acid production is reduced (Nordkvist, Jensen and Villadsen 2003). However, little is known regarding stress-induced metabolism related to carotenogenesis in LAB.

The carotenoid biosynthesis pathway and its upstream pathways such as the mevalonate and 2-C-methyl-d-erythritol 4-phosphate pathways have been well characterized in plants, photo-biosynthetic bacteria and other organisms (Takaichi and Mochimaru 2007; Cazzonelli and Pogson 2010; Heuston et al. 2012; Guggisberg, Amthor and Odom 2014). In LAB, a carotenoid, 4,4′-diaponeurosporene, produced by Enterococcus and Lactobacillus was identified and characterized (Taylor, Ikawa and Chesbro 1971; Breithaupt et al. 2001; Garrido-Fernández et al. 2010; Hagi et al. 2013). Fig. 1 illustrates the carotenoid biosynthesis pathway via an isoprenoid biosynthesis pathway in Gram-positive bacteria, including enterococci (Wilding et al. 2000; Garrido-Fernández et al. 2010). Acetyl-CoA is converted to isopentenyl diphosphate (PP) via the isoprene biosynthesis pathway, which includes the mevalonate pathway, due to the actions of six enzymes. In enterococci, there is a bi-functional protein that has both acetyl-CoA thiolase (atoB) and HMG-CoA reductase (hmgr) activity (Wilding et al. 2000). Isopentenyl-PP (one isoprene unit, five carbons) and its isomer dimethylallyl-PP are converted to farnesyl-PP by geranyltransferase (ispA). Finally, farnesyl-PP (three isoprene units, 15 carbons) is converted to 4,4′-diaponeurosporene (six isoprene units, 30 carbons) by dehydrodqsalene synthase (crTM) and dehydrodqsalene desaturase (crTN). The mevalonate pathway is one of the most important pathways for carotenoid production. To increase carotenoid production in Escherichia coli, metabolic engineering techniques were used to improve the mevalonate pathway (Das et al. 2007).

In carotenoid-producing Lactobacillus plantarum, carotenoid production levels were different among various strains when grown under static conditions (Garrido-Fernández et al. 2010). This implied that a constitutive promoter could influence carotenoid production levels. However, aerobic conditions induced expression of crtNM genes in E. gilvus (Hagi, Kobayashi and Nomura 2014), suggesting that aerobic conditions may have not only affected crtnm gene expression levels but also the biosynthesis pathways for carotenoid precursor synthesis, such as the isoprene biosynthesis pathway via mevalonate.

Thus, in this study, to determine whether oxidative stress could induce the mevalonate and isoprene biosynthesis pathways simultaneously, the expression levels of isoprenoid biosynthesis genes were compared between E. gilvus under both aerobic and anaerobic culture conditions.

Figure 1. Isoprenoid biosynthesis pathway for carotenoid production. Gene names are indicated with their historical designations and nomenclatures in bold. The isoprenoid biosynthesis pathway comprises three pathways: the mevalonate biosynthesis pathway (atoB, hmgr and hmgc), the isoprene biosynthesis pathway (mvk, pmvk, mpd, ipi and ispa) and the carotenoid biosynthesis pathway (crTM and crTN).

MATERIALS AND METHODS

Bacteria and growth conditions

Enterococcus gilvus CR1 was derived from raw cow’s milk (Hagi et al. 2013) and was anaerobically incubated at 30°C for 24 h in M17 medium (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5% glucose (GM17 medium). Cells from this preculture were inoculated into 200 mL of GM17 medium (0.5%, v/v) in an Erlenmeyer flask (500 mL). For aerobic conditions, an inoculated Erlenmeyer flask was statically incubated for 4 h in an AnaeroPack system (AnaeroPack, Mitsubishi Gas Chemical, Tokyo, Japan) and then shaken at 110 rpm at 30°C for 30 min without an AnaeroPack system. For aerobic conditions, a culture was statically incubated at 30°C for 4.5 h in an AnaeroPack system. To avoid oxygen being dissolved in the culture medium, an AnaeroPack was used as in our previous study (Hagi, Kobayashi and Nomura 2014). The yellow pigment of E. gilvus was extracted with methanol as in our previous study (Hagi et al. 2013). The amount of pigment was determined from the maximal absorption at 470 nm, which...
was normalized to the optical density of bacterial growth at a wavelength of 600 nm (OD$_{600}$) as previously reported (Umeno, Tobias and Arnold 2002).

