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Transcriptional Regulation of Xylose Utilization in *Enterococcus mundtii* QU 25 †

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Abstract

Enterococcus mundtii QU 25, a non-dairy lactic acid bacterium, produces optically pure L-lactic acid (≥ 99.9%) via homo-fermentation when cultured in the presence of xylose at high concentrations. However, as the xylose concentration decreases, a metabolic shift to hetero-lactic fermentation occurs in this strain. Furthermore, this strain preferentially metabolizes glucose when cultured in medium containing high concentrations of both glucose and xylose, indicating that a previously uncharacterized carbon-catabolite repression system may govern the regulation of these processes. Therefore, to increase the productivity of pure L-lactate by QU 25, it is necessary to investigate this regulatory process. In this study, we performed transcriptional analyses, including RNA sequencing to analyze the transcriptome of QU 25 cultivated in the presence of various glucose and/or xylose concentrations. Our results demonstrate that there was a gradual reduction in the expression of several genes in the xylose gene cluster as the glucose concentration increased, and that there was robust transcription of the genes involved in hetero-lactic fermentation under homo-lactic fermentation conditions. The former result indicates that transcriptional regulation of genes in the xylose gene cluster is involved in the catabolite repression observed in QU 25. The latter results show that the metabolic shift between homo- and hetero-lactic fermentation in QU 25 is not caused by the transcriptional regulation of related genes under the conditions tested. We therefore propose that a yet uncharacterized transcriptional regulation process is involved in the observed catabolite repression.

1. Introduction

Xylose is a major component of renewable plant-derived biomass. This aldopentose is typically found in hemicellulose, which is present along with cellulose and lignin in biomass. Currently, efforts have been focused on expanding the types of biomass used as feedstock for bioconversion. After saccharification of plant-derived biomass, nonselective utilization of xylose and glucose is highly desired. Green plastic poly-L-(+)-lactic acid is comprised of optically pure L-lactic acid, which can be obtained only by microbial fermentation. Furthermore, L-lactic acid has a number of valuable commercial uses, including as a food preservative.[1]

Enterococcus mundtii QU 25 is a non-dairy lactic acid bacterium (LAB), isolated from ovine feces, that ferments three aldopentoses (arabinose, ribose and xylose).[2] This strain produces high levels of L-lactic acid when cultured under optimal conditions using glucose or xylose as the sole carbon source, and its use for bioconversion of plant-derived biomass has been proposed.[3, 4] Recently, we analyzed and annotated the complete 3.02 Mb genome sequence of QU 25,[5] and demonstrated that this organism encodes the genes necessary for xylose utilization and homo- and hetero-lactic fermentation (Table 1).

In a previous study, when QU 25 was cultured in medium containing 50 $g \cdot L^{-1}$ glucose and 50 $g \cdot L^{-1}$ xylose, both sugars were utilized simultaneously. Notably, however, while glucose was preferred over xylose, this strain did not exhibit a diauxic two-step growth curve. Specifically, the maximum glucose consumption rate of QU 25 was 5.72 $g \cdot L^{-1} \cdot h^{-1}$, while that of xylose was 1.17 $g \cdot L^{-1} \cdot h^{-1}$, and only 42.9% of the xylose was metabolized.[6] This phenomenon cannot be explained by the well-characterized mechanism of carbon catabolite repression (CCR) exhibited by low G+C count Gram-positive bacteria

(*Firmicutes*). In CCR, genes encoding transporters and enzymes involved in the metabolism of a particular sugar(s) are repressed in the presence of a preferred sugar such as glucose. Subsequent exhaustion of the preferred sugar, however, triggers transcription of the repressed genes followed by uptake and metabolism of the second sugar. This metabolic process results in a canonical diauxic growth curve (reviewed in 7). While it is possible that QU 25 employs a type of CCR when cultured in the presence of glucose, we previously demonstrated that this strain consumed 98.0% of the xylose with a similar consumption rate as glucose when cultured in medium containing 25 $g \cdot L^{-1}$ glucose and 50 $g \cdot L^{-1}$ xylose, which is inconsistent with this hypothesis.[6]

In a previous study, QU 25 produced optically pure (≥ 99.9%) L-lactic acid via homo-fermentation when cultured in media containing xylose as the sole carbon source (100 $g \cdot L^{-1}$), and it was predicted that this process involved the pentose-phosphate (PP)/glycolytic pathway.[4] While no phosphoketolase (PK) activity, which comprises the first step in the hetero-lactic fermentative PK pathway, was detected at high xylose concentrations,[4] a metabolic shift to hetero-lactic fermentation was observed at concentrations less than 25 $g \cdot L^{-1}$, which was accompanied by the formation of byproducts, such as acetic acid, formic acid, and ethanol, as well as PK activity, and the activity of enzymes involved in the PP/glycolytic pathway.[4]

To increase the yield of pure L-lactate produced by QU 25 from biomass, it is crucial to investigate the CCR-like phenomenon that governs xylose metabolism and the metabolic shift to hetero-lactic fermentation employed by this strain. In this study, we utilized RNA sequencing (RNA-seq) to analyze the transcriptome of QU 25 in the presence of varying concentrations of glucose and xylose, as well as Northern hybridization analysis to assess

the expression of genes involved in xylose fermentation, and primer extension analysis of the xylose gene cluster to determine the transcriptional start site (TSS) of the xylose isomerase gene (*xylA*). Using these approaches, we demonstrate that transcriptional regulation is involved in the CCR observed in QU 25. However, the metabolic shift of QU 25 was not mediated by the transcriptional regulation of xylose metabolism-related genes under the conditions tested.

