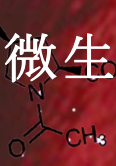
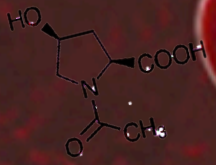
博士論文

Microbial production of *N*-acetyl *cis*-4-hydroxy-L-proline

(*N*-アセチル *cis*-4-ヒドロキシ-L-プロリンの微生物生産)





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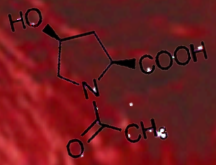

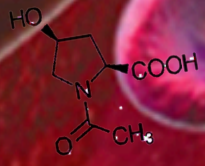

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Microbial production of *N*-acetyl *cis*-4-hydroxy-L-proline

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Contents

Abstract

The proline analogue *cis*-4-hydroxy-L-proline (CHOP), which inhibits the biosynthesis of collagen, has been clinically evaluated as a drug for anticancer, but its low water solubility and low molecular weight limits its therapeutic potential since it is rapidly excreted. In addition, CHOP is too toxic due primarily to its systematic effects on non-collagen proteins. To promote permeability as an oral drug or reduce the side effect of CHOP, *N*-acetylation of CHOP might be a novel approach as a prodrug, instead of the conjugation of poly(ethylene glycol-Lys) or the modification of *O*-acetylation.

In this study, I first found that *N*-acetyltransferase Mpr1 that detoxifies the proline analogue L-azetidine-2-carboxylic acid in the yeast *Saccharomyces cerevisiae* Σ1278b also converts CHOP into *N*-acetyl CHOP *in vitro* and *in vivo*. *Escherichia coli* BL21(DE3) cells overexpressing Mpr1 showed greater CHOP resistance than those carrying the control empty vector. Furthermore, I also found that *N*-acetyl CHOP production could be increased by the addition of NaCl into the medium that induces osmotic stress, which accelerates CHOP uptake into *E. coli* cells. As a result, the amount of *N*-acetyl CHOP production in Mpr1-overexpressing cells was 3.5-fold higher than that observed in the cells cultured in the absence of NaCl. The highest yield was achieved during the exponential growth phase of cells in the presence of 2% NaCl (52 μmol *N*-acetyl CHOP per g wet cell weight).

Next, I designed the study was designed to achieve the microbial production of *N*-acetyl CHOP from L-proline. Recently, the L-proline *cis*-4-hydroxylases SmP4H from the Rhizobium *Sinorhyrobium meliloti*, which belongs to a 2-oxoglutarate-dependent dioxygenase family, was shown to catalyze the

hydroxylation of free L-proline to CHOP. Also, as mentioned above, *N*-Acetyltransferase Mpr1 converts CHOP into *N*-acetyl CHOP. Therefore, I attempted to establish microbial production of *N*-acetyl CHOP by co-expression of SmP4H and Mpr1. I constructed the co-expression plasmid harboring both the SmP4H and Mpr1 genes and introduced it into *E. coli* BL21(DE3) or its L-proline oxidase gene-disrupted (Δ*putA*) strain. I found that *E. coli* Δ*putA* cells accumulated L-proline by approximately 2-fold compared to that in wild-type cells, but there was no significant difference in CHOP production between wild-type and Δ*putA* cells. The addition of NaCl and L-ascorbate resulted in 2-fold increase in *N*-acetyl CHOP production in the L-proline-containing M9 medium. The highest yield was achieved at 42 h cultivation in the optimized medium at 37°C. *N*-Acetyl CHOP, which was purified by anion-exchange chromatography, was detected on HPLC according to the modified fatty acid assay and negative ion mode of mass spectrometry.

Finally, for optimization of the *N*-acetyl CHOP production, I examined the cellular metabolism of *N*-acetyl CHOP in *E. coli*. Five unknown compounds were detected in the total protein reaction, probably due to the degradation of *N*-acetyl CHOP. Our results suggest that weakening of the degradation or deacetylation pathway improves the productivity of *N*-acetyl CHOP. However, *E. coli* deletion mutants of predicted deacetylase gene could not reduce the deacetylation of *N*-acetyl CHOP. It was also speculated that CHOP causes misfolding of *E. coli* proteins into which it is incorporated competitively with L-proline. Interestingly, the addition of high concentrations of CHOP induced pinkish brown color production in the culture medium and cells, suggesting that CHOP metabolism was dependent by unknown pathway(s). In conclusion, this study is the first to report the microbial production of *N*-acetyl CHOP as a new prodrug. Further

improvements in *N*-acetyl CHOP production are expected to weaken its degradation pathway and to engineer SmP4H with enhanced activity.

Abbreviations

cis-4-Hydroxy-L-proline	CHOP
Poly(ethylene glycol)	PEG
trans-4-Hydroxy-L-proline	THOP
L-Azetidine-2-carboxylic acid	AZC
L-Glutamate-γ-semialdehyde	GSA
L-Δ^1-Pyrroline-5-carboxylic acid	P5C
9-Anthrydiazonmethane	ADAM
1-Pyrenyldiazomethane	PDAM
GCN5-related *N*-acetyltransferase	GNAT
5-Thio-2-nitrobenzoic acid	TNB
N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride	EDC-HCl
Thin-layer chromatography	TLC
cis-4-Hydroxy-D-proline	CHOP (D)
Fluorodinitrophenyl-5-L-leucine amide	FDLA
Per g wet cell weight	g^{-1} WCW

Preface

Despite several decades of intensive research, cancer is still one of the most feared diseases in the world. Metastasis of cancerous cells can damage the nearby tissues and transfer other parts of body through the blood and lymph systems (Chambers et al., 2002; Hanahal and Weinberg, 2000; Finkel et al., 2007). Cancer therapies were included a combination of surgery, radiation, and chemotherapy. The surgery and radiation therapy would succeed only when malignant cells are confined to the treated area. Chemotherapy, the other essential treatment for non-metastatic or metastatic cancerous cells (Skeel, 2007), used drugs that are toxic agents with many side effects. These chemotherapeutic drugs are antimetabolites, alkylating agents, anthracyclines, topoisomerase inhibitors, plant alkaloids, and other antitumour agents (Singh et al., 2008; Skeel, 2007). A major challenge of developing cancer chemotherapeutic drugs is to achieve higher specific for cancer cells with less side effects.

A prodrug therapy is a useful approach to design less cytotoxic form of anticancer drugs (Rautio et al., 2008). A prodrug is an inactive substance covalently linked with a chemical moiety and undergoes transformation into a drug inside the body. The prodrug design was developed to overcome the disadvantages of anticancer drugs (Rautio et al., 2008; Testa et al., 2004; Ettmayer et al., 2004): such as limited solubility, instability, unacceptable taste or odor, pain upon injection, gastrointestinal irritability, too rapid or too slow transport of the drugs to the tumor and low therapeutic index. The major objective of prodrug design is to temporarily change the physicochemical properties of the drugs to enhance the therapeutic benefits such as prolongation of action, reduced toxicities and side effects, increased

specificity delivery, and improved aqueous solubility. The classical approach has been successfully used to design and develop prodrugs with improved pharmacokinetic and pharmacodynamic properties (Singh et al., 2008).

cis-4-Hydroxy-L-proline (CHOP) is an unnatural imino acid that can be obtained through a complex chemical synthesis procedure. In 1971, the first *in vitro* study for CHOP showed about 61% incorporation of CHOP into protocollagen. Collagen containing CHOP in place of proline and *trans*-4-hydroxy-L-proline (THOP) is not extruded at normal rate (Rosenbloom and Prockop, 1971). Recently, clinical observations showed the effect of CHOP on fibrous collagens such as type I, II, III, and V. Several studies revealed that CHOP can be used as anticancer drug to treat liver cancer, bladder cancer, prostate cancer, and two cases of renal pelvic cancer. However, these tumor cells are damaged in response to the treatment with CHOP via induction of endoplasmic reticulum stress and other mechanisms (Mueller et al., 2006; Olszewski et al., 2008). Like other proline analogues, CHOP can be randomly incorporated into L-proline residues in protein molecules (Joel and Derwin, 1971). This incorporation of CHOP may affect the functions of non-collagenous proteins and disturb cell functions leading to toxic side effects (Dickens et al., 2008; Eldridge et al., 1988).

A prodrug approach to decrease the toxicity of free CHOP has been developed to use poly(ethylene glycol) (PEG) as a carrier polymer to deliver CHOP to specific sites of active collagen formation, with the objective of maximizing the local concentration of CHOP relative to endogenous L-proline. CHOP has been attached in to high-molecular-weight PEG through an amide linkage or through an ester linkage (Poiani et al., 1994; 1997). PEG-L-Lysine (Lys)-CHOP amide seemed to inhibit the biosynthesis of collagen specifically, but there are several disadvantages

in using PEG: it crosses the cell membrane with difficulty, displays limited drug release, and irritates the mucous membrane (Rowe et al., 2009). I propose that the *N*-acetylation of CHOP promotes gastrointestinal permeability as an oral drug or decreases the toxicity because of the missing amine group that is required for CHOP incorporation into proline residues. *N*-acetyl CHOP is promising as a prodrug instead of the conjugation of poly (PEG-Lys).

In this thesis, my objective was to produce *N*-acetyl CHOP in bacteria. First, I found that CHOP is a new substrate of the yeast *N*-acetyltransferase Mpr1 *in vitro*. Also, *Escherichia coli* cells that overexpress Mpr1 could produce *N*-acetyl CHOP. Second, to develop a microbial production system for *N*-acetyl CHOP from L-proline, the Rhizobium L-proline *cis*-4-hydroxylase SmP4H and yeast Mpr1, which convert L-proline into CHOP and CHOP into *N*-acetyl CHOP, respectively, were co-expressed in the *E. coli* strain BL21(DE3) Δ*putA,* in which the L-proline degradation pathway was blocked. The highest yield of *N*-acetyl CHOP was achieved at 42 h cultivation in optimized medium. To improve *N*-acetyl CHOP productivity, I finally analyzed cellular metabolism of *N*-acetyl CHOP in *E. coli*, the results of which suggested that CHOP metabolism is dependent on an unidentified pathway(s). These results provide a promising approach to microbial production of *N*-acetyl CHOP as a new prodrug.

Chapter I

Production of *N*-acetyl *cis*-4-hydroxy-L-proline by the yeast *N*-acetyltranferase Mpr1

1.1. Introduction

Proline analogues are useful compounds for pharmaceutical and cosmetic applications. For instance, *trans*-4-hydroxy-L-proline (THOP) is an enhancer for procollagen synthesis that is used in cosmetic product (Aoki et al., 2012). In addition, the THOP derivative *N*-acetyl THOP (oxaceprol) is an atypical inhibitor of inflammation, and is useful for the treatment of diseases affecting the connective tissues such as osteoarthritis. Oxaceprol was established as a drug for joint disease without toxicity or side effects (Wright and Nolan, 2001). Other derivatives, such as *cis*-4-hydroxy-L-proline (CHOP), have been evaluated as anticancer drugs that could prevent procollagen from folding into a stable triple-helical conformation because the excessive decomposition of collagen occurs in fibrotic process of tumor cells, and consequently CHOP leads to inhibition of cancer development (Kerr et al., 1987; Poiani et al., 1997; Riley et al., 1980). However, CHOP can be incorporated into all noncollagen proteins instead of only critical L-proline residues, leading to the degradation of misfolding proteins and the impairment of cell functions (Mueller et al., 2006). As described above, modification of CHOP with PEG-Lys is thought to be one of the methods that decrease the toxicity of free CHOP, but I propose that *N*-acetylation of CHOP can overcome the disadvantages of free CHOP and *N*-acetyl CHOP can be used as an oral drug. However, *N*-acetyl CHOP has not been studied or commercialized yet.

In the yeast *Saccharomyces cerevisiae* Σ1278b, we discovered that the *MPR1* genes is involved in the detoxification of the proline analogue L-azetidine-2-carboxylic acid (AZC) (Takagi et al., 2000). The *MPR1* gene encodes an *N*-acetyltransferase that converts AZC into *N*-acetyl AZC (Fig. 1), which is no longer incorporated into proteins and therefore not toxic to cells (Shichiri et al., 2001). Many yeasts and fungi contain the *MPR1*-homologous genes (Wada et al., 2008). However, AZC is not a cellular substrate of Mpr1, because AZC occurs only in some plant species of the families of lily, fabaceae, and beets (Elijah and Leonard, 1980; Fowden, 1955). Therefore, the combination of *MPR1* and AZC provides a powerful tool as a selectable marker system for yeast and plant transformation (Ogawa et al., 2009; Tsai et al., 2010). Previously, Mpr1 has been shown to protect yeast cells by reducing the intracellular reactive oxygen species (ROS) levels under oxidative stress conditions (Takagi et al., 2000; Du and Takagi, 2005; 2007; Nomura et al., 2003). We recently revealed that Mpr1 converts L-glutamate-γ-semialdehyde (GSA), which is an equilibrium form of the L-proline catabolism intermediate L-Δ^1-pyrroline-5-carboxylic acid (P5C), into *N*-acetyl GSA, which is an L-arginine intermediate. It was also found that Mpr1-dependent L-arginine synthesis confers stress tolerance on yeast cells (Nomura and Takagi, 2004; Nishimura et al., 2010). Meanwhile, Mpr1, which is a novel member of the GCN5-related *N*-acetyltransferase (GNAT) superfamily (Shichiri et al., 2001), has no characterized proteins whose primary sequences are similar to that of Mpr1. In addition, AZC as the acetyl-receptor in the reaction of Mpr1 is a secondary amine, although primary amines are the acetyl-receptors of the proteins in the GNAT superfamily. These properties suggest that Mpr1 has a novel tertiary structure and unique mechanisms of catalytic reaction and substrate recognition.

In this study, I found that CHOP is a new substrate of Mpr1 (Fig. 1). Also, *Escherichia coli* cells that overexpress Mpr1 could produce *N*-acetyl CHOP, which is promising as a new anticancer prodrug.