**Gene expression level determination by real-time quantitative reverse transcription PCR**

Total RNA was extracted from harvested *E. gilvus* cells that had been grown aerobically and anaerobically. Harvested cells were treated with mutanolysin (20 U/200 μL) at 37°C for 1 h, following which total RNA was extracted using the RNase-Free DNase Set (Qiagen). Total RNA was extracted from three independent *E. gilvus* cell cultures for each culture condition.

Reverse transcription PCR was performed using a PrimeScript® II 1st strand cDNA Synthesis Kit (Takara, Otsu, Japan) as previously reported (Hagi, Kobayashi and Nomura 2014). cDNA was synthesized from 1 μg of total RNA. The resulting cDNA solutions were diluted with 60 μL of sterile distilled water and used as templates for real-time PCR.

Real-time quantitative PCR was performed using THUNDER-BIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) and the C1000 Thermal Cycler CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). For both assays, 2 μL of a diluted cDNA solution was added to 18 μL of a PCR mixture that included 10 μL of THUNDERBIRD SYBR qPCR Mix, 1.0 μL of each primer (10 pmol) and 6.0 μL of sterile distilled water. The thermal cycling conditions used were as follows: 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The melting program was 95°C for 10 s, 65°C for 5 s and 95°C for 50 s.

The primer sequences are listed in Table 1. The primers were designed using sequence information for *E. gilvus* (ASWH01000001) obtained from the National Center for Biotechnology Information (NCBI) database. The locus tags of targeted genes were as follows: NADH oxidase (locus tag or accession no. IS92_01535, gene name nox); superoxide dismutase (IS92_02089, sod); hydroxymethylglutaryl (HMG) -CoA synthase (IS92_01009, hmgS); HMG-CoA reductase (IS92_01010, hmgr); mevalonate kinase (IS92_00331, mvk); phosphomevalonate kinase (IS92_00329, pmvk); phosphomevalonate decarboxylase (IS92_00330, mvd); and geranyltransteransferase delta-isomerase type 2 (IS92_00218, ispa). In this study, acetyl-CoA thiolase (aotb) at the top part of the pathway (Fig. 1) was not evaluated because the HMG-CoA reductase gene of *E. gilvus* includes acetyl-CoA thiolase, which is a dual-function protein in *Enterococcus faecalis* (Hedl et al. 2002).

Standard curves (slope, efficiency = 10$^{-1/slope}$ – 1, y-intercept and correlation coefficient = $r^2$) and gene expression levels in *E. gilvus* were analysed using CFX Manager Software version 2.1 (Bio-Rad). Real-time quantitative reverse transcription PCR (qRT-PCR) was performed in triplicate wells for each sample as described previously (Hagi, Kobayashi and Nomura 2014). Gene expression data from qRT-PCR were analysed using the ΔΔCt method (Livak and Schmittgen 2001). Gene expression ratios were determined by the following equations: ΔΔCt (aerobic) = Ct (target gene, aerobic) – Ct (16S rRNA, aerobic); ΔΔCt (anaerobic) = Ct (target gene, anaerobic) – Ct (16S rRNA, anaerobic); and ΔΔCt = ΔCt (aerobic) – ΔCt (anaerobic). Gene expression ratios were normalized to 16S rRNA using the 2$^{-\Delta\DeltaCt}$ method. Relative gene expression levels in *E. gilvus* cells without aerobic treatment (anaerobic conditions) were set at 1 and those of cells with aerobic treatment were determined accordingly. Student’s t-tests were used for statistical comparisons. P-values <0.05 were considered statistically significant.