2. Results

2.1 RNA-seq analysis of QU 25 cultured under low sugar conditions

A previous study demonstrated that CCR of xylose utilization genes does not occur in the QU 25 strain when cultured in media containing xylose and glucose if the glucose concentration is below 25 $g \cdot L^{-1}$.[6] In addition, this strain exhibits hetero-lactic fermentation in the presence of low concentrations ($\leq 25\ g \cdot L^{-1}$) of xylose as a sole carbon source.[4] Therefore, to examine the transcriptome of this strain when cultured in the presence of low sugar concentrations, we performed RNA-seq analysis on QU 25 cultured in G5, X5, and G1X5 media (see Experimental 4.1). The results of this analysis are summarized in Table 2 (genes involved in xylose metabolism and CCR) and Supplementary Table S2 (all genes). Growth curves for cells cultured under these conditions are depicted in Supplementary Fig. S1. The results demonstrate that QU 25 grew more slowly in the X5 medium than in all other media evaluated.

2.1.1 Genes in the xylose gene cluster As shown in Table 2, high levels of transcription of the *xylA*, *xynB*, *araG*, and *xylB* genes, which are encoded in the xylose gene

cluster, were observed when QU 25 was cultured in the presence of 5 $g \cdot L^{-1}$ xylose (reads per kb of exon per million mapped reads [RPKM]; values ranged between 2,270.88 and 16,096.79). Furthermore, the expression levels of these genes were markedly higher when cultured in the X5 medium than in the G5 medium, as the X5/G5 values (generated by dividing the RPKM values obtained from cells cultured in the X5 medium by those obtained from cells cultured in the G5 medium) ranged from 10.83 to 18.87, indicating that expression of these loci was highly induced by xylose. Meanwhile, the G1X5/G5 expression values ranged from 8.04 to 28.30, demonstrating that the presence of glucose in the G1X5 medium did not affect the expression levels of these four genes. Conversely, the *xylR* gene, which was annotated as a ROK family xylose repressor[8] gene and is divergently transcribed from the other genes in the cluster (see Fig. 2), was neither induced by xylose nor repressed by glucose. The RPKM values obtained for this gene ranged between 122.39 and 214.78, and the X5/G5 and G1X5/G5 values were 1.75 and 1.60, respectively.

2.1.2 Genes in the PP/glycolytic and PK pathways In contrast to the xylose cluster, there was no discernible change in the expression of the genes involved in the PP/glycolytic pathway and the PK pathway, with the exception of *adhE*, under the sugar conditions tested; the X5/G5 and G1X5/G5 values for these genes ranged between 0.32–2.66 and 0.33–1.52, respectively (Table 2). Conversely, the *adhE* gene exhibited high X5/G5 (7.70) and G1X5/G5 values (3.46). Moreover, there was marked expression of the genes of both pathways in the X5 medium except for *tktA3*, a paralog of *tktA1* (RPKM values from 201.08–11,246.12), which is consistent with a previous report demonstrating that QU 25 exhibited hetero-lactic fermentation in the presence of xylose concentrations lower than 25 $g \cdot L^{-1}$.[4]

2.1.3 Genes involved in CcpA-dependent CCR Lastly, we examined the transcriptional levels of the genes encoding catabolite control protein A (CcpA), histidine-containing protein (HPr), and HPr kinase/phosphorylase, which are commonly involved in the CcpA-dependent CCR pathway of *Firmicutes* in response to glucose.[7] In strain QU 25, there was robust and constitutive expression of the *ptsH* gene (RPKM = 4,088.60–7,039.75), which encodes HPr, compared to that of the genes in the xylose gene cluster when cultured in the presence of xylose (X5/G5 and G1X5/G5 = 0.58 and 0.97, respectively). While the RPKM values from the *ccpA* and *hprK* (encoding HPr kinase/phosphorylase) genes were considerably lower than that from *ptsH* (167.39–535.85), these loci were also transcribed constitutively (X5/G5 and G1X5/G5 = 0.65–1.57), including the X5/G5 value for *ccpA* (1.57) (Table 2).

2.2 Northern hybridization analysis of the xylose gene cluster and identification of the *xylA* TSS under low sugar conditions

To confirm the results of our RNA-seq analysis, we performed Northern hybridization to examine the expression levels of the genes in the xylose gene cluster under identical culturing conditions.

As shown in Fig. 1A, the *xylA* probe detected three mRNA molecules expressed by QU 25 that were approximately 1.3, 6.4, and 10 kilobases (kb) in size, when cultured in X5 and G1X5 media. However, this probe did not detect any transcripts in the G5 medium, indicating that the transcripts detected were induced by xylose and not repressed by glucose in the medium containing both sugars. These results were therefore consistent with the gene expression patterns obtained by our RNA-seq analysis. The 10 kb mRNA molecule that was

detected by the *xylA* probe was also detected in the QU 25 RNA samples by the *xynB*, *araG*, and *xylB* probes after culturing in the X5 and G1X5 media. Likewise, the 6.4 kb mRNA was detected by the *xylA*, *xynB*, and *araG* probes, but not by the *xylB* probe, in these samples. Given that the *xylA* coding sequence is 1,308 base pairs (bp) in length (see Table 1), the two larger mRNA molecules were likely polycistronic mRNAs, while the 1.3 kb mRNA contained only the *xylA* sequence. In addition to the 10 kb band, the *xylB* probe detected a 5.7 kb mRNA in the samples from cells cultured in the X5 and G1X5 media, which was larger than the *xylB* gene (1,485 bp in length; Table 1). Therefore, *xylB* seems to also be transcribed as another operon that does not include *xylA*, *xynB*, or *araG*. Lastly, the *xylR* probe detected a single 1.1 kb mRNA in the samples obtained from all three cultures. As the *xylR* gene is 1,152 bp in length (see Table 1), this finding is consistent with the monocistronic transcription of this gene. Furthermore, the transcriptional level of *xylR* in the G5 medium was less than that observed in the other two xylose-containing media, which also corresponds to the results of the RNA-seq analysis.