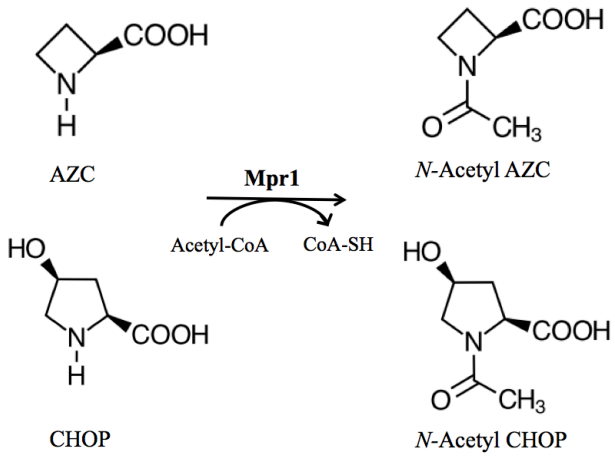


Fig. 1. Proposed scheme for the AZC or CHOP acetyltransferase reaction by Mpr1.

1.2. Materials and methods

1.2.1. Strains and media

The *S. cerevisiae* strain L5685 with a Σ1278b background was supplied by G. Fink (Massachusetts Institute of Technology, Cambridge, MA) and used as the wild-type strain in this study. The *mpr*-disrupted strain LD1014 (Shichiri et al., 2001). LD1014 was harboring the 2μ-based high-copynumber plasmid pYES-Mp1, which contains *URA3* was used to express *MPR1*. We also used the *S. cerevisiae* S288c background lacking MPR1 supplied by C. Kaiser (Massachusetts Institute of Technology).

The *E. coli* strain BL21(DE3) (F^- *dcm ompT* $hsdS_B(r_B^- m_B^-)$ *gal* λ(DE3))was used as a host for plasmid construction and Mpr1 expression. The *E. coli* recombinant cells were spotted on M9 agar medium plus 2% casamino acid (M9CA) containing 50

μg/ml kanamycin and 5 mg/ml CHOP (Tokyo Chemical Industry, Tokyo) or AZC (Sigma-Aldrich, St. Louis, MO). For *N*-acetyl CHOP prodution, the *E. coli* recombinant strain was cultivated on LB medium containing 5 mM CHOP, 50 μg/ml kanamycin, and 0.5%, 1%, 1.5% or 2% NaCl at 37°C for 24 h with vigorous shaking (200 rpm).

1.2.2. Construction of expression plasmid for Mpr1

PCR was performed using pQE-MPR1 (Shichiri et al., 2001) as a template with primers FseI-MPR (5'-CGA GGC CGG CCA TGG ATG CGA ATC CAT CGA ATG G -3') corresponding to the 5'-end of the *MPR1* gene containing the *Fse*I site (underlined) and KpnI-MPR (5'-GCT GGT ACC TTA TTC CAT GGA GAG GAA TTG GG -3') corresponding to the 3'-end of the *MPR1* gene containing the *Kpn*I site (underlined). The amplified 690-bp fragments were digested with *Fse*I and *Kpn*I, ligated at the *Fse*I and *Kpn*I sites of the isopropyl thiogalactopyranoside (IPTG)-inducible plasmid pRSF(Duet)-1 (Merck, Darmstadt, Germany). The plasmid pRSF(Duet)-1 has been used for expressing two genes *MPR1* gene and another gene (SmP4H) in Chapter II. I used pRSF(Duet)-1 as the cloning vector to facilitate the comparison of all the recombinant strains. In this chapter, I subcloned *MPR1* gene into the second multi cloning site of pRSF(Duet)-1. The constructed pRSF(Duet)-Mpr1 was introduced into *E. coli* strain BL21(DE3). The resultant recombinant cells were used for *in vivo* production of *N*-acetyl CHOP.

1.2.3. Overexpression and purification of recombinant Mpr1

An *E. coli* strain, JM109, which was transformed with the previously described pQE-MPR1 (Shichiri et al., 2001), was grown at 37°C in M9CA medium. When

absorbance at 600 nm reached 0.1, IPTG was added to the culture medium to a final concentration of 0.1 mM to induce gene expression. After cultivation for 18 h at 18°C, the cells were harvested by centrifugation and suspended with ice-cold 50 mM Tris buffer (pH 8.0) containing 1 X protease inhibitor cocktails (Sigma-Aldrich) and disrupted by sonic oscillation under cooling. After centrifugation (20 min at 15,000 × g), the supernatant was used as the soluble fraction. The His-tagged fusion proteins in the soluble fraction were then purified using a Ni-NTA His·Bind Resin (Merck) in accordance with the manufacturer's protocol.

1.2.4. Enzyme assay

The acetyltransferase activity of Mpr1 purified from *E. coli* cells harboring pQE-MPR1 was assayed at 30°C by monitoring the increase in 5-thio-2-nitrobenzoic acid (TNB) as described previously (Shichiri et al., 2001). The increase in absorbance at 412 nm was measured in the presence of the reaction mixture (final volume 1 ml) containing 50 mM $H_3BO_3/Na_2B_4O_7$ buffer (pH 8.5) or 50 mM Tris buffer (pH 7.0), 1 mM AZC or CHOP, 0.1 mM acetyl-CoA, and enzyme solution. The reaction rate was calculated using an extinction coefficient for TNB of 15,570 M^{-1} cm^{-1}. One unit is defined as the amount of enzyme that catalyzes the formation of 1 mmol TNB per minute. The kinetic parameters k_{cat} and K_m were obtained from initial rate measurements by monitoring the absorbance increase at 412 nm with a DU-640 spectrophotometer (Beckman Coulter, Fullerton, CA). When the apparent K_m value for AZC or CHOP was determined, the AZC or CHOP concentration was varied from 0.5 mM to 5 mM in the presence of a fixed concentration of acetyl-CoA (1 mM). Protein concentrations were determined with a Bio-Rad protein assay kit (Hercules, CA) with bovine serum albumin as the standard protein.

1.2.5. *In vivo* CHOP conversion

The cell-free extracts of the *E. coli* strain overexpressing Mpr1 were prepared by sonic oscillation under cooling. The Dowex 1X8 Cl^- form (Sigma-Aldrich) was converted to hydroxide form and then washed with distilled water. The cell-free extract was applied to an anion-exchange column of the converted Dowex 1X8 resin. After washing with distilled water, 0.5M NaCl was used to elute 50% of *N*-acetyl CHOP. Then, 70 μl containing *N*-acetyl CHOP was incubated with 50 μl of 25 mM 2-nitrophenylhydrazine (2-NPH) (Tokyo Chemical Industry) in the presence of 50 μl of 25 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC-HCl) (Thermo Fisher Scientific, Rockford, IL) (Miwa et al., 1985) at 80°C for 5 min. After the addition of 100 μl of 1 M KOH, the mixture was further heated at 80°C for 5 min and was centrifuged at maximum speed for 5 min. The supernatant containing *N*-acetyl CHOP was analyzed by reverse-phase HPLC (LaChrom Elite; Hitachi, Tokyo, Japan). The HPLC separation was made using a C18 column (YMC, Kyoto, Japan) and a gradient of elution A 10% and elution B 60% acetonitrile in 25 mM sodium phosphate buffer (pH 6.0). The flow rate was kept at 0.5 ml/min. Derivatives of *N*-acetyl CHOP were detected at 420 nm at 45°C. The standard curve for *N*-acetyl CHOP was built from chemically synthesized *N*-acetyl CHOP with or without the cell-free extract.

1.2.6. Production of *N*-acetyl CHOP

The reaction mixture (5 ml) containing 5 mM CHOP and 2 mM acetyl-CoA with or without 0.5 mg of purified Mpr1 in 25 mM borate buffer was incubated at 30°C overnight. The reaction mixture was then kept at -25°C for 10 h. The frozen and

dried sample was dissolved in 0.1 ml of chilled 70% ethanol. The solution was collected and then concentrated (Concentrator 5301, Eppendorf, Hamburg, Germany). This step was repeated three times to remove all borate salt in the sample. The resultant samples were analyzed with a negative-ion-mode mass spectrophotometry (Q-TOF Global Ultima, Micromass, Manchester, UK).

The *E. coli* recombinant cells harboring pRSF(Duet)-Mpr1 were cultivated on LB medium containing 5 mM CHOP, 50 μg/ml kanamycin in the absence or presence of 0.5%, 1%, 1.5%, and 2% NaCl. The *N*-acetyl CHOP content was calculated as μmol *N*-acetyl CHOP per g wet cell weight (g^{-1} WCW).

1.2.7. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

After cultivation of the *E. coli* recombinant cells harboring pRSF(Duet)-Mpr1, cell cultures (OD_{600} = 2) were dissolved in a solubilization buffer containing 1% (wt/vol) SDS, 2.5% (vol/vol) β-mercaptoethanol, 20% (vol/vol) glycerol, and 16 mM Tris-HCl (pH 6.8) and incubated in a boiling water bath for 5 min. Fifteen microliter of samples was loaded on 12% SDS-polyacrylamide gels.

1.2.8. Thin-layer chromatography analysis

Thin-layer chromatography (TLC) was performed on 10 x 10 cm plates (Silicagel 60 F_{245}, Merck). Five microliters of the standard solution (50 mM for proline, CHOP, THOP, and *cis*-4-hydroxy-D-proline, and 2 mM for acetyl-CoA) in 25 mM borate buffer, and 20 μl of the investigated sample were spotted on the plates. One-dimensional TLC analysis was performed with *n*-butanol: acetic acid: water in a volume ratio of 3:1:1 as a mobile phase. Spots were observed under UV light at 366 nm before spraying with 5% permanganate dissolved in acetone. The reactions

were incubated at 30°C for overnight under various conditions (Table 2) and analyzed by TLC. The conversion of CHOP catalyzed by Mpr1 was then measured.

1.2.9. Determination of intracellular CHOP content

E. coli recombinant cells cultivated in LB medium for 24 h were harvested, washed twice with water, and resuspended in 500 μl of distilled water (OD_{600} = 20). The suspension was transferred to boiling water, and intracellular amino acids were extracted for 20 min. Debris was removed by centrifugation, and the supernatant was applied to the filter (0.2 μm; Millex-LG, Nihon Millipore; Osaka, Japan) to remove the proteins. The supernatants were subjected to CHOP measurement by an amino acid analyzer (JLC-500, JEOL; Tokyo, Japan).

1.3. Results

1.3.1. CHOP acetyltransferase activity of Mpr1

Our previous reports showed that Mpr1 catalyzes the *N*-acetylation of AZC (Shichiri et al., 2001) and P5C or its equilibrium compound GSA (Nomura and Takagi, 2004) with acetyl-CoA. We examined the acetyltransferase activities of Mpr1 toward various L-proline-related compounds, such as CHOP, THOP, and *cis*-4-hydroxy-D-proline (CHOP (D)). Interestingly, in addition to AZC, acetyltransferase activity toward CHOP was clearly detected at pH 8.5 in borate buffer (Table 1). Kinetic analysis showed that the apparent K_m and k_{cat} values for CHOP were lower than those for AZC (Table 1). These results suggest that CHOP is a novel substrate of Mpr1, although Mpr1 did not acetylate the other hydroxyproline derivatives, THOP and CHOP (D).

Table 1. Specific activities and apparent kinetic constants of Mpr1.

Substrate	Specific activity (mU/mg)	k_{cat} (sec^{-1})	K_m (mM)	k_{cat} / K_m (sec^{-1} mM^{-1})	Conversion (%)
AZC	11.77 ± 0.40	13.57 ± 0.05	1.62 ± 0.08	8.38 ± 0.56	85
CHOP (L)	13.88 ± 0.82	5.19 ± 0.02	0.38 ± 0.04	13.66 ± 1.33	75
CHOP (D)			ND		
THOP			ND		
L-Proline			ND		

Assays were performed in 25 mM Tris-HCl (pH 8.5) for AZC and in 25 mM borate (pH 8.5) for CHOP at 30°C using 0.7 mg of purified enzyme. The values are means ± standard deviations from three independent experiments. ND; Not detected.

1.3.2. Effects of various reaction conditions on Mpr1

Figure 2 shows the acetyltransferase activity of Mpr1 toward AZC and CHOP with various reaction conditions. In terms of specific activities toward both substrates, borate and Tris buffers are more preferable than sodium and potassium buffers (Fig. 2A). In the range 20-45°C, the highest activity was observed at 40-45°C in both substrates, AZC and CHOP (Fig. 2B). Therefore, temperature is an important environmental factor, which affects the acetyltransferase activity of Mpr1 toward CHOP. In a previous report (Nomura et al., 2003), the optimum temperature of Mpr1 was approximately 35°C, thus I chose the temperature for the enzyme reaction at 37°C. As shown in Fig. 2C, the optimal pH of Mpr1 was about 8.5 for both substrates, AZC and CHOP. A previous report (Nomura and Takagi 2004) showed that the optimum pH of Mpr1 was between 8.0-8.5. In this study, the Mpr1 activity in borate buffer was higher than that observed in Tris buffer, thus I chose 50 mM borate buffer (pH 8.5) for further experiments. The conversion percentages for AZC and CHOP by Mpr1 under optimal conditions were 85% and 75%,

respectively (Table 1).