**Table 1. Primer sequences.**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
<th>Amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Hydroxymethylglutaryl-CoA synthase</td>
<td>Hmgs F</td>
<td>CACATTGTTCTTGAGCAGAT</td>
<td>128</td>
<td>Present study</td>
</tr>
<tr>
<td>2 Hydroxymethylglutaryl-CoA reductase</td>
<td>Hmgs R</td>
<td>AGGGCTAGCTGCAGGAACCAA</td>
<td>122</td>
<td>Present study</td>
</tr>
<tr>
<td>3 Mevalonate kinase</td>
<td>Mvk F</td>
<td>ATTCGCGATGCCTATGACAT</td>
<td>126</td>
<td>Present study</td>
</tr>
<tr>
<td>4 Phosphomevalonate kinase</td>
<td>Pmvk F</td>
<td>TCGCTATATTGGCCAGATTG</td>
<td>123</td>
<td>Present study</td>
</tr>
<tr>
<td>5 Phosphomevalonate decarboxylase</td>
<td>Mpd F</td>
<td>GCCTGGTGTCAGAATCTGACC</td>
<td>89</td>
<td>Present study</td>
</tr>
<tr>
<td>6 Isopentenyl-diphosphate delta-isomerase type 2</td>
<td>Ipi F</td>
<td>ATTCGGGATCTATGTGCTAGAT</td>
<td>126</td>
<td>Present study</td>
</tr>
<tr>
<td>7 Geranyltransteransferase</td>
<td>Ispa R</td>
<td>GGATCAGACCGAATATGTCATTG</td>
<td>121</td>
<td>Present study</td>
</tr>
<tr>
<td>8 Dehydrodrosqualene synthase</td>
<td>CrtM F</td>
<td>TGGGTCAGATTGGTAGAAGCTTG</td>
<td>148</td>
<td>Present study</td>
</tr>
<tr>
<td>9 Dehydrodrosqualene desaturase</td>
<td>CrtR F</td>
<td>GACAGTGCGGCTTTCAACTCCT</td>
<td>179</td>
<td>Present study</td>
</tr>
<tr>
<td>10 16S rRNA gene</td>
<td>341F</td>
<td>CTTACTGAGGAGACGACGG</td>
<td>195</td>
<td>Hagi et al. 2014</td>
</tr>
<tr>
<td>11 Superoxide dismutase</td>
<td>I592 518R</td>
<td>GACAGTGCGGCTTTCAACTCCT</td>
<td>184</td>
<td>Present study</td>
</tr>
<tr>
<td>12 NADH oxidase</td>
<td>I592 12N</td>
<td>CTTGCGTAGTCGGTGAGGAGATA</td>
<td>173</td>
<td>Present study</td>
</tr>
</tbody>
</table>

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RESULTS

Superoxide dismutase (sod), NADH oxidase (nox), dehydrosqualene synthase (crtM) and dehydrosqualene desaturase (crtN) gene expression levels in *E. gilvus*

After *E. gilvus* was subjected to aerobic conditions, carotenoid production was increased and yellow-pigmented cells were observed (Fig. 2a). Absorbance levels at 470 nm of the yellow pigments extracted from *E. gilvus* cells under anaerobic and aerobic conditions were 0.003 and 0.012, respectively (Fig. 2b). Fig. 3 shows the relative gene expression levels of nox, sod, crtM and crtN. Based on PCR primer performance, the resulting standard curves using 10-fold serial dilutions of *E. gilvus* DNA were as follows: target gene hmg, slope = −3.288, y-intercept = 17.635, efficiency = 101%, r² = 0.999; hmgr, slope = −3.245, y-intercept = 17.463, efficiency = 103%, r² = 0.999; muk, slope = −3.144, y-intercept = 16.778, efficiency = 108%, r² = 0.999; pmuk, slope = −3.204, y-intercept = 16.929, efficiency = 105%, r² = 0.999; mps, slope = −3.305, y-intercept = 17.488, efficiency = 101%, r² = 0.999; ipi, slope = −3.478, y-intercept = 15.546, efficiency = 94%, r² = 0.997; and ispA, slope = −3.026, y-intercept = 21.382, efficiency = 114%, r² = 0.989.