Using primer extension analysis, we determined that the TSS of the *xylA* gene is a thymidine (T) that is 63 bp upstream of the *xylA* translational start codon (Fig. 1B and 1C). Within the whole genome sequence, this thymidine is base 2,917,497. As such, the 10 kb transcript detected by Northern hybridization is estimated to start at this position and to terminate near the end of the EMQU_2802 (base 2,906,796) locus, a region encompassing the *xynB*, *araG*, EMQU_2807, EMQU_2806, *xylB*, EMQU_2804, and EMQU_2803 genes (Fig. 2). Similarly, the 6.4 kb mRNA is predicted to comprise the sequences encoded at the locus between this TSS and near the end of the EMQU_2806 (base 2,910,773), which includes the *xynB*, *araG*, and EMQU_2807 genes. The predicted promoter region of *xylA* is

192 indicated by double bars in Fig. 1C. Based on the results of the Northern hybridization and
193 primer extension analyses, we deduced the structure of the operon encoding the xylose gene
194 cluster (Fig. 2).
195
196 **2.3 RNA-seq analysis of QU 25 cultured under high sugar conditions**
197 In previous analyses, QU 25 exhibited CCR when cultivated under high sugar conditions
198 (i.e. consumed glucose faster than xylose and left a portion of the xylose unused), such as in
199 medium containing a 50 g L^{-1} glucose and 50 g L^{-1} xylose mixture.[6] QU 25 also exhibited
200 homo-lactic fermentation when cultured with 100 g·L^{-1} of xylose as the sole carbon source.[4]
201 Therefore, to examine the gene expression profile of QU 25 under high sugar conditions, we
202 performed a second RNA-seq analysis of cells cultured in the following media: G150, X150,
203 G10X50, G25X50, G50X50, and G100X50 (see Experimental 4.1). Table 3 summarizes the
204 data regarding the genes involved in xylose metabolism and CCR while Supplementary
205 Table S3 contains the data for all genes. The growth curves of cells under the described
206 conditions are shown in Supplementary Fig. S2. The results show that QU 25 grew more
207 slowly in the X150 medium, compared to all other media evaluated.
208 **2.3.1 Genes in the xylose gene cluster** As shown in Table 3, there was robust
209 expression of all genes in the xylose gene cluster when QU 25 was cultured in the X150
210 medium (RPKM values for these genes ranged from 387.34–6,503.77).
211 However, a gradual reduction in the transcriptional expression levels of *xylA*, *xynB*, and
212 *araG* occurred in a glucose concentration-dependent manner. Indeed, while the
213 G10X50/G150 values for these genes ranged from 107.22 to 208.62, these values decreased
214 to 51.45–106.20 (G25X50/G150), 41.08–82.09 (G50X50/G150), and 8.62–37.19

(G100X50/G150) as the glucose concentration increased. Meanwhile, the RPKM values for the *xylB* gene were nearly identical in the G10X50 and X150 media (1,851.55 and 1,930.46, respectively); however, as the glucose concentration increased, the relative values for this gene exhibited a reduction similar to that observed with the *xylA*, *xynB*, and *araG* genes. Specifically, the G10X50/G150, G25X50/G150, G50X50/G150, and G100X50/G150 values were 44.82, 21.01, 14.41, and 2.74, respectively. It is therefore conceivable that glucose or a glucose metabolite negatively regulates transcription of the xylose gene cluster, resulting in the CCR effect observed in QU 25. Notably, the transcriptional levels of the *xylR* gene, which was annotated as a repressor, were nearly constant regardless of the glucose concentration of the medium (G10X50/G150, G25X50/G150, G50X50/G150, and G100X50/G150 = 2.78, 2.99, 2.79, and 3.22, respectively).

2.3.2 Genes in the PP/glycolytic and PK pathways As shown in Table 3, there was robust expression of all genes in the PP/glycolytic and PK pathways when QU 25 was cultured in X150 medium (RPKM values for these genes ranged from 13.33–4,982.09). Of the genes in the PP/glycolytic pathway, the X150/G150 values for the *tktA1*, *tktA3*, and *talC* genes were between 2.65–5.08, and the expression levels of each gene were highest in the X150 medium (RPKM =507.90, 13.33, and 81.77, respectively). However, there was stable expression of these genes, regardless of the glucose concentration, in mixed media.

Similar to the genes of the PP/glycolytic pathway, the highest expression levels of the PK pathway genes *xfpA* and *ack*, which are involved in hetero-lactic fermentation, were observed in the X150 medium (RPKM = 256.22 and 1,463.87, respectively), and the transcript levels of these genes were not affected by changes in the glucose concentration. Conversely, while *eutD* and *pflB* also exhibited the highest transcript levels in the X150

medium (RPKM = 1,676.58 and 4,982.09, respectively), the expression of these genes decreased in a manner similar to that of the xylose gene cluster, albeit more moderately, as the glucose concentration in the medium increased. The highest expression level of the *adhE* gene was observed in the G10X50 medium (RPKM = 4,204.20), and the relative RPKM values in the other xylose-containing media (compared to that in the G150 medium) were between 1.26 and 2.67. It was noteworthy that multiple genes, including *xfpA*, demonstrated such robust expression in the X150 medium despite the fact that QU 25 exhibited homo-lactic fermentation. Furthermore, no phosphoketolase activity was previously detected in the presence of high (100 $g \cdot L^{-1}$) xylose concentration.[4]

2.3.3 Genes involved in CcpA-dependent CCR Concerning the expression of the three CCR-related genes, the transcript levels of *ptsH* were consistently high (RPKM = 6,777.32–10,533.67). In contrast, the transcriptional levels of *ccpA* and *hprK* were markedly lower (RPKM = 356.97–829.44) and were similar to the expression levels of these genes under low sugar conditions. Meanwhile, the relative values of each were between 0.89 and 1.64, including the X150/G150 value for *ccpA* (1.14) (Table 3).