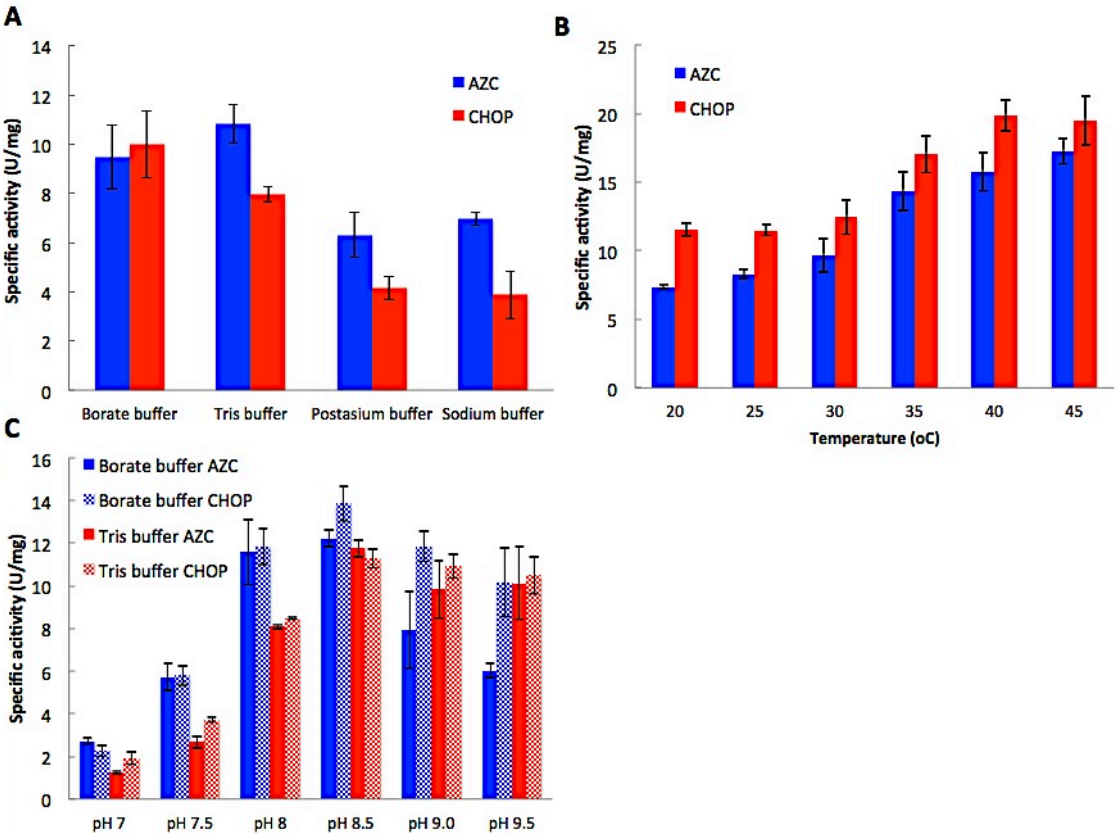


Fig. 2. Effects of buffer (**A**), temperature (**B**), and pH (**C**) on acetyltransferase activity of Mpr1 toward AZC and CHOP. The reaction mixture (final volume 1 ml) containing 50 mM $H_3BO_3/Na_2B_4O_7$ (Borate), Tris, K_2HPO_4/KH_2PO_4 (Potassium) or Na_2HPO_4/NaH_2PO_4 (Sodium) buffer (pH 7.5), 1 mM AZC or CHOP, 0.1 mM acetyl-CoA, and enzyme solution.

1.3.3. *In vitro N*-acetyl CHOP production

To demonstrate that Mpr1 can convert CHOP into *N*-acetyl CHOP, *in vitro* enzymatic analysis was carried out with the purified recombinant Mpr1 in the presence of CHOP and acetyl-CoA. By analyzing the reaction mixture with negative-ion-mode mass spectrophotometry, the expected *N*-acetyl CHOP and CHOP were detected with ions at m/z = 172.06 $(M\text{-}H)^-$ and m/z= 130.05 $(M\text{-}H)^-$, respectively (Fig. 3A). As a negative control, the sample without Mpr1 showed only

the mass spectrum at m/z =130.05 $(M\text{-}H)^-$, corresponding to CHOP. Judging from the spectrum data, approximately 25% of CHOP was converted into its *N*-acetyl form by Mpr1.

We also confirmed the Mpr1-catalyzed conversion from CHOP into *N*-acetyl CHOP by TLC analysis (Fig. 3B). Various compounds (L-proline, CHOP, *cis*-4-hydroxy-D-proline, THOP, and *N*-acetyl THOP) were spotted and characterized by the R_f -values. Acetyl-CoA and CoA can be visualized at R_f = 0 under UV light before spraying permanganate. The R_f value of Mpr1 itself was 0.488. The Mpr1-derived spots were also seen in all of the reaction samples. This is probably because the imidazole used in the purification of the recombinant Mpr1 still remained. There was no significant difference in the R_f values between the isomers CHOP and THOP. It was shown that the R_f value of *N*-acetyl CHOP from chemical synthesized (provided by Dr. Matsuo) and enzymatic converted by Mpr1 was approximately 0.38, which is lower than that of *N*-acetyl THOP (0.434). However, chemical synthesized *N*-acetyl CHOP was unstable under mobile solvent with unknown reason thus it occurred the second spot on TLC plate (Fig. 3C) that migrated similar as R_f value of Mpr1 itself and of *N*-acetyl THOP. The higher of acetyl-CoA concentration in the reaction mixture resulted the higher conversion rates of CHOP into *N*-acetyl CHOP with 7%, 11%, 30% and 55% under the conditions 1, 2, 3 and 6, respectively. Conditions 5 and 7 gave the same conversion rate (35%). The conversion rate under the condition 4 was 11% similar to that under the condition 2, even though the substrate concentration in the condition 4 was twice that of the condition 2. These results suggest that *N*-acetyl CHOP production depended on the concentration of acetyl-CoA, not that of CHOP or Mpr1. To purify *N*-acetyl CHOP from TLC plates, the condition 6 was optimized in this study (Fig.

3C). However, I did not succeed in the purification of intact *N*-acetyl CHOP from TLC plates. The similar degradation pattern of chemically or enzymatically synthesized samples were obtained in positive ion mode mass spectrometry.

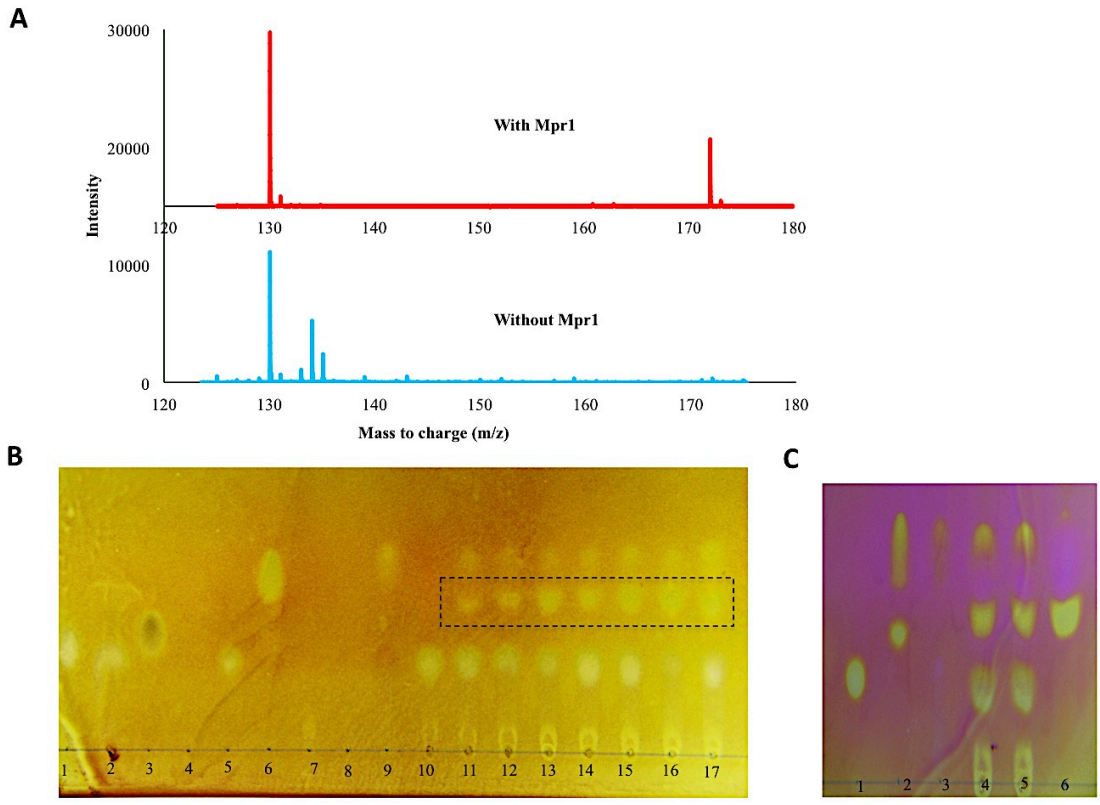


Fig. 3. *N*-Acetyl CHOP production *in vitro*. (**A**) Analyzed negative-ion-mode mass spectral of reaction with Mpr1 (red line) or without Mpr1 (blue line). (**B**) TLC chromatogram of the standards and investigated reaction. Ten microliter of 1 mM standards such as, *cis*-4-hydroxy-D-proline (R_f = 0.241) (lane 1), THOP (R_f = 0.270) (lane 2), proline (R_f = 0.289) (lane 3), borate buffer (lane 4), CHOP (R_f = 0.220) (lane 5), *N*-acetyl THOP (R_f = 0.434) (lane 6), acetyl-CoA (lane 7), CoA (lane 8), Mpr1 (0.14 mg/ml) (lane 9), and the reaction mixture without (lane 10) or with Mpr1 (lanes 11-17) were loaded on TLC plate. Lanes 11-17 contained the sample reacted under the conditions described in Table 2. (**C**) TLC chromatogram of chemically synthesized *N*-acetyl CHOP, optimized reaction mixture with Mpr1 and purified *N*-acetyl CHOP from TLC. CHOP (lane 1), chemically synthesized *N*-acetyl CHOP (lane 2) (R_f = 0.475 or R_f = 0.375), Mpr1 (lane 3), and the reaction mixture 6 (lane 4 and 5) and purified *N*-acetyl CHOP from TLC (lane 6). Most compounds were migrated in *n*-butanol:acetic acid:H_2O (3:1:1) except for acetyl CoA and CoA. Spots were detected under oxidation of 5 % permanganate.

Table 2. Reaction conditions in Fig. 3B

Condition	Mpr1 (μg)	Acetyl-CoA (μmol)	CHOP (μmol)	Lane in Fig. 3B
1	6	2	10	11
2	6	4	10	12
3	6	8	10	13
4	6	4	20	14
5	8	8	20	15
6	6	10	10	16
7	10	10	20	17

1.3.4. Toxicity of CHOP for *S. cerevisiae* and *E. coli* cells

Proline analogues AZC and CHOP have been reported to inhibit the growth of microorganisms. I examined the growth of *S. cerevisiae* and *E. coli* cells on agar plates containing AZC and CHOP. The *S. cerevisiae* strains expressing *MPR1* (Σ1278b and LD1014 harboring pYES2-Mpr1) showed greater AZC resistance than that of *S. cerevisiae* strains S288C and LD1014 lacking *MPR1*, which is sensitive to AZC (Fig. 4). In contrast, CHOP (up to 5 mg/ml) did not show a significant toxicity to all of yeast strains (Fig. 4).

Next, I investigated the effect of the wild-type Mpr1 and its mutants on detoxification of AZC and CHOP in *E. coli* (Fig. 5). *E. coli* cells carrying the vector only were sensitive to a low concentration of CHOP (0.6 mg/ml) as well as AZC. However, overexpression of Mpr1 conferred tolerance to AZC and CHOP on *E. coli* cells, suggesting that Mpr1 is involved in the resistance of *E. coli* to CHOP. A previous study reported that the K63R or F65L mutation in Mpr1 enhanced the

antioxidant activity or the thermal stability of Mpr1, respectively (Iinoya et al., 2009). The mutational analysis and molecular modeling suggest that an a-helix containing Lys63 and Phe65 has important roles in the function of Mpr1 (Iinoya et al., 2009). As shown in Fig. 5, mutant-type Mpr1 whose Leu117 and Phe65 are substituted with Val and Leu, respectively, exhibited a lower anti-toxic activity for CHOP than that of the wild-type Mpr1.

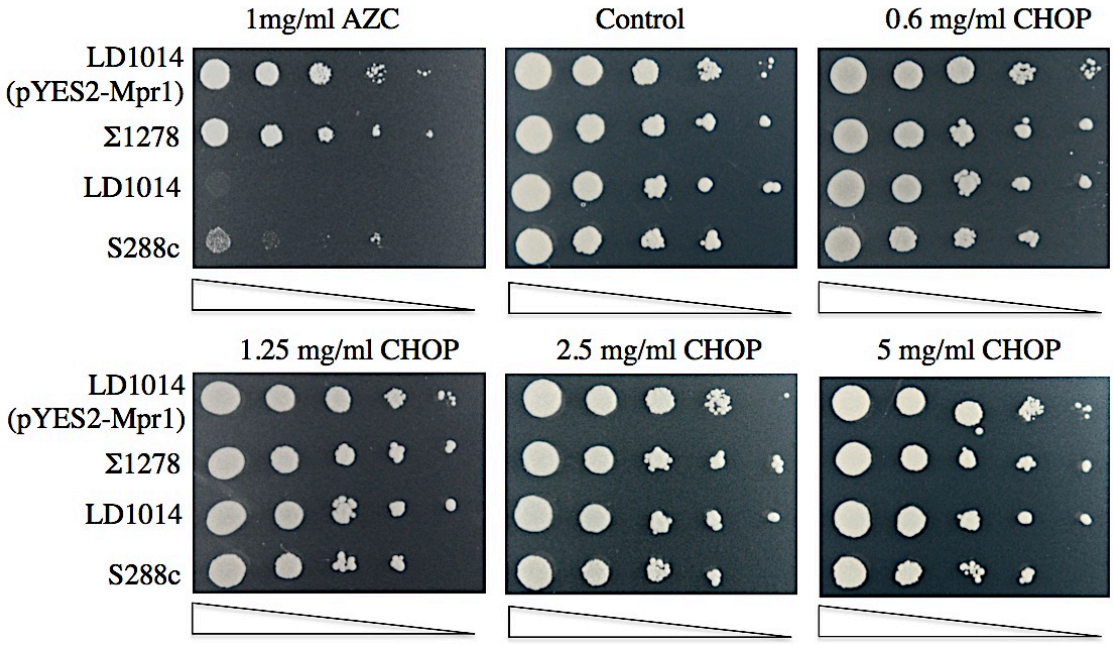


Fig. 4. Growth phenotype of *S. cerevisiae* strains. After cultivation in liquid SD medium at 30°C for 48h, approximately 10^6 cells of *S. cerevisiae* Σ1278 possessing *MPR1*, *mpr1*-disrupted strain LD1014, LD1014 carrying pYES2-Mpr1, and S288c lacking *MPR1*, and serial dilution of 10^{-1} to 10^{-5} were spotted onto SD medium in the absence (control) or presence of various concentrations of AZC and CHOP.