In the mevalonate pathway, hmg gene expression levels under aerobic conditions tended to be higher than under anaerobic conditions (1.2-fold, *P* < 0.1). The expression level of the hmgr gene under aerobic conditions was slightly higher than under anaerobic conditions (1.2-fold, *P* < 0.05). Based on the isoprene biosynthesis pathway, the expression levels of the muk, pmuk and mps genes under aerobic conditions were 2.3-, 1.9- and 2.0-fold higher, respectively, than under anaerobic conditions (*P* < 0.005). Under aerobic conditions, the gene expression levels of ipi and ispA, which convert isopentenyl-PP to farnesyl-PP (isoprene condensation), were 2.3- and 1.3-fold higher, respectively, than under anaerobic conditions (*P* < 0.005).

DISCUSSION

Some LAB, including some *Enterococcus* and *Lactobacillus* species, can produce the carotenoid 4,4'-diaponeurosporene (Taylor, Ikawa and Chesbro 1971; Martinez-Murcia and Collins 1991; Garrido-Fernández et al. 2010). This carotenoid in LAB is thought to increase their tolerance to oxidative stress, photo stress and other types of stress (Maraccini, Ferguson and Boehm 2012; Hagi et al. 2013). It has recently been found that aerobic conditions increased carotenoid production and expression levels of carotenoid biosynthesis genes (crtNM) (Hagi, Kobayashi and Nomura 2014). Under aerobic conditions, metabolism mediated through glycolysis, pyruvate production and the lactate acid pathway in LAB can change dramatically. However, little is known regarding stress-induced metabolism related to carotenogenesis in LAB. In Gram-positive bacteria, carotenoids are produced from acetyl-CoA via the mevalonate, isoprene and carotenoid pathways.
biosynthesis pathways (Wilding et al. 2000). In this present study, we investigated whether aerobic conditions might affect the expression levels of carotenoid biosynthesis genes via the mevalonate and isoprene biosynthesis pathways in E. gilvus using qRT-PCR.

Under aerobic conditions, oxygen can alter the redox balance in LAB (Nordkvist, Jensen and Villadsen 2003; Kang, Korber and Tanaka 2013) and increase the expression levels of antioxidant genes such as nox and sod. In this study, as a control for aerobic conditions, we assessed nox and sod gene expression levels in E. gilvus. Our qRT-PCR results showed that the expression levels of both these genes under aerobic conditions were 7.2- and 8.0-fold higher, respectively, than under anaerobic conditions (Fig. 3). In addition, the expression levels of the carotenoid biosynthesis genes crtm and ctn in E. gilvus under aerobic conditions were also increased (5.3- and 5.9-fold, respectively). These results suggest that carotenoids are strongly related to oxidative stress, as are NADH oxidase and superoxide dismutases.

There are two isoprenoid biosynthesis pathways: the mevalonate pathway and the alternative 2-C-methyl-d-erythritol 4-phosphate pathway. In enterococci, a carotenoid precursor (isoprene) is synthesized through the mevalonate pathway (Heuston et al. 2012). In the mevalonate pathway, the HMG-CoA reductase gene of E. gilvus includes acetyl-CoA thiolase, which is a dual-function protein in E. faecalis (Hedl et al. 2002). Thus, gene expression levels of the mevalonate biosynthesis pathway shown in Fig. 1 were investigated. When these oxidative stress response genes and carotenoid biosynthesis genes were up-regulated, the expression levels of five genes in the isoprene biosynthesis pathway (from mevalonate kinase to geranyltranstransferase in Fig. 1), in which mevalonate is converted to farnesyl-PP via isopentenyl-PP, were concomitantly and significantly increased under aerobic conditions (Fig. 4). Cafaro et al. (2014) reported that the expression level of the geranylgeranyl pyrophosphate synthase gene, which converts isopentenyl-PP and dimethylallyl-PP to geranylgeranyl pyrophosphate involved in the isoprene condensation pathway in Oenococcus ceni, was increased under ethanol stress. These results suggested that several types of stress could induce the isoprenoid biosynthesis pathway in carotenoid-producing LAB to ameliorate environmental stress.