2.3.4 Aldopentose transporter genes Notably, we found that several aldopentose transporter genes were significantly repressed under high glucose conditions, as determined by RNA-seq. These transporters consisted of three sets of gene clusters encoding the D-xylose, ribose, and L-arabinose transport system proteins, as well as another three ribose transport-related genes, EMQU_1846, EMQU_2382, and EMQU_2381 (Tables 1 and 4). In particular, the EMQU_2808–2806 ribose transporter gene set, which includes the *araG2* (EMQU_2808) located in the xylose gene cluster (Fig. 2), exhibited high levels of expression in X150 and G10X50 media (RPKM = 1,882.41–2,921.43 and 1,589.56–

2,555.99, respectively). However, the expression of these genes was gradually repressed in a glucose concentration-dependent manner (G10X50/X150 = 0.81–0.87, G25X50/X150 = 0.31–0.39, G50X50/X150 = 0.26–0.31, and G100X50/X150 = 0.03–0.07) (Table 4). Furthermore, the G150/X150 values of these genes (0.01) were the lowest of all genes examined (Table 4). Meanwhile, the other two transport systems and the three ribose transport-related genes exhibited low transcript levels (RPKM = 2.89–109.57) in media containing both glucose and xylose.

2.4 Northern hybridization analysis of *xylA* under high sugar conditions

To confirm these results, we again performed Northern hybridization analysis of *xylA* gene expression using RNA samples harvested from cells cultivated in G10X50, G25X50, G50X50, and G100X50 media. As observed under low sugar conditions, the *xylA* probe detected three mRNA species (1.3, 6.4, and 10 kb in size, respectively) after culturing in the high xylose-containing media (Fig. 3A), and there was a gradual reduction in the densities of these bands as the glucose concentration in the medium increased (Fig. 3B). These results are therefore consistent with the RNA-seq data summarized in Tables 3 and S3 for all genes, indicating the reproducibility and accuracy of our results.

3. Discussion

In this study, we utilized RNA-seq analysis to examine the gene expression profile of *E. mundtii* QU 25, which is an efficient L-lactic acid-producing LAB in the presence of varying glucose and xylose concentrations. Furthermore, we performed Northern

hybridization analysis to assess the expression of genes involved in the xylose gene cluster as well as their transcriptional activity. The results of the Northern analysis (Figs. 1 and 3) were consistent with those of the RNA-seq analyses (Tables S2 and S3 for all genes), indicating the reproducibility and accuracy of our results. Moreover, our results, particularly those of the RNA-seq analysis of QU 25 cultured under high sugar conditions, indicate that i) the CCR observed previously in QU 25[6] was controlled at the transcriptional level, while ii) the metabolic shift between homo- and hetero-lactic fermentation, according to the initial concentration of xylose as a sole carbon source in the media, was not under transcriptional control under the conditions tested in this study.

Fujita categorized CCR in *Firmicutes* into two groups: CcpA-dependent and CcpA-independent CCR.[7] The former involves the CcpA complex including the seryl-phosphorylated form of HPr (P-Ser-HPr), which is a negative repressor. This complex then binds to specific sites (*cre* sites) near promoter regions that function as operators to regulate transcriptional expression. In this typical CCR, the bacterium exhibits a diauxic two-step growth curve. However, CcpA-independent CCR mechanisms involve either the transcriptional repressors CggR or CcpN, or the histidyl-phosphorylated form of HPr (P-His-HPr), which is involved in the phosphotransferase transport system (PTS) (reviewed in 7). However, *cggR* and *ccpN* are not present in the QU 25 genome, indicating that this CCR mechanism may not occur in this organism.

Therefore, we examined the possibility that CcpA-dependent CCR may regulate xylose and glucose utilization in QU 25. We identified a putative *cre* sequence (ATGAAAGCGTATACTA) in the *xylA* promoter region, indicated by a dashed line in Fig. 1C, that exhibits 92% identity (excluding Ns) with the consensus *cre* sequence

(WTG$_3$NNARC$_8$G$_9$NWWWC$_{14}$AW, where W stands for A or T, R for A or G, and N for any base).[9] Notably, however, G_3, C_8, G_9, and C_{14} are the only bases that are completely conserved within experimentally verified *cre* sites.[9] The sequence identified in this study also contains these four conserved bases, which supports the notion that this region is a *cre* site. Furthermore, the 3′-end of this sequence included the -10 region of the predicted *xylA* promoter. It is therefore possible that CcpA is involved in the CCR-mediated regulation of xylose utilization by QU 25. In a previous study, however, Zomer *et al*. (2007) reported several genes that contained a putative *cre* site but did not exhibit observable CCR regulation.[10] Therefore, it is also conceivable that the *xylA* gene in QU 25 might be an example of such a gene.

Next, we examined the phenomenon reported by Asanuma *et al*. (2004) that the transcriptional expression level of *ccpA* was higher in media containing a more rapidly utilizable energy source in *Streptococcus bovis* that exhibits CcpA-dependent CCR.[11] In contrast to these findings, there were similar expression levels of *ccpA* in QU 25 cultured under low or high sugar conditions (X5/G5 and X150/G150 = 1.57 and 1.14, respectively; Tables 2 and 3). Moreover, QU 25 grew faster in media containing glucose only than in media containing xylose (Supplementary Figs. S1 and S2). Therefore, the putative involvement of CcpA in the CCR of QU 25 was unclear.

It was evident from our findings that the genes involved in xylose transport and metabolism were transcribed in media containing a mixture of glucose and xylose. However, the transcript levels of these genes decreased as the glucose concentration of the medium increased. These observations, as well as the simultaneous consumption of glucose and xylose by QU 25, are not consistent with a model of hierarchical metabolism of these sugars

via a CcpA-dependent CCR pathway. Therefore, we propose the existence of a CcpA-independent CCR mechanism in QU 25 that regulates the simultaneous metabolism of xylose and glucose. There is precedence for this phenomenon, as Mortera *et al*. (2012) reported the coexistence of both CcpA-dependent and CcpA-independent CCR mechanisms in *Enterococcus faecalis*.[12]

Notably, QU 25 harbors more genes that encode transporters related to lactic acid fermentation, including both ABC transporters and a PTS, than other enterococcal species or even the other *E. mundtii* strain.[13] Xylose is a member of the aldopentose group, which includes arabinose, ribose, and xylose. In QU 25, the aldopentose transporter genes were highly repressed under high glucose conditions (Table 4). Furthermore, the ribose ABC transporter genes in the xylose gene cluster (EMQU_2806–2808) were highly transcribed in the presence of xylose but were gradually repressed as the glucose concentration increased, indicating that these proteins could participate in the CCR of QU 25. That these genes exhibited the lowest G150/X150 values among the transporter genes examined (Table 4) strengthens this supposition. Conversely, no PTS genes specific to aldopentose transport were identified in QU 25. We therefore predict that the ribose ABC transporter genes in the xylose gene cluster contribute to the CCR observed in QU 25 under high glucose conditions, and we summarize a model for the xylose fermentation pathway of QU 25 in Fig. 4.