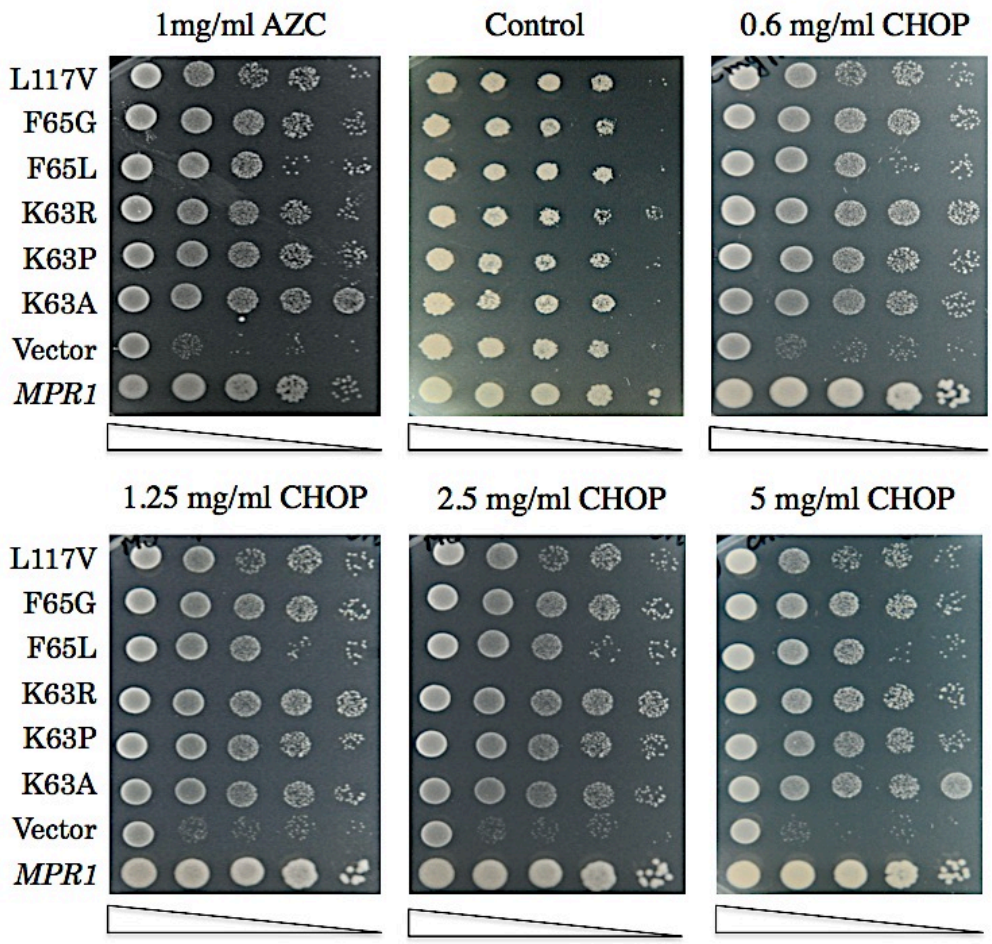


Fig. 5. Growth phenotype of *E. coli* strains expressing Mpr1. After cultivation in liquid M9CA medium containing 50 μg/ml ampicillin at 37°C for 18h, approximately 10^6 cells of *E. coli* BL21(DE3) strain carrying the empty vector or expressing each Mpr1, and serial dilution of 10^{-1} to 10^{-4} were spotted onto M9CA medium containing 0.1 mM and 50 μg/ml ampicillin in the absence (control) or presence of various concentrations AZC or CHOP.

1.3.5. Effect of Mpr1 overexpression on CHOP resistance in *E. coli* cells

We examined the growth of *E. coli* strains on M9 agar plates containing the toxic L-proline analogues AZC and CHOP (Fig. 6A). *E. coli* cells overexpressing Mpr1 showed greater resistance to both AZC and CHOP than those carrying only the vector. This result suggests that Mpr1 can detoxify AZC and CHOP by catalyzing the *N*-acetylation reaction. It should be noted that AZC is more toxic than CHOP to

E. coli cells (Fig. 6A).

We also measured the growth curve of *E. coli* cells in LB liquid medium (Fig. 6B). As the result, overexpression of Mpr1 enhanced the growth of *E. coli* but did not confer significant detoxification effect for CHOP (Fig. 6B). One of the reasons why Mpr1 enhanced the growth is that Mpr1 protects yeast cells from various oxidative stresses by regulating the level of intracellular reactive oxygen species (Nishimura et al., 2010). In addition, *E. coli* cells were more sensitive to CHOP in M9 medium than in LB medium, probably because in LB medium contain more exogenous L-proline than M9 medium. It is suggested that L-proline uptake from the medium reduces CHOP toxicity to *E. coli* cells. (Supplementary Fig. 1).

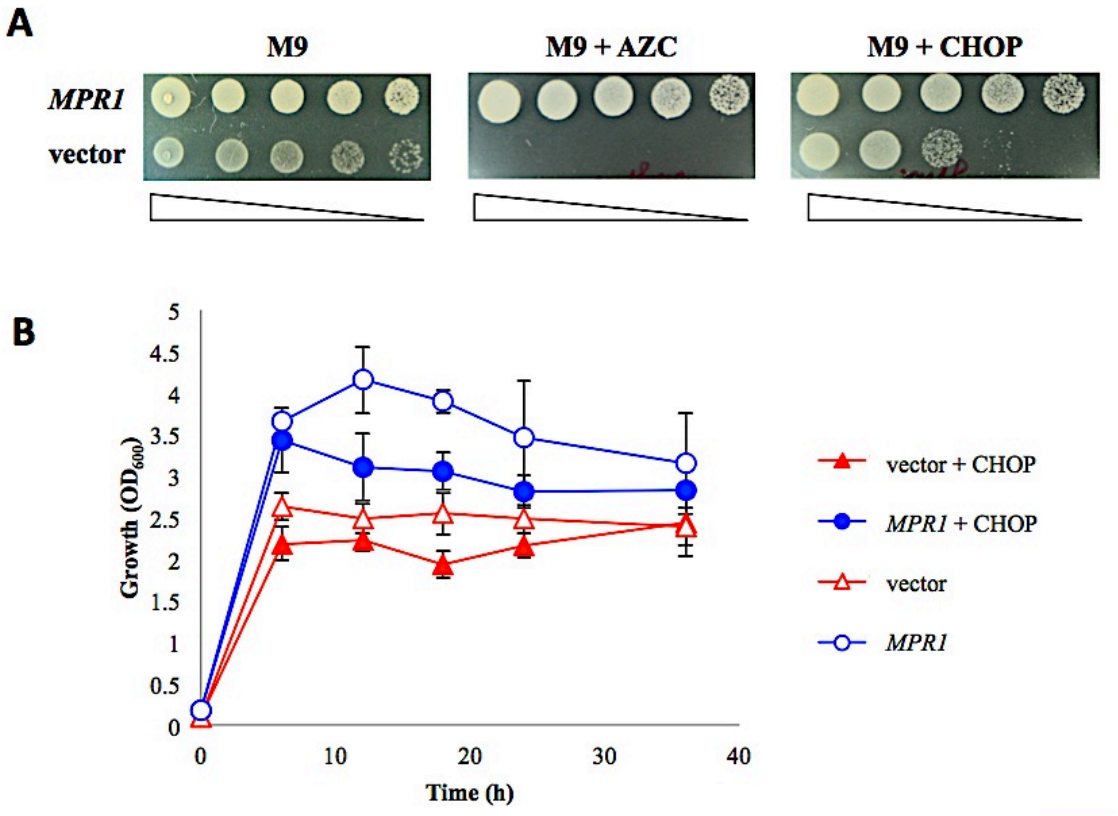


Fig. 6. Toxicity of AZC and CHOP for *E. coli* cells. (**A**) Approximately 10^6 cells of *E. coli* cells carrying pRSF(Duet)-MPR1 (*MPR1*) and pRSF(Duet)-1 (vector) and serial dilution of 10^{-1} to 10^{-5} were spotted onto M9CA medium containing 50 mg/ml kanamycin (left), plus 5 mg/ml

AZC (center) and 5 mg/ml CHOP (right). (**B**) Growth curve of *E. coli* cells carrying pRSF(Duet)-MPR1 (*MPR1*) and pRSF(Duet)-1 (vector) in the absence or presence of 5 mM CHOP in LB medium. The values are the means and standard deviations of results from three independent experiments.

1.3.6. CHOP uptake in *E. coli* cells

The transport system for THOP has been reported in *Pseudomonas* sp. (Gryder and Adams, 1970). In that species, the uptake system and metabolic enzymes for hydroxyproline are induced in cells grown in the presence of hydroxyproline. In *E. coli* and *Salmonella* sp., proline analogues can enter the cells via the proline transport system (Tristram et al., 1968), but the hydroxyproline metabolism has not been clarified (Elijah and Leonard, 1980). CHOP and THOP are not utilized as the sole nitrogen or carbon source in *E. coli* cells that overexpress Mpr1. As shown in Table 3, 0.1 μmol CHOP per g wet cell weight could be detected in *E. coli* cells carrying the empty vector, but not in Mpr1-overexpressing cells after 24 h of cultivation in LB medium, due to the conversion of CHOP into *N*-acetyl CHOP by Mpr1 (4.48 μmol). When 2% NaCl was added to LB medium that induces osmotic stress, the CHOP content increased significantly (approximately 6- and 7-fold) in *E. coli* cells carrying the vector and expressing Mpr1, respectively. As a result, the *N*-acetyl CHOP production in Mpr1-overexpressing cells was 3.5-fold higher than that in cells cultured in the absence of NaCl (Table 3). Interestingly, *E. coli* cells cultured in CHOP (2-5 mM)-containing LB media showed pinkish-brownish color in cells and the media after 18 h of cultivation at 37°C. However, this pigment was not observed in Mpr1-overexpressing cells or in cells cultured in L-proline- and THOP-containing LB medium.

Table 3. Intracellular contents of CHOP and *N*-acetyl CHOP under various conditions

LB medium containing	Plasmid	μmol/g wet cell weight	
		CHOP	*N*-acetyl CHOP
5 mM CHOP	Vector	0.10 ± 0.056	0
	MPR1	0	4.48 ± 1.53
5 mM CHOP + 2% NaCl	Vector	0.75 ± 0.082	0
	MPR1	0.60 ± 0.078	15.98 ± 2.34

CHOP and *N*-acetyl CHOP in *E. coli* cells carrying pRSF(Duet)-1 (vector) and pRSF(Duet)-MPR1 (*MPR1*) was detected after 24 h of cultivation at 37°C in 50 ml of LB medium containing 5 mM (6.5 mg/ml) CHOP in the absence or presence of 2% NaCl. The values are means ± standard deviations from three independent experiments.

1.3.7. *In vivo N*-acetyl CHOP production

The samples containing *N*-Acetyl CHOP produced *in vivo* and *in vitro* showed a peak within a retention time of 7.5-8.2 min by HPLC , which was consistent with those for the authentic *N*-acetyl forms of CHOP and THOP.

Since the addition of NaCl was effective for *N*-acetyl CHOP production (Table 3), effect of NaCl concentrations on *N*-acetyl CHOP production and the growth was examined using *E. coli* cells carrying pRSF(Duet)-Mpr1. Although NaCl addition to the medium inhibited the cell growth (Fig. 7A), when NaCl at concentrations of 1-2% was added to the medium, the *N*-acetyl CHOP production was 2- to 3-fold higher than that with 0.5% NaCl in the early stationary phase (Fig. 7B). The highest yield was 52 μmol *N*-acetyl CHOP per g wet cell weight in the presence of 2% NaCl at the stationary growth phase. Surprisingly, the yields of *N*-acetyl CHOP

showed 2- and 3-fold decreases at the end of the stationary and death phases, respectively, suggesting that *N*-acetyl CHOP could be deacetylated or degraded in *E. coli* cells.

SDS-PAGE analysis showed the recombinant Mpr1 (26 kDa) was overexpressed in *E. coli* cells after 6 h of cultivation in LB medium containing 2% NaCl and 5 mM CHOP (Fig. 8). The 26-kDa band was not observed in the extract of *E. coli* without Mpr1 gene (data not shown). The protein amount did not change event after 42 h of cultivation. This result suggests that the *MPR1* gene was induced in *E. coli* cells by IPTG at the exponential growth phase with *N*-acetyl CHOP production. It should be noted that *N*-acetyl CHOP production was reduced at the stationary growth phase of cells, although the Mpr1 proteins still remained in the cells. It was suggested that one of the substrates for Mpr1, acetyl-CoA, which is essential intermediate in numerous biosynthetic and energy yielding metabolic pathways, was low level at stationary growth phase because lacking energy source or other carbon sources.