Our qRT-PCR results showed that aerobic conditions resulted in markedly increased expression levels of three genes (muk, pmuk and mpd) involved in the next step of the mevalonate biosynthesis pathway and that the expression level of hmgr, which converts 3-hydroxy-3-methylglutaryl-CoA to mevalonate, was only slightly increased under aerobic conditions (1.2-fold; \( P < 0.05 \)). In addition, the expression level of hmgS, which converts acetyl-CoA and acetocacetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA, was not significantly increased under aerobic conditions (1.2-fold; \( P < 0.1 \)).

In Staphylococcus aureus, which can produce carotenoids via the mevalonate pathway, a deficiency of the HMG-CoA synthase or HMG-CoA reductase gene results in up-regulation of their downstream genes, including those for mevalonate kinase, phosphomevalonate kinase and phosphomevalonate dehydrogenase (Balibar, Shen and Tao 2009). Balibar, Shen and Tao (2009) also showed that a deficiency of the mevalonate kinase gene resulted in down-regulation of its downstream genes; however, its upstream genes, for HMG-CoA synthase and HMG-CoA reductase, were not responsive to muk deficiency. This suggests that the gene expression patterns between the mevalonate biosynthesis pathway and its downstream pathway (muk, pmuk and mpd) are different.

Our results showed that subjecting E. gilvus to aerobic conditions up-regulated not only oxidative stress response genes (sod and nox) but also carotenoid biosynthesis genes (crtNM) and other isoprenoid biosynthesis genes (from hmgr to ispA). In our previous study, we showed that carotenoid production increased oxidative stress tolerance to hydrogen peroxide (Hagi et al. 2013). Furthermore, we demonstrated that increased carotenoid production, which resulted from aerobic conditions, improved the oxidative stress tolerance of E. gilvus (Hagi, Kobayashi and Nomura 2014). These results indicate that the isoprenoid biosynthesis pathway for carotenoid production can be up-regulated to protect against oxidative stress damage in E. gilvus cells. Several regulatory genes, such as the catabolite control protein (ccp) and global regulator (spx), are associated with the regulation of oxidative stress genes (nox, sodA, thioredoxin reductase trxB, etc.) in Enterococcus and other Gram-positive bacteria (Gaudu et al. 2003; Nakano et al. 2003; Engman et al. 2012; Kajfasz et al. 2012). Catabolite control protein A (ccpA: IS92_00780) and regulatory protein Spa (spx: IS92_02651) are also found in the genomic sequence of E. gilvus (ASWH01000001). Zomer et al. (2007) reported that the expression level of the muk gene in lipid metabolism was decreased in a ccpA mutant of L. lactis. This result implies that ccpA can act as an activator in the mevalonate pathway. On the other hand, accumulation of Spa could lead to increased pigmentation of cells in S. aureus (Engman et al. 2012). These transcriptional regulators may alter mevalonate and isoprene metabolism under oxidative stress conditions.

LAB are catalase-negative bacteria that are sensitive to oxidative stress. Thus, some studies have used molecular technology to confer oxidative or other types of stress tolerance to LAB (Bruno-Barcena et al. 2005; Rallu et al. 2000; Rochat et al. 2005). In our previous study, we showed that carotenoid production increased multistress tolerance against hydrogen peroxide, acid, bile acids and lysozyme (Hagi et al. 2013). To acquire stress-tolerant LAB, mutagenesis in the isoprenoid biosynthesis pathway is considered to be a useful means to increase the amounts of carotenoids associated with stress tolerance. Furthermore, LAB that produce high levels of carotenoids would contribute to the supply of fermented products by LAB with antioxidants.

In summary, subjecting E. gilvus to aerobic conditions increased the expression levels of genes involved in the isoprenoid biosynthesis pathway, which converts acetyl-CoA to 4,4′-diaponeurosperone. Our results indicate that E. gilvus can...
increase its carotenoid production by up-regulating these genes to avoid oxidative damage. These results provide novel insights into the aerobic metabolism of LAB.

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Conflict of interest. None declared.

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