We propose that the metabolic shift between homo- and hetero-lactic fermentation in the presence of xylose as a sole carbon source is regulated in QU 25 downstream of the transcriptional level. This conclusion is supported by our findings that transcription of the genes involved in the PK pathway was maintained under homo-fermentation conditions, indicating that the shift to hetero-lactic fermentation is not under transcriptional control;

however, the mechanism governing this switch remains unclear. For efficient production of optically pure lactic acid, homo-lactic fermentation of pentoses and hexoses is highly desired. In the case of pentoses, such as xylose, up to five moles of lactic acid are synthesized from three moles of xylose via the homo-fermentative PP/glycolytic pathway. As such, the theoretical yield of lactic acid produced per xylose consumed is 1.67 mol/mol. In a previous study, the maximal yield of L-lactic acid produced by QU 25 was 1.51 mol/mol when cultured with 50 $g \cdot L^{-1}$ xylose.[4] Considering that sugars are used to increase cell mass rather than to produce lactate during exponential growth of bacteria, this is a remarkable yield and strengthens the importance of QU 25 in the green plastic industry.

The role of the XylR repressor cannot be ignored when investigating the transcriptional regulation of the xylose gene cluster. Like other known xylose gene clusters that are repressed by XylR in the absence of xylose,[14] the expression of the genes within the xylose gene cluster of QU 25 was also repressed in the absence of xylose and induced in its presence. The putative XylR-binding site, GTTAGTTTGTTAGATAAACTAAC, which is indicated by a dotted line in Fig. 1C, exhibited 95% identity (excluding Ns) to the consensus sequence GTTWGTTTATNNNATAAACWAAC among all *Firmicutes*, except *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactococcus lactis*, and *Clostridium acetobutylicum*.[15] However, the position of the first base in this site in QU 25 was at +7, which is markedly further downstream than that of other XylR regulons in *Firmicutes*.[15, 16] Dahl *et al*. (1995) reported that XylR contributes to glucose repression because glucose 6-phosphate is an anti-inducer that prevents xylose-mediated induction in *Bacillus subtilis, B. megaterium*, and *B. licheniformis*.[17] Accordingly, this possibility should also be considered for QU 25.

4. Experimental

4.1 Bacterial strain and culturing conditions

E. mundtii QU 25, isolated from ovine feces,[2] was cultured in M17-2 medium (M17 medium lacking yeast extract and lactose)[18] supplemented with various concentrations of glucose and/or xylose at 37°C. Media were named according to the type and concentration of sugars used: 5 $g \cdot L^{-1}$ glucose (G5); 150 $g \cdot L^{-1}$ glucose (G150); 5 $g \cdot L^{-1}$ xylose (X5); 150 $g \cdot L^{-1}$ xylose (X150); 1 $g \cdot L^{-1}$ glucose and 5 $g \cdot L^{-1}$ xylose (G1X5); 10 $g \cdot L^{-1}$ glucose and 50 $g \cdot L^{-1}$ xylose (G10X50); 25 $g \cdot L^{-1}$ glucose and 50 $g \cdot L^{-1}$ xylose (G25X50); 50 $g \cdot L^{-1}$ glucose and 50 $g \cdot L^{-1}$ xylose (G50X50); and 100 $g \cdot L^{-1}$ glucose and 50 $g \cdot L^{-1}$ xylose (G100X50).

4.2 RNA preparation

Logarithmic phase cultures (OD_{600} of 0.8) were utilized for RNA extraction. Cultures (8 mL) were collected by centrifugation and frozen at -80°C. Total RNA was then extracted using the QIAGEN RNeasy Mini Kit (Qiagen, Venlo, Netherlands).

4.3 RNA-seq

The total RNA from each culture was prepared for analysis as follows: rRNA was removed from each sample using the Ribo-Zero™ Magnetic Kit for Gram-Positive Bacteria (Epicentre [an Illumina company], Madison, WI, USA) and cDNA libraries were generated using the

NEBNext mRNA Library Prep Reagent Set for Illumina (New England BioLabs, Ipswich, MA, USA). Libraries prepared from cultures under low sugar conditions (Table 2) were then analyzed using the GAIIx Next-Generation Sequencing (NGS) platform (Illumina, San Diego, CA, USA) by 50-base-paired-end sequencing. Meanwhile, libraries prepared from cultures under high sugar conditions (Table 3) were analyzed using the HiSeq 2000 system (Illumina) by 50-base-single-end sequencing. Each sequence was mapped to the QU 25 genome (deposited in GenBank/DDBJ/EMBL under accession numbers AP013036–AP013041) using the CLC Genomics Workbench software version 7.0.4 (length fraction = 0.5, similarity fraction = 0.8, maximum number of hits for a read = 1) (CLC bio, Aarhus, Denmark). Mapped reads of each gene were normalized using the RPKM approach.[19]

