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Carbon Catabolite Repression in *Leuconostoc mesenteroides* SY1, a Strain Isolated from Kimchi

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In most Gram (+) bacteria including LAB (lactic acid bacteria), CCR (carbon catabolite repression) is mediated through CcpA (catabolite control protein A) (Henkin, 1996; Titgemeyer and Hillen, 2002; Warner and Lolkema, 2003). CcpA is the most important regulator in CCR and it functions as negative regulator by binding to cis-acting sequence known as *cre* (catabolite responsive element) site preceding catabolite-controlled genes or operons. Binding of CcpA to *cre* is aided by co-repressor, P-Ser-HPr (HPr phosphorylated at the 46th serine). Besides its repressor function, CcpA sometimes functions as an activator, as has been reported for *B. subilis genes encoding acetate kinase, α-acetolactate synthase, phosphotransacetylase, and glutamate synthase, and for Lactococcus lactis las* operon encoding phosphofructokinase, pyruvate kinase, and L-lactate dehydrogenase (Faires et al. 1999; Luesink et al. 1998). The function of CcpA and other regulatory proteins are best understood for *B. subtilis* but not much is known for other Gram (+) bacteria. *L. mesenteroides* SY1 was isolated from cabbage Kimchi. When α - and β -galactosidase activities of SY1 were measured, they decreased significantly when SY1 was grown on media containing glucose, indicating the operation of CCR in *L. mesenteroides* SY1 (Park et al. 2005; Kim et al. 2005) (Fig. 1). Accordingly, genes involved in CCR such as *ccp*A, *pts*HI, and *hpr*K (encoding HPr kinase/phosphorylase) were examined for their presence by PCR. PCR primers were designed based on the homologous gene sequences of *L. mesenteroides* ATCC 8293 (AABH02000011), which was isolated from fermented olive and genome project has been undergone (Klaenhammer et al. 2002). Since *L. mesenteroides* ATCC 8293 and SY1 were isolated from plant materials, it was expected that both strains might share various properties including metabolic capacities in utilizing carbon sources. Expected size of DNA fragments containing above genes were successfully amplified, and their DNA sequences were determined. Table 1 summarizes the results obtained after sequencing cloned genes involved in CCR. The *ccpA* gene was shown to be transcribed as a monocistronic operon. A catabolite-responsive element (*cre*) was found in the promoter region of *ccpA*, suggesting that *ccpA* transcription in *L. mesenteroides* SY1 is autogenously regulated (Fig. 2). *ccpA* is divergently transcribed from *pepQ* encoding a dipeptidase which specifically cleaves X-prolyl peptide bonds, and this gene organization is common among LAB (Mahr et al. 2000). The amount of *ccpA* transcripts was seemingly constant on different carbon sources (1% fructose, sucrose, and raffinose), although it was less in cells grown on glucose (1%). Similar result was reported for *L. plantarum* (Muscariello et al. 2001). *ccpA*, *ptsH*, and *ptsH* mutant genes were overexpressed in *E. coli* by using pET26b(+) vector and His-tagged proteins (at the C-termini) were purified