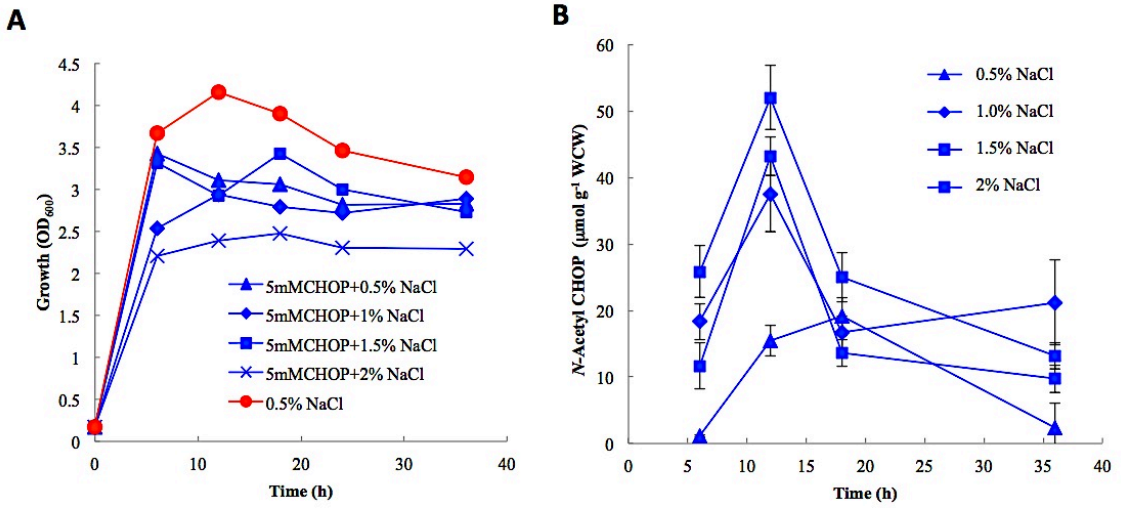


Fig. 7. Growth and *N*-acetyl CHOP production of *E. coli* cells that overexpress Mpr1 under various conditions. (**A**) Growth curve of *E. coli* cells overexpressing Mpr1 at 0.5% (triangle), 1% (diamond), 1.5% (square), and 2% (cross) of NaCl in LB medium containing 5 mM CHOP. As a control, *E. coli* cells carrying pRSF(Duet)-MPR1 were cultivated at 0.5% NaCl in the

absence of CHOP (circle). The values are the means from three independent experiments. (**B**) Time course of *N*-acetyl CHOP production by *E. coli* cells carrying pRSF(Duet)-MPR1 at 0.5% (triangle), 1% (diamond), 1.5% (square), and 2% (cross) of NaCl in LB medium containing 5 mM CHOP. *N*-Acetyl CHOP production was determined by HPLC. The values are the means and standard deviations of results from three independent experiments.

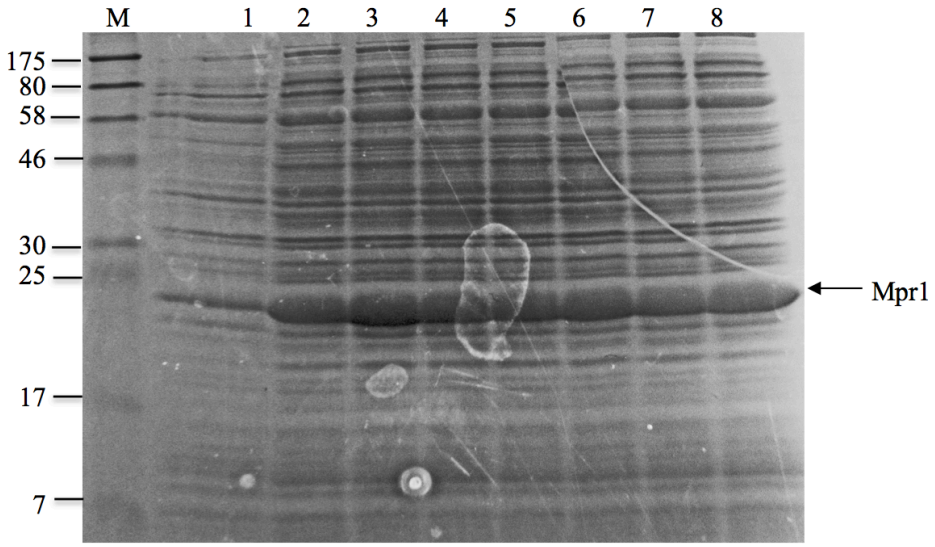


Fig. 8. SDS-PAGE of total proteins from *E. coli* cells that overexpress Mpr1. *E. coli* cells harboring pRSF(Duet)-MPR1 were cultured in LB medium containing 2% NaCl and 5mM CHOP. After cultivation, cell cultures were dissolved in a solubilization buffer and incubated in a boiling water bath for 5 min. Each sample (10 μg of protein) was loaded on 12% SDS-polyacrylamide gels. Lanes 1-8 show the samples after 0, 6, 12, 18, 24, 28, 32, and 42 h of cultivation, respectively. The positions of molecular mass standards (kDa) are shown on the left (lane M). The arrow indicates the position of the Mpr1 protein.

1.4. Discussion

In this study, we showed the first evidence of microbial production of *N*-acetyl CHOP directly from free CHOP, a novel substrate of the yeast *N*-acetyltransferase Mpr1. The *MPR1* genes found in the *S. cerevisiae* Σ1278b background strain are missing in other *S. cerevisiae* strains, including the genome project strain S288C and sake yeast strain K-9 (Takagi et al., 2000). Mpr1 was shown to have

acetyltransferease activity to detoxify the proline analogue AZC, but toxic AZC occurs only in plants belonging to the Lilaceae family. We recently found that Mpr1 can acetylate the intermediate of the L-proline metabolic pathway (P5C/GSA), leading to the regulation of P5C-mediated ROS generat1ion under oxidative stress conditions (Nomura and Takagi 2004). In addition, *N*-acetyl GSA can enter into the L-arginine synthetic pathway (Nishimura et al., 2010).

Table 2 shows the acetyltransferase activities of Mpr1 toward various hydroxyproline isomers, *cis* or *trans*- and D- or L- isomerization of 4-hydroxyl proline. Among the hydroxy proline isomers tested, Mpr1 acetylated CHOP. The specific activity of Mpr1 for CHOP was higher than that for AZC. It appears that Mpr1 does not acetylate other hydroxy proline compounds. The question arises as why Mpr1 can catalyze CHOP but not CHOP isomers as THOP. To answer this question, it is necessary to clarify the mechanism underlying the catalytic reaction and the substrate recognition. We are currently attempting to determine its crystal structure.

The results in Fig. 4 and 5 suggest that hydroxylproline analogues exhibit different inhibition to *S. cerevisiae* and *E. coli* cells. There are several potential explanations why these analogues are nontoxic to *S. cerevisiae* but toxic to *E. coli*: i) proline permease in yeast and *E. coli* might have different binding affinities for these analogues, ii) the analogues could be transported and then rapidly metabolized in the cells, iii) the analogues could be transported but not be efficiently recognized by prolyl-tRNA synthetase, leading to the failure of incorporation into the nascent proteins, and iv) the analogues may be incorporated into the proteins but not disrupt the protein function. However, as described below, I decided *E. coli* as host cells that overexpress Mpr1 to produce *N*-acetyl CHOP in further

examination. *S. cerevisiae* showed little sensitivity to CHOP, suggesting that yeast is a promising host cell for producing *N*-acetyl CHOP. However, *E. coli* is a versatile host cell for the production of heterologous proteins and lacks hydroxyproline metabolic pathways. It also seems to be advantageous that proline permeases of *E. coli* can transport the proline analogues into cells (Adam and Leonard, 1980; Elijah and Leonard, 1980).

Under the osmotic stress conditions caused by adding 2% NaCl into the medium, CHOP uptake into *E. coli* cells clearly increased (Table 3). This suggests that L-proline permease putP, a Na^+/L-proline symporter enhances uptake the extracellular proline and proline analogues. CHOP includes a free carboxyl head group, a small polar side-chain (hydroxyl group), and an amino head group in the a position. It appears that the distance between the charged carboxyl and amino groups permits its transport into the cell. After 24 h of cultivation, a large amount of CHOP was accumulated in Mpr1-overexpressing cells (Table 3). However, the decrease in *N*-acetyl CHOP production commenced after 12 h of cultivation, probably because of the deacetylation or degradation of *N*-acetyl CHOP by unidentified enzyme(s) in *E. coli* (Fig. 7B).

Like other proline analogues, CHOP is randomly incorporated into protein leading to inhibit collagen synthesis and reduces cell differentiation and extracellular matrix in mammalian cells (Zuckerman et al., 1985). It has been used as anticancer drug for cancer cells required excessive fibrosis formation of collagen I, II and III. It is unclear why CHOP is less toxic against bacteria than mammalian cells. One of the possible reasons is that bacteria do not have biosynthesis pathway for collagen. However, competitive CHOP incorporation of CHOP with L-proline into protein may affect the polypeptide chain conformation leading to nonfunctional

protein formation and an overall deleterious effect in bacteria. *N*-acetylation at the amine group of CHOP could inhibit random incorporation of CHOP into protein. Thus overexpression of Mpr1 that acetylated CHOP to *N*-acetyl CHOP would be effective to avoid the toxicity of CHOP in *E. coli* cells (Fig. 6A).

Chapter II

Microbial production of *N*-acetyl *cis*-4-hydroxy-L-proline by co-expression of the Rhizobium L-proline *cis*-4-hydroxylase and the yeast *N*-acetyltransferase Mpr1

2.1. Introduction

The industrial process for the production of hydroxyproline isomers was limited until enantioselective L-proline hydroxylases were discovered. It was found that several microorganisms produce L-proline *trans*-4- or *cis*-3-hydroxylase. These enzymes were applied to the industrial production of *trans*-4- and *cis*-3-hydroxy-L-proline (Katz et al., 1978; Shibaki et al., 2000). Hara and Kino (2009) isolated L-proline *cis-4*-hydroxylase. This was the first report on an enzymatic conversion of free L-proline to CHOP.

In mammalian systems, prolyl hydroxylase is described as 2-oxoglutarate-dependent dioxygenase that hydroxylates peptidyl proline, but not free L-proline. In contrast, bacterial L-proline 4-hydroxylase that converts free L-proline to free THOP has been found in *Streptomyces griseoviridus, Dactylosporangium* sp., and *Amycolatopsis* sp. (Lawrence et al., 1996; Shibasaki et al., 1999; 2000a; 2000b). Some bacterial strains, such as *Bacillus* and *Streptomyces* spp. (Mori et al., 1996; 1997) have L-proline 3-hydroxylase that converts free L-proline to *cis*-3-hydroxy-L-proline. Hara and Kino (2009) found L-proline *cis*-4-hydroxylases from the Rhizobium *Sinorhyrobium meliloti* and *Mesorhizobium loti* that hydroxylate free L-proline to CHOP. L-Proline *cis*-4-hydroxylases from *S. meliloti* (SmP4H) and *M. loti* (MIP4H), which belong to a 2-oxoglutarate-dependent

dioxygenase family, have similar enzyme activity properties despite the low amino-acid sequence identity between the two enzymes (66%).

In the previous chapter, I found that Mpr1 could be used for *N*-acetyl CHOP synthesis from CHOP *in vivo* and *in vitro*. In this chapter, to develop a microbial production system for *N*-acetyl CHOP directly from L-proline, I constructed a coexpression plasmid harboring the genes encoding SmP4H and Mpr1 from *S. meliloti* and *S. cerevisiae*, respectively, and introduced it into *E. coli* BL21(DE3) Δ*putA* strain in which the L-proline degradation pathway was blocked.

2.2. Materials and methods

2.2.1. Strains and plasmids

E. coli strain BL21(DE3) (F^- *dcm ompT hsdS*$_B$(r_B^- m_B^-) *gal* λ(DE3))was used as a host in this study. Strain BL21(DE3) Δ*putA* was constructed by P1 transduction from strain JW0999 (Δ*(araD-araB)567* Δ*lacZ4787*(::rrnB-3) $λ^-$ *rph-1* Δ*(rhaD-rhaB)568 hsdR514* Δ*putA::kan*) (Baba et al., 2006) to strain BL21(DE3). Plasmid pPC20 carrying the FLP recombinase gene was used to remove the kanamycin resistant gene.

To obtain the SmP4H gene, a polymerase chain reaction (PCR) was performed with a set of primers, 5'-CGA GAG CTC ATG GGC ACC CAT TTC TTG GGC-3' and 5'-CGT AAG CTT TTA GTA TGT CAT CAC CTC GCC ACG-3' with pET-SmP4H as a template. The primers introduced *Sac*I and *Hin*dIII sites (underlined) into the fragment (Hara and Kino, 2009). PCR product was digested with *Sac*I and *Hin*dIII, and subsequently cloned into the isopropyl thiogalactopyranoside (IPTG)-inducible pRSF(Duet)-1 vector (Merck, Darmstadt, Germany) to construct pRSF(Duet)-SmP4H. A region encoding the Mpr1 gene was amplified on a 0.7-kb

fragment from pQE2-Mpr1 (Nomura and Takagi, 2004) with a set of primers, 5'-CGA GGC CGG CCA TGG ATG CGG AAT CCA TCG AAT GG-3' and 5'- GCT GGT ACC TTA TTC CAT GGA GAG GAA TTG GG-3'. The primers introduced *Fse*I and *Kpn*I sites (underlined) into the fragment, which was ligated to the pRSF(Duet)-1 and pRSF(Duet)-SmP4H to construct pRSF(Duet)-Mpr1 and pRSF(Duet)-SmP4H-Mpr1, respectively.

2.2.2. Media and cultivation

Luria-Bertani (LB) medium (10 g l^{-1} polypeptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) and M9 minimal medium (100 mM potassium phosphate buffer, 0.5 g l^{-1} NaCl, 0.246 g l^{-1} $MgSO_4$, 1 g l^{-1} NH_4Cl, 4 g l^{-1} glucose as the carbon source) were used for the general cultivations. When absorbance at 600 nm reached 0.1, IPTG was added to the culture medium to a final concentration of 0.4 mM to induce gene expression. If necessary, ampicillin (50 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) were added. The pH was adjusted to 7.0 with 5 M NaOH. *E. coli* cells were cultured aerobically by vigorous shaking (200 rpm) at 34°C or 37°C. Growth was monitored by measurement of the optical density at 600 nm (OD_{600}). Glucose concentration was measured by Glucose C2 kit (Wako Pure Chemical Industries, Osaka, Japan).