4.4 Northern hybridization analysis

Chemically labeled RNA probes were prepared using the DIG RNA Labeling Kit (SP6/T7) (Roche, Basel, Switzerland) by *in vitro* transcription using T7 RNA polymerase of the cloned region into the pSPT18 vector. Oligonucleotide primers used for PCR amplification of the corresponding DNA fragments for cloning into the vector were synthesized by Hokkaido System Science Co., Ltd. (Sapporo, Japan; shown in Supplementary Table S1). RNA (10 μg) was denatured by incubation at 65°C for 10 min and then subjected to agarose gel electrophoresis on a 1% agarose gel containing 2.22% formaldehyde and 1X 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (20 mM MOPS, 1 mM ethylenediaminetetraacetic acid [EDTA], and 5 mM sodium acetate [pH 7.0]). Perfect RNA Markers (0.2–10 kb; Novagen [a brand of Merck KGaA], Darmstadt, Germany) was used as

419 a size marker. The RNA was then transferred to Hybond N^+ membranes (GE Healthcare,
420 Little Chalfont, United Kingdom) by capillary action using 20X SSC (1X SSC = 0.15 M
421 NaCl and 0.015 M sodium citrate) as transfer buffer and by placing a weight on the
422 gel-contacted membrane and incubating at room temperature (r. t.) overnight. Hybridization
423 was performed at 60°C overnight using DIG Easy Hyb Granules (Roche), followed by
424 washing twice at r.t. for 5 min with 2X SSC-0.1% SDS solution. This was then followed by
425 another two washes at 60°C for 15 min with 0.5X SSC-0.1% SDS solution. An
426 anti-Digoxigenin-AP Conjugate (Roche) treatment was then performed and the membrane
427 was washed twice at r.t. for 15 min with 2X SSC-0.1% SDS solution. The density of each
428 band was quantified using Image Lab™ software (version 2.0; Bio-Rad, Hercules, CA,
429 USA).

430

431 **4.5 Primer extension method for identification of the *xylA* TSS**

432 RNA was extracted from 8 mL of cells cultured in M17-2 medium supplemented with 5 $g \cdot L^{-1}$
433 xylose. Primer extension analysis was performed using *Bca*BEST™ RNA PCR Kit Ver.1.1
434 (Takara Bio Inc., Otsu, Japan) by annealing 10 pmol of IRDye800-labeled primer (MS
435 TechnoSystems, Osaka, Japan) to 15 μg of RNA. This was followed by incubation at 58 °C
436 for 1 h. The GATC sequence ladder was produced using the DNA fragment that was
437 amplified by PCR using two oligonucleotide primers (Supplementary Table S1), the same
438 primer described above, and Ex Taq DNA polymerase (Takara Bio). Following an initial
439 5-min denaturation at 95°C, 30-cycle amplifications were performed with denaturation at 95°C
440 for 30 sec, annealing at 55°C for 30 sec, and extension at 70°C for 1 min. A 6% Long Ranger

441 running gel (Long Ranger™ Gel Solutions [Lonza], Basel, Switzerland) was used for
442 analysis.

443

444 **4.6 RNA-seq data accession numbers**

445 The RNA-seq data obtained in this study were deposited in the DNA Data Bank of Japan
446 Sequence Read Archive (DRA)/National Center for Biotechnology Information Sequence
447 Read Archive (SRA)/European Bioinformatics Institute Sequence Read Archive (ERA)
448 under the accession number DRA002964 .

449

450

451 **5. Conclusion**

452 In this paper, we examined the atypical CCR mechanism employed by the QU 25 strain
453 under high glucose conditions, and provided evidence that the transcriptional regulation of
454 the genes in the xylose gene cluster played a role in this CCR process. We propose that this
455 regulation pathway involves a CcpA-independent mechanism. Furthermore, we speculate
456 that the ribose ABC transport system encoded in the xylose gene cluster may contribute to
457 this process. We also demonstrated that the observed metabolic shift between homo- and
458 hetero-lactic fermentation in QU 25 is regulated downstream of the transcriptional
459 expression of the enzymes involved in xylose metabolism.

460

461

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505 **Table 1** Feature IDs, gene names, chromosomal regions, strand directions, GC%, and predicted function of the gene

506 products described in this study

Feature ID	Gene name	Chromosomal region		Strand direction	GC (%)	Predicted function
		Start	End			
Genes concerned in xylose fermentation and carbon catabolite repression (CCR) (examined in Tables 2 and 3)						
Xylose gene cluster						
EMQU_2811	*xylR*	2917694	2918845	+	40.54	xylose repressor
EMQU_2810	*xylA*	2916127	2917434	-	41.13	xylose isomerase
EMQU_2809	*xynB*	2914407	2916023	-	42.86	xylan β-1,4-xylosidase
EMQU_2808	*araG*	2912855	2914366	-	40.41	ribose transport system ATP-binding protein
EMQU_2805	*xylB*	2909196	2910680	-	44.18	D-xylulose kinase
Pentose-phosphate/glycolytic pathway						
EMQU_1275	*tktA1*	1334628	1336622	+	45.61	transketolase

EMQU_2812	*tktA3*	2918972	2920966	-	46.22	transketolase
EMQU_2814	*talC*	2921874	2922527	-	44.19	transaldolase
Phosphoketolase pathway						
EMQU_1837	*xfpA* (*ptk*)[a]	1913814	1916189	-	43.73	D-xylulose 5-phosphate/D-fructose 6-phosphate phosphoketolase
EMQU_2620	*ackA*	2690711	2691892	-	37.23	acetate kinase
EMQU_2119	*eutD* (*pta*) [a]	2204462	2205442	-	39.45	phosphotransacetylase
EMQU_1120	*pflB*	1175401	1177632	+	40.37	formate acetyltransferase
EMQU_0224	*adhE*	231590	234187	+	40.99	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
CCR proteins						
EMQU_0954	*ptsH*	989353	989619	+	38.95	PTS system transporter protein HPr
EMQU_1943	*ccpA*	2032634	2033635	-	38.42	catabolite control protein A