by affinity column chromatography with HiTrap Chelating HP column (Amersham Biosciences, Uppsala, Sweden). Purified CcpA successfully bound to a PCR-amplified DNA fragment containing *cre* from *aga* gene (encoding α-galactosidase) but BSA did not (- control) (see Fig. 3). From the *cre* sequence (AGTAACCGTTTACA), C at the 6th position (underlined) was changed into A, G, or T by PCR. CcpA bound efficiently to mutated *cre*, indicating substitution of a central nucleotide was not critical for *in vitro* binding of CcpA. For the binding of CcpA to *cre*, CcpA is first bound with co-repressor, P-Ser-HPr. HPr kinase/phosphorylase is responsible for the phosphorylation of HPr at the 46th serine, and the same enzyme dephosphorylates P-Ser-HPr, generating HPr (Kravanja et al. 1999; Frey et al. 2003). Thus HPr K/P is a special enzyme with dual functions. Some metabolites such as FBP (fructose 1,6-bisphosphate) and pyrophosphate increase the phosphorylation reaction rate and Pi increases dephosphorylation reaction rate carried by this enzyme (Poncet et al. 2004). Purified HPr K/P from *L. mesenteroides* SY1 phosphorylated HPr and HprH16A, but failed to phosphorylate HPr S47A. Thus the result confirmed that phosphorylation by HPr K/P occurs at Ser residue as known for other Gram (+) bacteria. The position of Ser is 47th in SY1 unlike most other HPrs (46th) from Gram (+) bacteria. His is the 16th amino acid in SY1 rather than the 15th as in other HPrs. Thus it is likely that one additional amino acid was inserted into the HPr of SY1. *ccpA* gene was introduced into *L. mesenteroides* SY1 by using a plasmid vector, pSJE, which was constructed based on a cryptic plasmid (4,661 bp) isolated from another *L. mesenteroides* strain isolated from Kimchi. Increase in the *ccpA* transcript level was confirmed by slot blot analysis for RNA samples obtained from SY1 cells harboring pSJEccpA. 2-D gel analyses for protein extracts from SY1 cells harboring pSJEccpA showed that the intensity of some protein spots changed when compared with those from SY1 control cells. Further detailed studies for these protein spots are necessary to evaluate the effect of increased transcription of *ccpA*. Also, efforts are being made to obtain a mutant where chromosomal *ccpA* gene is knocked out. If the knock-out mutant obtained, it will be used to locate genes which are under direct control of CcpA by 2-D gel analyses. It is likely that *L. mesenteroides* SY1 has the same CCR mechanisms operating in many other Gram (+) organisms, but it is not known yet whether SY1 has any unique features. More studies are necessary to understand the CCR in LAB isolated from Kimchi.

Table. 1 PCR cloned CCR related genes and *aga* which is under CCR from *L. mesenteroides* SY1

Gene	Protein	size	MW, pI	
ccpA	CcpA	335 aa	36738.6, 4.74	*cre* (TGTAAGCGTTTGCA)
ptsH	HPr	89 aa	9212.4, 4.68	
ptsI	EnzI	571 aa	62519.5, 4.72	
hprK	HPr K/P	322 aa	35295.9, 4.66	
aga	α-galactosidase	740 aa	84321.2 5.42	*cre* (AGTAACCGTTTACA)

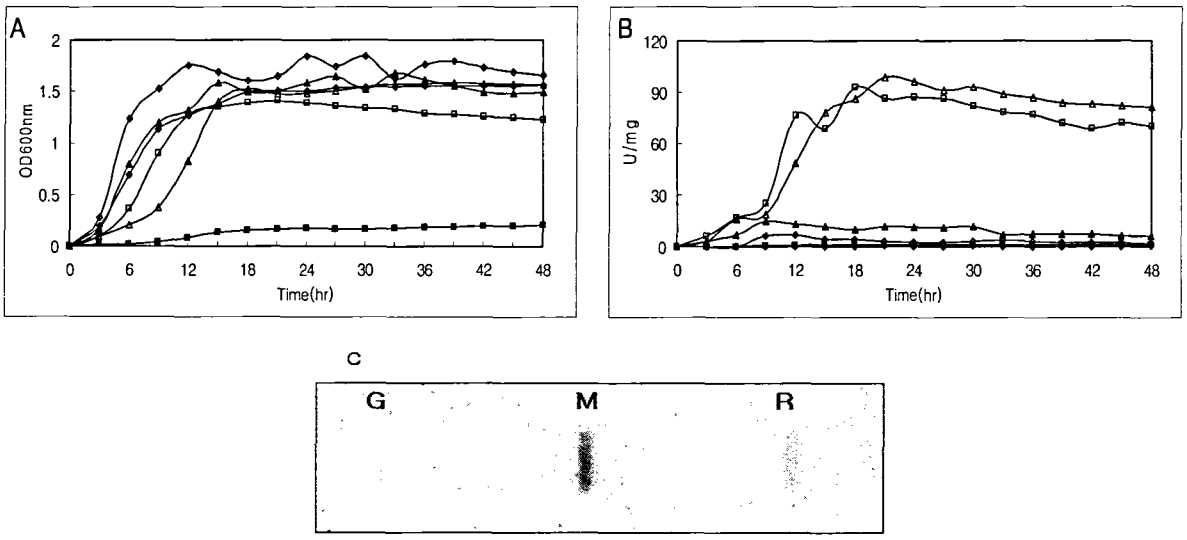

Fig. 1 Growth and α-Gal activity of *L. mesenteroides* SY1.

(A). Growth curve of *L. mesenteroides* SY1 in MRS media containing different carbon sources (1%).