2.2.3. *N*-Acetyl CHOP assay

Cells were disrupted on ice for 20 sec × 10 times with the interval of 7 min with sonicator (Sonifier 450; Branson, Danbury, CT, USA), and cell debris was removed by centrifugation (13,000×*g*) at 4°C for 20 min. *N*-Acetyl CHOP was analyzed by derivation with 50 ml of 25 mM 2-nitrophenylhydrazine in the presence of 50 ml of 25 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) hydrochloride (Miwa

et al., 1985; Peter et al., 2004; Yomota and Ohnishi, 2007) at 80°C for 5 min. After addition of 100 ml of 1 M KOH, the mixture was further heated at 80°C for 5 min. The reaction was centrifuged at 13,000 × *g* for 5 min. The *N*-acetyl CHOP derivative was analyzed by HPLC (Elite LaChrom, Hitachi, Japan) using Hydrosphere C18 column (YMC, Kyoto, Japan). The mobile phase A consisted of 10% acetonitrile and mobile phase B consisted of 60% acetonitrile in 25 mM sodium phosphate buffer (pH 6.0). The flow rate was kept at 0.5 ml min^{-1}. Derivatives of *N*-acetyl CHOP were detected at 420 nm at 45°C. The linearly gradient started at 10% B to 100% B in 20 min. The standard curve for *N*-acetyl CHOP was built using chemically synthesized *N*-acetyl CHOP (provided by Dr. Matsuo).

2.2.4. Determination of intracellular L-proline and CHOP contents

The intracellular L-proline and CHOP contents were assayed by the same method described in Chapter I.

2.2.5. Determination of proteins in *E. coli* recombinant strains

Cells (OD_{600}=2) were dissolved in a solubilization buffer containing 1% (wt/vol) SDS, 2.5% (vol/vol) β-mercaptoethanol, 20% (vol/vol) glycerol, and 16 mM Tris-HCl (pH 6.8) and incubated in a boiling water bath for 5 min. Fifteen microliters of the protein sample were loaded on 12% SDS-polyacrylamide gels. After electrophoresis, the gel was transferred to nitrocellulose membrane and subjected to Western blot analysis. Anti-His_6 antibody and anti-Mpr1 polyclonal antibodies (Shichiri et al., 2001) were used as the primary antibodies for SmP4H and Mpr1, respectively. And then anti-mouse IgG and anti-rabbit IgG were employed as the second antibodies for His_6 and anti-Mpr1 antibody, respectively. The Phototope-Star Detection kit (New England Biolabs, Ipswich, MA, USA) was used for chemiluminescent

detection.

2.2.6. Purification of *N*-acetyl CHOP

N-Acetyl CHOP was purified by anion exchange chromatography. At each stage of purification, eluted fraction with *N*-acetyl CHOP productivity was identified with *N*-acetyl CHOP assay (see above). *E. coli* transformant cells overexpressing SmP4H and Mpr1 genes were collected from 5 l of optimized M9 medium containing 5 mM L-proline, 5 mM L-ascorbate, and 1% NaCl, and the crude extract was obtained as described above. Protein was removed from the crude extract by 80% acetone. The solution containing *N*-acetyl CHOP was subjected to Dowex 1X8 (Cl^- form) column. The column was washed with distilled water and *N*-acetyl CHOP was eluted with the range of 0.1-0.5 M NaCl. *N*-acetyl CHOP-containing fractions were collected and dried by freeze dry. Then, the sample was dissolved in 100% methanol to remove NaCl. This step was repeated several times to remove NaCl completely. Purified *N*-acetyl CHOP was analyzed with a positive and negative ionization mass spectrophotometry (Q-TOF Global Ulitma, Micromass, Manchester, UK)

2.2.7. Liquid chromatography-mass spectrometry (LC/MS)

Samples were reacted with NPH-HCl assay. Asymmetry C18 (150x4.6 mm I.D.) column was used for hydrophobic separation at 42°C. Mobile phase A consisted of 10% acetonitrile and mobile phase B consisted of 60% acetonitrile. The flow rate was 0.5 ml min^{-1} and the gradient started at 10% linearly increased to 100% B in 5 min. Next the gradient was linearly reduced to 100% A in 15 min and kept at isocratic conditions for 0.1 min. Finally the gradient was allowed to reach the initial condition in 5 min and then equilibrated for 5 min. The total cycle time was thus 50

min. The sample from RT 8.7-9.2 min was collected for LC/MS. The capillary voltage of ESI interface was 1 kV and the cone voltage was 80 V. The temperature of ion source was 100°C. Data were obtained between m/z 100 and 800 in positive and negative ionization.

2.3. Results

2.3.1. Identification of *N*-acetyl CHOP produced by *E. coli* recombinant cells

N-Acetyl CHOP converted from CHOP by Mpr1 had been identified with Q-TOF-MS, TLC, and HPLC (Bach et al., 2012). A clear peak for *N*-acetyl CHOP was detected at the retention time (RT) of around 9 min in *N*-acetyl CHOP as a standard mixed with cell-free extract carrying the empty vector pRSF(Duet)-1 (Fig. 9A). The peak of *N*-acetyl CHOP was observed in the crude extract of *E. coli* recombinant cells overexpressing two enzymes, SmP4H and Mpr1 (Fig. 9D). However, *E. coli* cells harboring pRSF(Duet)-1 (Fig. 9B) and pRSF(Duet)-SmP4H (Fig. 9C) did not show the peak corresponding to *N*-acetyl CHOP. We confirmed by liquid chromatography-mass spectrometry (LC/MS) that the peak at about 9.2 min contained *N*-acetyl CHOP (Fig. 10). L-Proline and CHOP could not be analyzed by HPLC, because EDC-modified carboxyl group of amino acids reacts with the amine group itself instead of 2-NPH. Thus, we detected intracellular L-proline and CHOP with amino acid analyzer as described in Materials and Method.

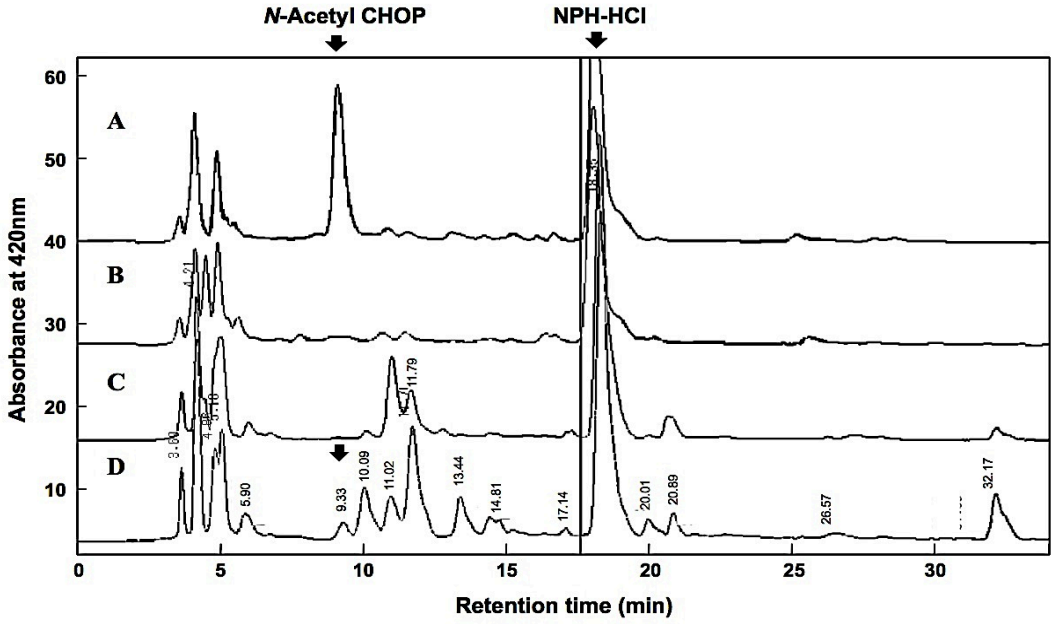


Fig. 9. HPLC analyses of *N*-acetyl CHOP. Crude extract of BL21(DE3) Δ*putA* harboring pRSF(Duet)-1 with 1 mM *N*-acetyl CHOP (**A**), BL21(DE3) Δ*putA* harboring pRSF(Duet)-1 (**B**), BL21(DE3) Δ*putA* harboring pRSF(Duet)-SmP4H (**C**), and BL21(DE3) Δ*putA* harboring pRSF(Duet)-SmP4H-Mpr1 (**D**). *E. coli* recombinant cells were cultivated at 37°C for 24 h, and the crude extracts were obtained and derivatized as described in Materials and methods. Ten ml of each derivative was subjected to HPLC. Arrows indicate *N*-acetyl CHOP and nitrophenylhydrazine (NPH)-HCl.

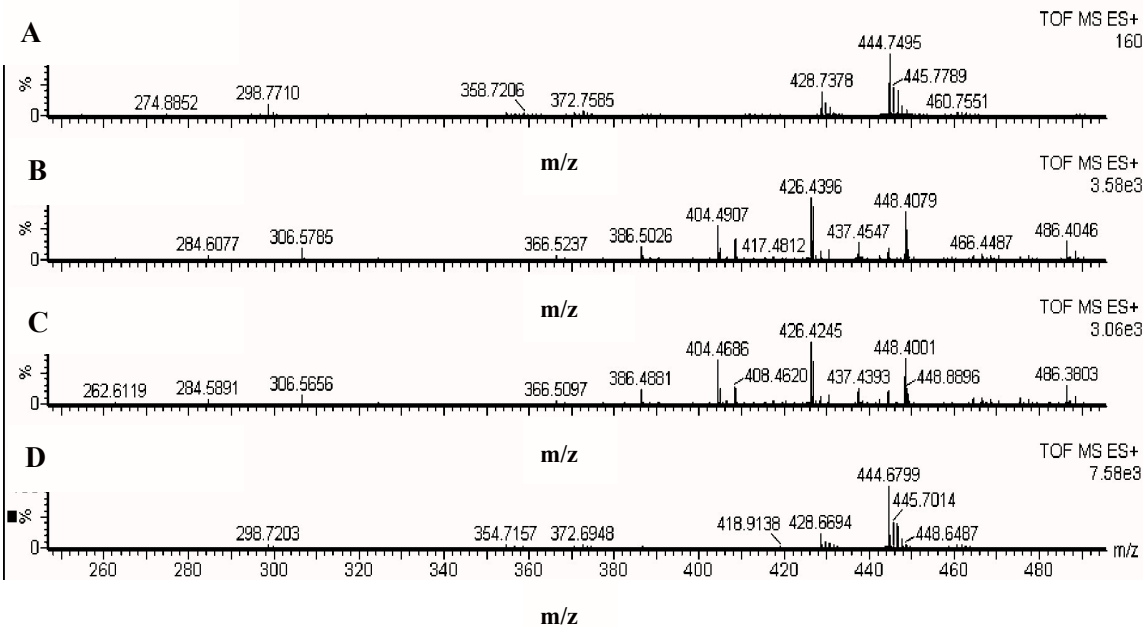


Fig. 10. LC-MS (ESI) spectra of NPH derivatives. Positive ion spectrum of 2-NPH derivative of *N*-acetyl CHOP at ionization voltage from 2 to 9.1 kV, discharge mode of 40 mA,

scan range of m/z 250-500. Retention time at 8.75 -9.2 min of crude extract pRSF(Duet) (**A**), 1mM *N*-acetyl CHOP including crude extract of pRSF(Duet)-1 (**B**), crude extract of pRSF(Duet)-SmP4H-Mpr1 (**C**) and pRSF(Duet)-SmP4H (**D**) analyzed by Micromass Q-TOF API (Micromass, England).

2.3.2. Effect of L-proline oxidase deficiency on *N*-acetyl CHOP production in *E. coli*

Table 4 shows the amounts of CHOP and *N*-acetyl CHOP in *E. coli* wild-type and Δ*putA* cells grown in LB and M9 medium containing 5 mM L-proline. There was no significant difference in L-proline contents between the wild-type cells carrying pRSF(Duet)-1 in LB or M9 medium containing L-proline. In wild-type cells carrying pRSF(Duet)-SmP4H or pRSF(Duet)-SmP4H-Mpr1, both CHOP and *N*-acetyl CHOP productivity showed an approximately 2-fold increase in M9 medium as compared to that in LB medium.

To examine whether the L-proline oxidase gene (*putA*) involved in the L-proline degradation pathway in *E. coli* affects the productivity of *N*-acetyl CHOP, I introduced pRSF(Duet)-1 carrying the genes encoding SmP4H and/or Mpr1 and the empty vector pRSF(Duet)-1 into the Δ*putA* strain. The L-proline content showed a twofold increase in the Δ*putA* mutant carrying pRSF(Duet)-1 compared to that in wild-type strain carrying pRSF(Duet)-1 (Table 4). There was no significant difference in CHOP or *N*-acetyl CHOP production between the wild-type and Δ*putA* strains harboring pRSF(Duet)-SmP4H-Mpr1. Interestingly, Δ*putA* cells carrying pRSF(Duet)-SmP4H accumulated more intracellular L-proline compared to the wild-type cells overexpressing SmP4H.

Table 4. Effect of L-proline oxidase deficiency on *N*-acetyl CHOP production in *E. coli.*

Medium	Strain	Plasmid	L-Proline	CHOP	*N*-Acetyl CHOP
			(μmol g^{-1} wet cell weight)		
LB	WT	pRSF(Duet)-1	0.28 ± 0.09	0	0
		pRSF(Duet)-SmP4H	0.13 ± 0.04	0.25 ± 0.08	0
		pRSF(Duet)-SmP4H-Mpr1	0.10 ± 0.06	0	1.5 ± 0.34
M9	WT	pRSF(Duet)-1	0.39 ± 0.12	0	0
		pRSF(Duet)-SmP4H	0.09 ± 0.02	0.46 ± 0.15	0
		pRSF(Duet)-SmP4H-Mpr1	0.12 ± 0.08	0	2.8 ± 0.52
M9	Δ*putA*	pRSF(Duet)-1	0.70 ± 0.15	0	0
		pRSF(Duet)-SmP4H	1.10 ± 0.10	0.39 ± 0.16	0
		pRSF(Duet)-SmP4H-Mpr1	0.30 ± 0.15	0	3.2 ± 0.64

E. coli BL21(DE3) (WT) and BL21(DE3) Δ*putA* (Δ*putA*) harboring pRSF(Duet)-1, pRSF(Duet)-SmP4H and pRSF(Duet)-SmP4H-Mpr1, respectively, were cultured at 37˚C for 24 h in LB or M9 containing 5 mM L-proline, 30 μg/ml kanamycin and 0.4 mM IPTG.