EMQU_1951	*hptK*	2039941	2040879	-	42.07	HPr kinase/phosphorylase
Aldopentose transporter genes (examined in Table 4)						
D-xylose transport system proteins						
EMQU_1845		1925107	1926198	-	39.93	D-xylose transport system substrate-binding protein
EMQU_1844	*araG*	1923280	1924812	-	40.77	D-xylose transport system ATP-binding protein
EMQU_1843	*araH*	1922078	1923280	-	42.39	D-xylose transport system permease protein
Ribose transport system proteins (included in xylose gene cluster)						
EMQU_2808	*araG*	2912855	2914366	-	40.41	ribose transport system ATP-binding protein
EMQU_2807		2911848	2912813	-	39.03	ribose transport system permease protein
EMQU_2806		2910773	2911822	-	41.52	ribose transport system substrate-binding protein
Other ribose transport system proteins						
EMQU_1846		1926425	1927390	+	39.65	ribose transport system substrate-binding protein
EMQU_2382		2453523	2453918	-	38.64	ribose transport protein RbsD
EMQU_2381		2452618	2453505	-	41.78	ribose uptake protein

L-arabinose transport system proteins						
EMQU_1582		1640343	1641632	-	41.01	lactose/L-arabinose transport system substrate-binding protein
EMQU_1581	*lacF2*	1639118	1639987	-	35.29	lactose/L-arabinose transport system permease protein
EMQU_1580	*lacG2*	1638243	1639121	-	37.77	lactose/L-arabinose transport system permease protein

507 [a]Aliases are described in parenthesis.

508 **Table 2** RNA-seq data for genes involved in xylose fermentation and carbon catabolite repression (CCR) under low sugar conditions

Gene name	RPKM[a] (G5)	RPKM (X5)	RPKM (G1X5)	X5/G5[b]	G1X5/G5[b]
Xylose gene cluster					
xylR	122.39	214.78	195.41	1.75	1.60
xylA	856.04	16,096.79	13,383.75	18.80	15.63
xynB	594.43	10,023.59	12,473.84	16.86	20.98
araG	323.81	6,110.76	9,164.44	18.87	28.30
xylB	282.55	3,059.00	2,270.88	10.83	8.04
Pentose-phosphate/glycolytic pathway					
tktA1	120.15	201.08	116.81	1.67	0.97
tktA3	37.04	11.68	12.40	0.32	0.33
talC	218.56	208.95	160.38	0.96	0.73
Phosphoketolase pathway					

xfpA	158.58	421.98	196.78	2.66	1.24
ackA	920.55	595.13	483.45	0.65	0.53
eutD	716.65	585.16	895.70	0.82	1.25
pflB	4,501.62	11,246.12	6,833.48	2.50	1.52
adhE	1,089.53	8,384.88	3,772.75	7.70	3.46
CCR proteins					
ptsH	7,039.75	4,088.60	6,796.87	0.58	0.97
ccpA	209.62	329.06	167.39	1.57	0.80
hptK	504.83	327.44	535.85	0.65	1.06

[a]RPKM stands for reads per kb of exon per million mapped reads.

[b]X5/G5 and G1X5/G5 indicate the RPKM (X5)/RPKM (G5) and RPKM (G1X5)/RPKM (G5) values, respectively.

512 **Table 3** RNA-seq data for genes involved in xylose fermentation and carbon catabolite repression (CCR) under high sugar conditions

Gene name	RPKM[a] (G10X50)	RPKM (G25X50)	RPKM (G50X50)	RPKM (G100X50)	RPKM (G150)	RPKM (X150)	G10X50/ G150[b]	G25X50/ G150[b]	G50X50/ G150[b]	G100 X50/ G150[b]	X150 /G150[b]
Xylose gene cluster											
xylR	285.70	306.97	286.65	330.83	102.60	387.34	2.78	2.99	2.79	3.22	3.78
xylA	4,645.56	2,364.84	1,828.10	828.19	22.27	6,503.77	208.62	106.20	82.09	37.19	292.06
xynB	2,888.53	1,581.22	1,264.01	420.03	19.65	3,328.79	146.99	80.47	64.32	21.38	169.40
araG	1,837.77	881.84	704.14	147.71	17.14	2,265.47	107.22	51.45	41.08	8.62	132.17
xylB	1,851.55	868.18	595.27	113.08	41.31	1,930.46	44.82	21.01	14.41	2.74	46.73
Pentose-phosphate/glycolytic pathway											
tktA1	364.42	490.99	462.83	333.90	191.32	507.90	1.90	2.57	2.42	1.75	2.65
tktA3	7.36	5.50	4.49	4.70	2.63	13.33	2.80	2.09	1.71	1.79	5.08
talC	31.94	14.78	20.72	13.77	23.35	81.77	1.37	0.63	0.89	0.59	3.50

Phosphoketolase pathway											
xfpA	110.50	93.15	137.87	147.80	135.94	256.22	0.81	0.69	1.01	1.09	1.88
ackA	973.39	1,150.10	922.43	1,129.22	954.51	1,463.87	1.02	1.20	0.97	1.18	1.53
eutD	1,487.04	1,435.64	1,242.74	1,210.44	1,170.24	1,676.58	1.27	1.23	1.06	1.03	1.43
pflB	3,903.36	3,811.22	2,596.54	1,900.55	1,211.73	4,982.09	3.22	3.15	2.14	1.57	4.11
adhE	4,204.20	1,986.76	2,483.65	2,388.84	1,577.48	2,582.87	2.67	1.26	1.57	1.51	1.64
CCR proteins											
ptsH	9,658.17	10,533.67	9,079.38	9,383.88	7,610.33	6,777.32	1.27	1.38	1.19	1.23	0.89
ccpA	631.45	829.44	800.99	737.95	506.26	577.99	1.25	1.64	1.58	1.46	1.14
hptK	390.59	356.97	418.84	412.58	371.45	374.78	1.05	0.96	1.13	1.11	1.01

513 [a]RPKM stands for reads per kb of exon per million mapped reads.

514 [b]G10X50/G150, G25X50/G150, G50X50/G150, G100X50/G150, and X150/G150 indicate the RPKM (G10X50)/RPKM (G150), RPKM

515 (G25X50)/RPKM (G150), RPKM (G50X50)/RPKM (G150), RPKM (G100X50)/RPKM (G150), and RPKM (X150)/RPKM (G150)

516 values, respectively.