(B). α -Gal activity of *L. mesenteroidese* SY1 in MRS media containing different carbon sources. ◆, glucose; ■, galactose; ▲, fructose; ◇, sucrose; □, melibiose; △, raffinose. (C). Slot-blot hybridization of total RNA extracted from *L. mesenteroides* SY1 cells grown on different carbon sources (G, glucose; M, melibiose; R, raffinose). Internal fragment of *aga* (800 bp) was obtained by PCR (agaF-1: 5'-TGCGTGAACGTCAAATGGAGC-3' and agaR-1: 5'-GTCAAACGATCCGCCAAATCATA-3') and used as the probe.

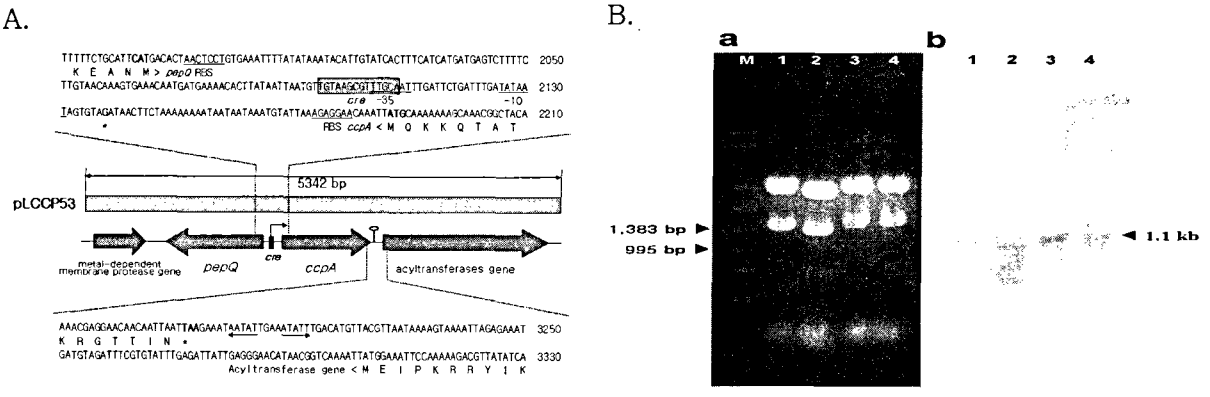

Fig. 2 Genetic organization of the *ccpA* and surrounding genes in *L. mesenteroides* SY1.

(A). The size and orientation of ORFs were deduced from the nucleotide sequence. Nucleotide sequence of the upstream regulatory region of *ccpA* is shown. Transcriptional start site is in the bold face and is marked by asterisk underneath. RBS and putative RNA polymerase binding sites (-10 and -35 region) in the sequence are underlined. *cre* motif is shown as a gray colored box. The N-terminal amino acid sequence of PepQ and CcpA are shown.

(B). Northern blot of total RNA (15 μg) separated on a 1.2 % agarose-formaldehyde gel and hybridized with ^{32}P-labelled 645 bp *ccpA* probe. Cells grown on MRS medium with 1% glucose (lane 1), fructose (lane 2), sucrose (lane 3), and raffinose (lane 4). a, agarose-formaldehyde gel; b, autoradiogram.

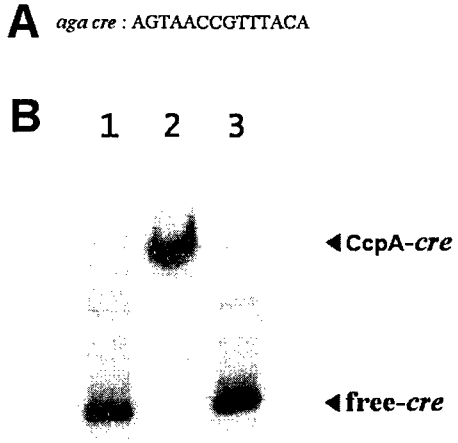

Fig. 3 Binding of CcpA to the *cre* site of *aga*.
CcpA was tested for the gel mobility shift of the 388 bp DNA fragment containing the *cre* site of *aga* using a 6% polyacrylamide gel. (A) aga sequence of L. mesenteroides SY1. (B) gel mobility shift experiment. 1, no protein added; 2, 1 μg of CcpA added; 3, 1 μg of BSA added (- control).

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