2.3.3. Effect of L-proline and NaCl on *N*-acetyl CHOP production in *E. coli*

As shown in Fig. 11A, the cellular L-proline contents of the wild-type and Δ*putA* strain were increased by approximately two- and fivefold, respectively, under the osmotic stress condition. There was no significant difference in L-proline content at higher concentrations of NaCl (Supplementary Fig. 2A). Fig. 11B shows that *N*-acetyl CHOP production in the Δ*putA* strain was increased by approximatey 50% when 1% NaCl was added. There was no significant difference in *N*-acetyl CHOP production in the Δ*putA* strain in the presence or absence of L-proline in M9 medium (Fig. 11C). However, no addition of L-proline resulted in growth inhibition of the cells, whereas L-proline enhanced the cell growth under the osmotic stress condition (Fig. 13B). Thus, we expected that the addition of L-proline to the medium could improve *N*-acetyl CHOP production, and we used 5 mM L-proline for further analyses.

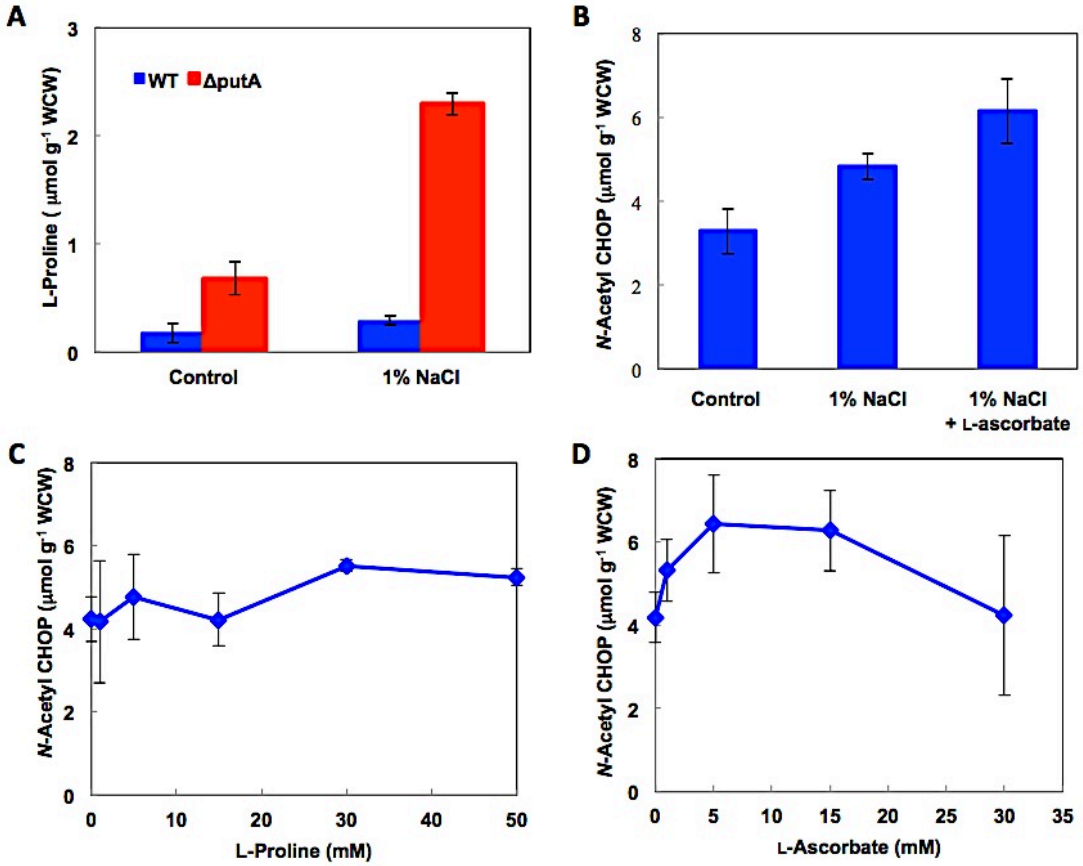


Fig. 11. Changes in L-proline and *N*-acetyl CHOP contents under various conditions. **(A)** Effect of *putA* disruption on intracellular L-proline contents. **(B)** Effect of NaCl and L-ascorbate on *N*-acetyl CHOP production. **(C)** Effect of L-proline on *N*-acetyl CHOP production. **(D)** Effect of L-ascorbate on *N*-acetyl CHOP production. Wild-type and Δ*putA* strains harboring the empty vector pRSF(Duet)-1 (**A**), and Δ*putA* strain harboring pRSF(Duet)-SmP4H-Mpr1 (**B-D**) were cultivated in 15 ml of M9 medium at 37°C for 24 h under various conditions indicated in the panels. L-Proline (**A**) and *N*-acetyl CHOP (**B-D**) contents were measured as described in Materials and methods.

2.3.4. Effect of L-ascorbate on *N*-acetyl CHOP production

L-Ascorbate is not necessarily required for the L-proline hydroxylation reaction catalyzed by SmP4H, but it enhances the reaction rate (Hara and Kino. 2009; Myllyla et al., 1978). *E. coli* cells can use L-ascorbate as the carbon source under aerobic conditions via the *yiaK-S* operon, although L-ascorbate is not synthesized de novo (Campos et al., 2007). Here, we examined the effect of L-ascorbate on *N*-acetyl

CHOP production (Fig. 11D). At low concentrations of L-ascorbate, *N*-acetyl CHOP production was increased by 30% to 60% as compared to that observed in the absence of L-ascorbate. However, higher than 15 mM L-ascorbate decreased *N*-acetyl CHOP production, inhibited cell growth and induced brown color production, which was probably due to the oxidation of L-ascorbate (Fig. 12B). Thus, I used 5 mM L-ascorbate for the optimized medium. The addition of 5 mM L-ascorbate resulted in a 20% increase in *N*-Acetyl CHOP production under the osmotic stress condition (Fig. 11B and Fig. 12A). Higher concentrations of NaCl with 5 mM L-ascorbate reduced the *N*-acetyl CHOP production (Supplementary Fig. 2B). As the results, 1% NaCl and 5 mM L-ascorbate were used in the optimized medium for further experiment.

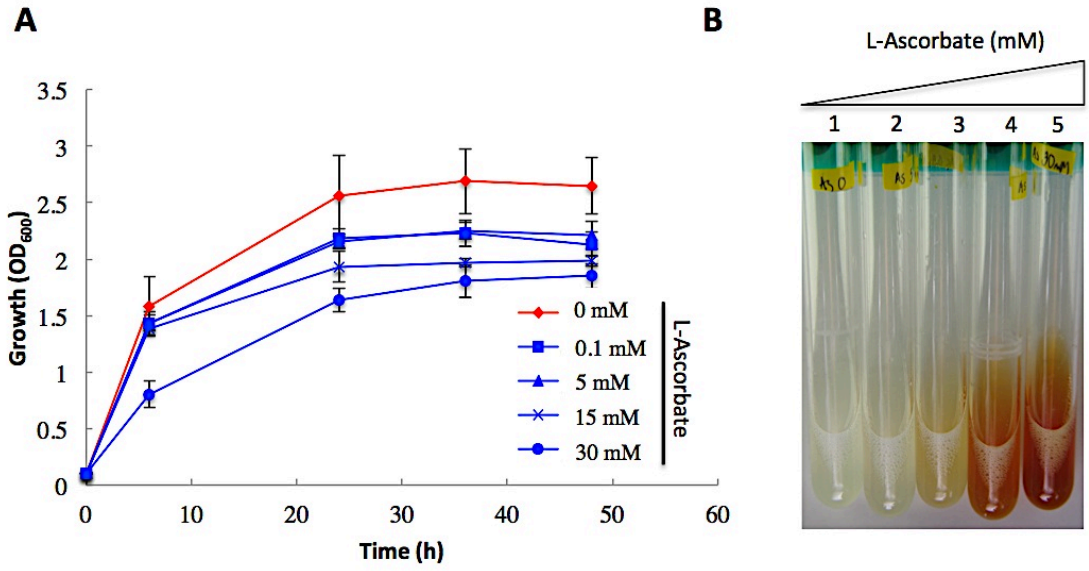


Fig. 12. Growth and color formation of *E. coli* cells that co-overexpress SmP4H and Mpr1 under L-ascorbate containing medium. (**A**) Growth curve of *E. coli* cells co-overexpressing SmP4H and Mpr1 in 0.1 mM (diamond), 0.1 mM (square), 5 mM (triangle), and 15 mM (cross) of L-ascorbate in M9 medium containing 5 mM L-proline and 1% NaCl. As a control, *E. coli* cells carrying pRSF(Duet)-SmP4H-MPR1 were cultivated in the absence of L-ascorbate (circle). The values are the means from three independent experiments. (**B**) Brown color production in *E. coli* Δ*putA* cells harboring pRSF(Duet)-SmP4H-MPR1 in M9 medium containing 5 mM L-proline, 1% NaCl and 0, 0.1, 5, 15, and 30 mM L-ascorbate (tubes #1, 2, 3,

4, and 5,respectively).

2.3.5. Effect of carbon source on *N*-acetyl CHOP production

It was expected that the addition of 2-oxoglutarate enhanced *N* -acetyl CHOP production because this compound is another substrate for SmP4H. Furthermore, I also considered that 2-oxoglutarate is a member of TCA cycle and could be effectively supplied by the addition of glucose. So, effect of glucose and 2-oxoglutarate on the optimal production of *N*-acetyl CHOP was examined (Fig. 13). The combination of 2-oxoglutarate with glucose showed the highest yield of 7.0 μmol *N*-acetyl CHOP per g wet cell weight (Fig. 13A). The addition of L-proline did not increase the productivity of *N*-acetyl CHOP (Fig. 13A), but enhanced the cell growth under the oxidative stress condition caused by L-ascorbate (Fig. 13B). When L-ascosbate is utilized as the sole carbon source (in the absence of glucose), the growth and *N*-acetyl CHOP productivity was significantly reduced (Fig. 13A and 13D). These results suggest that utilization of not only currently used glucose but also 2-oxoglutarate as an alternative carbon source is effective for the production of *N*-acetyl CHOP in *E. coli* cells. 2-Oxoglutarate, one of the substrates of these hydroxylation reactions, is supplied from glucose in the reaction medium via the Embden-Meyerhof pathway and TCA cycle in *E. coli*, and the product succinate is recycled (Fig. 16). When the *E. coli* strain that overproduces L-proline is used as the host cell, the large-scale production of CHOP from glucose will be possible. Thus, glucose was selected to optimize the medium. Next, I examined the effect of glucose concentration on *N*-acetyl CHOP production (Fig. 13C). At low concentration of glucose, *N*-acetyl CHOP product was a 2.5-fold increase as compared with that in the medium in the absence of glucose. There was no significant difference in

N-acetyl CHOP productivity at high concentrations of glucose and 0.4 % glucose was used for further analyses.