517

518 **Table 4** RNA-seq data for the aldopentose transporter genes that are highly repressed under high sugar conditions

Feature ID	RPKM[a] (G10X50)	RPKM (G25X50)	RPKM (G50X50)	RPKM (G100X50)	RPKM (G150)	RPKM (X150)	G10 X50/ X150[b]	G25 X50/ X150[b]	G50 X50/ X150[b]	G100 X50/ X150[b]	G150/ X150[b]
D-xylose transport system proteins											
EMQU_1845	46.11	3.50	7.23	5.43	8.25	107.76	0.43	0.03	0.07	0.05	0.08
EMQU_1844	20.10	4.01	7.38	6.86	8.20	70.03	0.29	0.06	0.11	0.10	0.12
EMQU_1843	17.96	4.85	10.99	8.41	12.83	72.50	0.25	0.07	0.15	0.12	0.18
Ribose transport system proteins (included in xylose gene cluster)											
EMQU_2808	1,837.77	881.84	704.14	147.71	17.14	2,265.47	0.81	0.39	0.31	0.07	0.01
EMQU_2807	1,589.56	576.10	490.50	76.12	17.79	1,882.41	0.84	0.31	0.26	0.04	0.01
EMQU_2806	2,555.99	993.96	750.61	95.35	22.27	2,921.43	0.87	0.34	0.26	0.03	0.01
Other ribose transport system proteins											
EMQU_1846	31.26	20.64	21.16	23.61	20.06	47.03	0.66	0.44	0.45	0.50	0.43

EMQU_2382	107.58	81.37	81.75	84.64	261.49	224.77	0.48	0.36	0.36	0.38	1.16
EMQU_2381	109.57	73.36	83.26	93.44	252.13	212.93	0.51	0.34	0.39	0.44	1.18
L-arabinose transport system proteins											
EMQU_1582	7.78	4.21	7.16	6.98	12.10	31.92	0.24	0.13	0.22	0.22	0.38
EMQU_1581	4.99	2.89	5.67	5.81	6.35	16.04	0.31	0.18	0.35	0.36	0.40
EMQU_1580	5.41	5.04	6.50	8.46	9.17	20.64	0.26	0.24	0.31	0.41	0.44

519 [a]RPKM stands for reads per kb of exon per million mapped reads.

520 [b]G10X50/X150, G25X50/X150, G50X50/X150, G100X50/X150, and G150/X150 indicate the RPKM (G10X50)/RPKM (X150), RPKM

521 (G25X50)/RPKM (X150), RPKM (G50X50)/RPKM (X150), RPKM (G100X50)/RPKM (X150), and RPKM (G150)/RPKM (X150)

522 values, respectively.

Figure legends

Fig. 1 Analysis of the expression and construction of the xylose gene cluster of *Enterococcus mundtii* QU 25

(A) Northern hybridization analysis was utilized to examine the expression levels of the genes involved in the xylose gene cluster under low sugar conditions. Lanes 1, 2, and 3 represent the samples harvested from cells cultured in M17-2 medium containing 5 $g \cdot L^{-1}$ glucose (G5), 5 $g \cdot L^{-1}$ xylose (X5), and 1 $g \cdot L^{-1}$ glucose and 5 $g \cdot L^{-1}$ xylose (G1X5), respectively. Arrowheads indicate the bands corresponding to the transcripts of each respective gene, and the subsequent numbers denote the size of each band in kilobases. Below each lane, ethidium bromide-stained 16S rRNA is included as a loading control. (B) Primer extension analysis was performed to identify the transcriptional start site of the *xylA* gene. T, A, C, G, and S indicate the corresponding nucleotide bases and a sample, respectively. The arrowhead denotes the transcriptional start point. (C) Graphic depiction of the DNA sequence located between the *xylR* and *xylA* ORFs. The transcriptional start site, the predicted promoter region of *xylA*, the putative CcpA-binding site, and the predicted XylR-binding site are denoted by the bent arrow, the double lines, the dashed line, and the dotted line, respectively. The partial ORFs of *xylR* and *xylA* are bracketed with single lines.

Fig. 2 Structure of the operon encoding the genes in the xylose gene cluster

Fig. 3 Northern analysis of *xylA* gene expression under high sugar conditions

(A) *Enterococcus mundtii* QU 25 was cultured in M17-2 medium containing 50 $g \cdot L^{-1}$ xylose and either 10 $g \cdot L^{-1}$ (G10X50), 25 $g \cdot L^{-1}$ (G25X50), 50 $g \cdot L^{-1}$ (G50X50), or 100 $g \cdot L^{-1}$ (G100X50) glucose. Arrowheads indicate the bands corresponding to *xylA* transcripts, and the adjacent values denote the sizes of each band. Below each lane, ethidium bromide-stained 16S rRNA is included as a loading control. (B) Quantification of the density of the three bands corresponding to *xylA* transcripts in (A). The *xylA* expression value, in arbitrary units, obtained from each sample was divided by that from the G10X50 sample and used as the relative transcriptional amount.

Fig. 4 Map of the xylose fermentation pathway in *Enterococcus mundtii* QU 25

The genes that exhibited glucose-dependent variations in transcriptional expression in media containing high sugar concentrations are indicated with bold type (see Tables 2 and 3). Thick arrowheads denote that there was a marked decrease in the transcriptional expression levels of the corresponding genes under high glucose conditions. Conversely, for genes where there was minimal change or fluctuation in expression under these conditions, the arrow was omitted. Abbreviations in this figure are as follows: P, phosphate; GAP, glyceraldehyde 3-phosphate. Dotted lines indicate the phosphoketolase (PK) pathway, which exerts only in the hetero-fermentation conditions. Solid lines indicate the other pathways including the pentose-phosphate (PP)/glycolytic pathway, which exert both in the homo- and hetero-fermentation conditions.