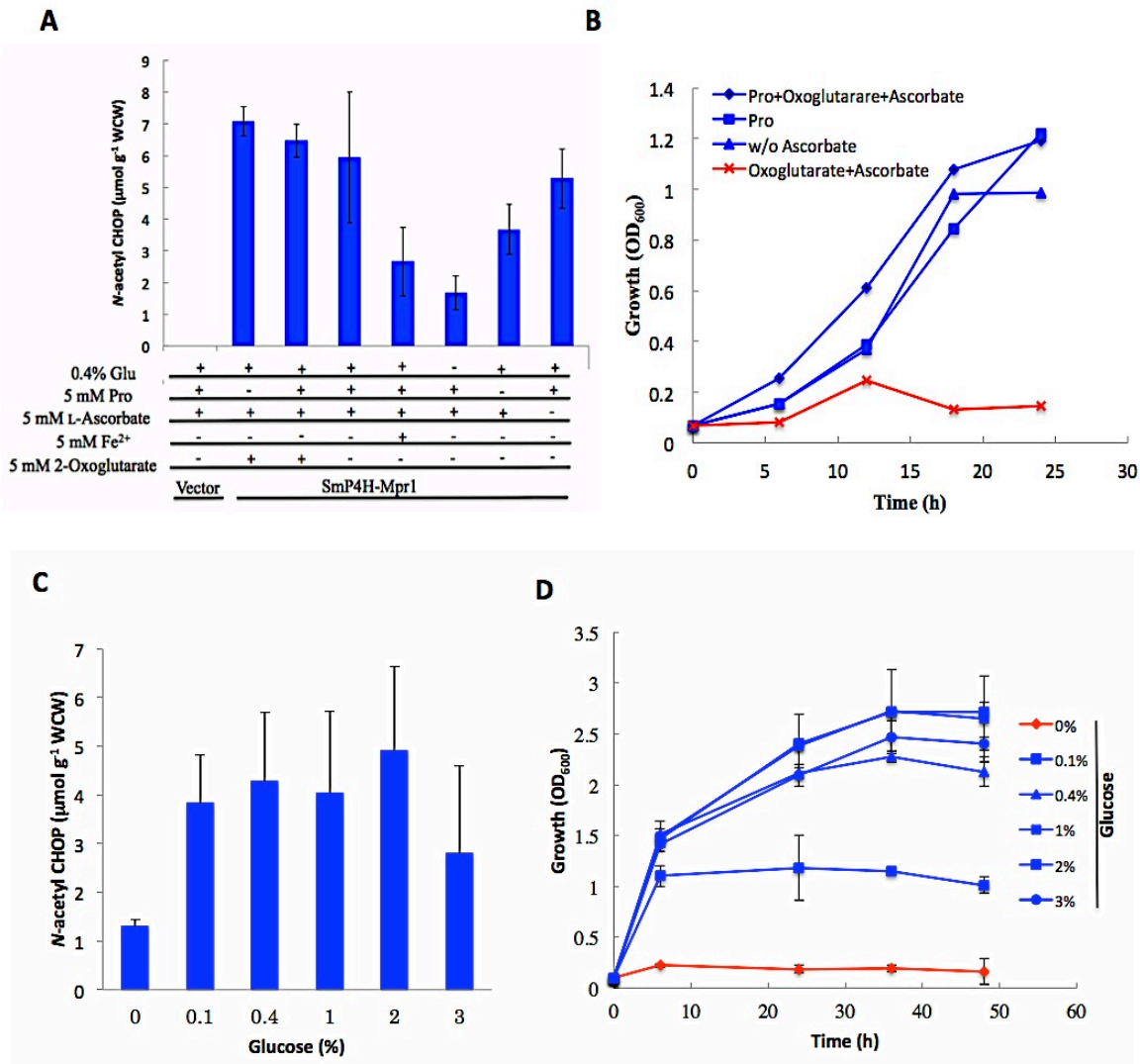


Fig. 13. Effect of carbon sources on *N*-acetyl CHOP productivity and cell growth. Effects of glucose and 2-oxoglutarate on *N*-acetyl *cis*-4-hydroxy L-proline synthesis (**A**) and cell growth (**B**) ability under L-ascorbate addition. (**C**) Glucose dependence of *N*-acetyl CHOP production in optimized M9 medium. (**D**) Growth curve of *E. coli* Δ*putA* cells co-expressing SmP4H and Mpr1 at 0.1% (close square), 0.4% (triangle), 1% (cross), 2% (open square) and 3% (circle) of gluocose in M9 medium containing 5 mM L-proline, 5mM L-ascorbate and 1% NaCl. As a control, *E. coli* Δ*putA* cells carrying pRSF(Duet)-SmP4H-MPR1 were cultivated at 5 mM L-proline and L-ascorbate in the absence of glucose (diamond). The values are the means from three independent experiments.

2.3.6. Production of *N*-acetyl CHOP in *E. coli* cells under the optimized conditions

The *E. coli* Δ*putA* strain harboring pRSF(Duet)-SmP4H-Mpr1 was cultivated at 37°C in an optimized M9 medium containing 5 mM L-proline, 5 mM L-ascorbate, 0.1 mM IPTG and 1% NaCl (pH 7.5), and we examined the growth and the *N*-acetyl CHOP productivity. As shown in Fig. 14A, the *N*-acetyl CHOP level was slightly increased during 24 h fermentation. After 24 h, the productivity was dramatically increased, and the highest yield of *N*-acetyl CHOP production was 26.5 μmol g^{-1} wet cell weight (g^{-1} WCW) at 42 h. Western blot analysis revealed that the SmP4H protein level gradually increased from 0 h to 24 h (Fig. 14B). High-level expression of SmP4H and Mpr1 was observed after 24 h, suggesting that the *N*-acetyl CHOP productivity depends on the expression level of SmP4H. However, the decrease of the intracellular *N*-acetyl CHOP level commenced after 42 h of cultivation, probably due to the *N*-acetyl CHOP degradation in *E. coli* cells.

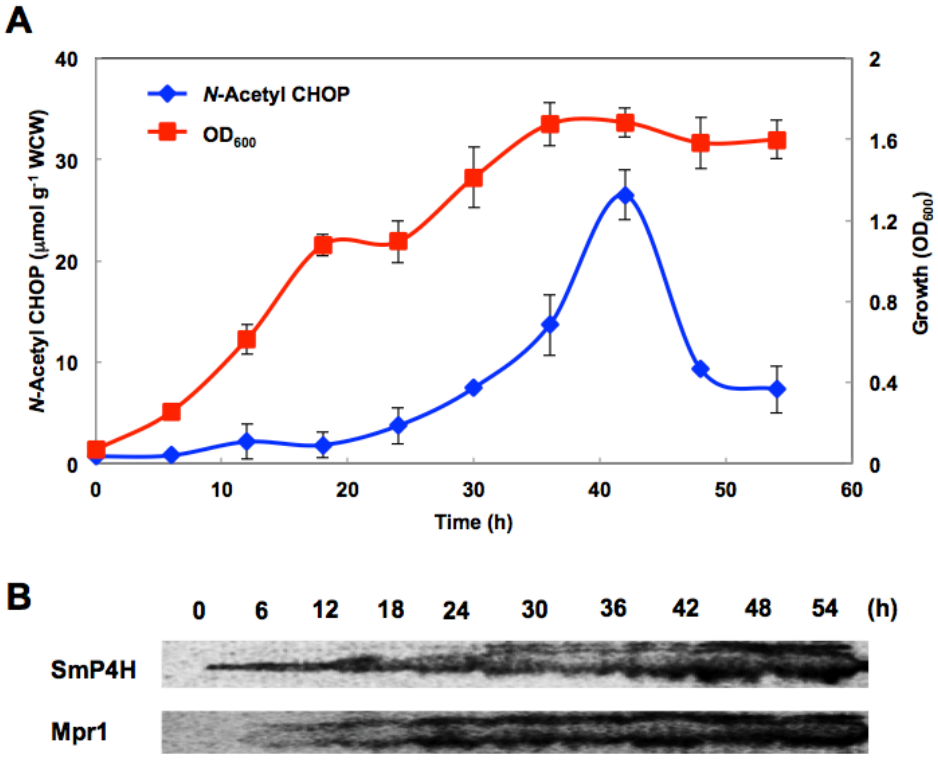


Fig. 14. Time course of *N*-acetyl CHOP production under the optimized conditions. (**A**) *E. coli* Δ*putA* strain harboring pRSF(Duet)-SmP4H-Mpr1 was cultivated at 37°C in the optimized medium, and the cells were taken at the period indicated and the growth (square) and *N*-acetyl CHOP production (diamonds) were measured. (**B**) Western blot analysis was performed to confirm the expression of SmP4H and Mpr1. 10 μg proteins in the total cellular fraction were subjected to 12.5% SDS-polyacrylamide gel electrophoresis. The SmP4H and Mpr1 proteins were detected using His-tag and Mpr1 polyclonal antibody, respectively.

To confirm *N*-acetyl CHOP production in *E. coli* recombinant strains, we purified *N*-acetyl CHOP from the cells cultivated in 5 l of the optimized medium (1.2 ± 0.4 g^{-1} WCW l^{-1}). The purification yield was 10%, but the additional desalting step required for MS analysis decreased the yield to 0.4%. The purity of *N*-acetyl CHOP was approximately 50%. Chemically synthesized (0.17 mg) and purified *N*-acetyl CHOP (0.1 mg) was subjected to quadruple time of flight mass spectrometry (Q-TOF MS) using the negative ion mode. Fig. 15B indicates that the fragment pattern of the purified *N*-acetyl CHOP from *E. coli* Δ*putA* cells harboring pRSF(Duet)-SmP4H-Mpr1 was consistent with that of the standard *N*-acetyl CHOP (Fig. 15A) with a major ion peak at m/z=172.07 $(M-H)^-$. The negative control with 50% methanol showed various peaks at m/z=89.04, 93.05, 130.07, 157.14, 212.1, 219.1, and 255.25 (Fig. 15C). These peaks were also found in standard and the purified *N*-acetyl CHOP.

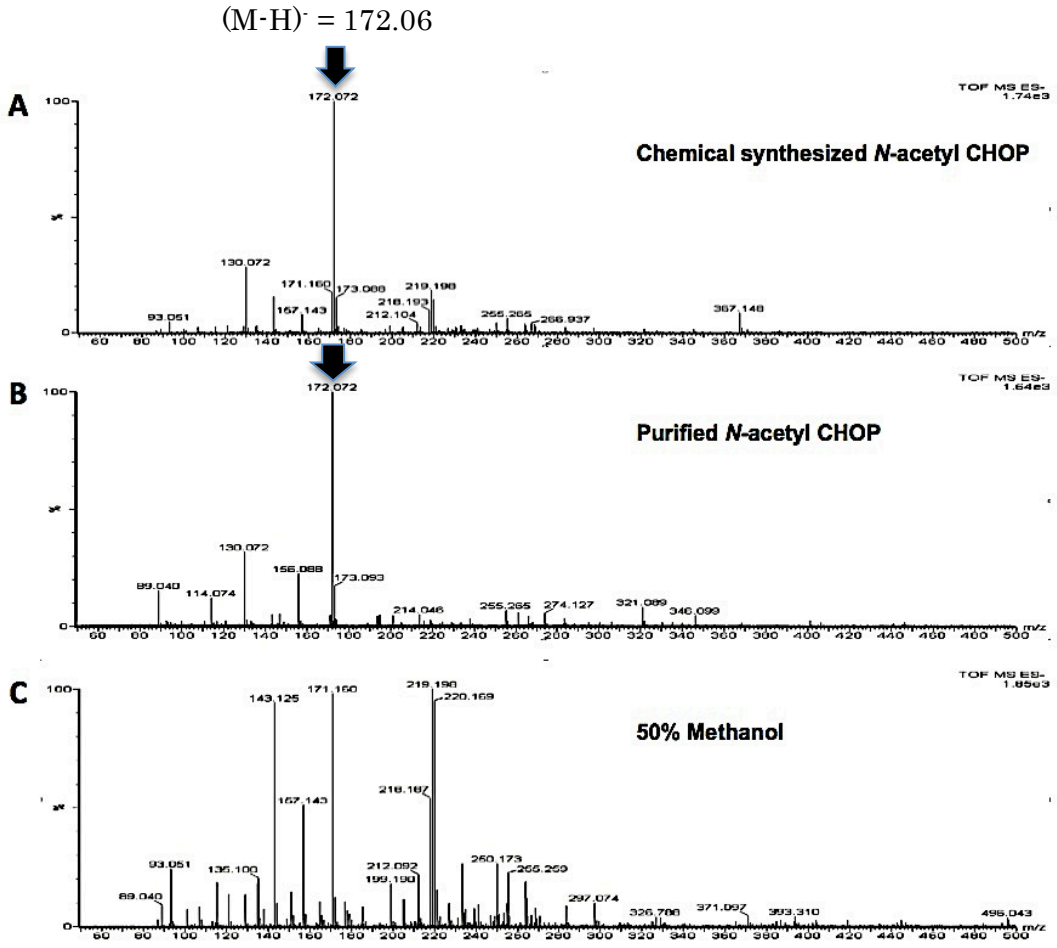


Fig. 15. Q-TOF MS analysis of chemically synthesized (**A**) and purified (**B**) *N*-acetyl CHOP, and (**C**) 50% methanol (control).

2.4. Discussion

The free *N*-acetylamino acids have no reactive groups other than a carboxyl group. The free carboxyl group was derivatized with 9-anthrydiazonmethane (ADAM) or 1-pyrenyldiazomethane (PDAM) (Kawakamin et al., 1992). Unfortunately, ADAM and PDAM are expensive and unstable regents for detecting free *N*-acetylamino acid in biological samples. Thus, 2- nitrophenylhydrazine (2-NPH) is widely used for the derivatization of a carboxyl group in industrial and biological samples (Miwa et al., 1985; Peter et al., 204; Yomota and Ohnishi, 2007). As show in Fig. 9, *N*-acetyl CHOP was drivatized with 2-NPH-HCl to the corresponding carboxyl group. The derivatized *N*-acetyl CHOP was separated by

optimized HPLC. However, the first limit of detection for *N*-acetyl CHOP in crude extract is the contaminated carboxyl group from small compounds and proteins. Thus, 0.07 to 10 mmol of *N*-acetyl CHOP could be detected in crude extract (OD =20). The second limit of detection for derivative *N*-acetyl CHOP with LC-MS is a side product from the reaction of EDC and 2-NPH specifically catalyzed by cyclic amide (Peter et al., 2004). Derivatives of *N*-acetyl CHOP could be detected similar to tested samples (pRSF(Duet)-SmP4H-Mpr1), but not as control samples (blank, pRSF(Duet)-1, and pRSF(Duet)-SmP4H).

The *E. coli* L-proline oxidase PutA is a bifunctional enzyme with both proline dehydrogenase and pyrroline-5-carboxylate dehydrogenase activities. PutA is an FAD-dependent and membrane-associated enzyme which transfers electrons from L-proline to the respiratory chain. In addition to its enzymatic activities, the PutA protein acts as a repressor of *putA* and *putP* in response to extracellular L-proline (Ling et al., 1994; Wood, 1987). Thus, we expected that deletion of *putA* would increase the intracellular L-proline level in *E. coli*, and we used the Δ*putA* strain as the host. As a result, L-proline and *N*-acetyl CHOP contents were increased by five- and 1.5-fold in Δ*putA* cells under the osmotic stress condition, respectively (Fig. 11B and Table 4). However, *N*-acetyl CHOP productivity was not affected by the addition of L-proline to the medium under the osmotic stress conditions (Fig. 14B and C), although L-proline improved the cell growth and consequently the total amount of *N*-acetyl CHOP was higher than that without the addition of L-proline (Fig. 13B). These results suggest that L-proline biosynthesis is accelerated in Δ*putA* cells under osmotic stress conditions. Thus, intracellular L-proline was used for the synthesis of *N*-acetyl CHOP and is also required to protect cells from osmotic stress (Grothe et al., 1986).

In the present study, we established a novel method for *N*-acetyl CHOP production from L-proline. It is considered that *N*-acetyl CHOP is produced by conversion from L-proline, but 2-oxoglutarate and acetyl CoA are supplied through the pathways of glycolysis and TCA cycle in *E. coli* (Fig. 16). Thus, glucose-containing medium induces CHOP and *N*-acetyl CHOP production (Table 4). However, L-proline accumulation in the *putA* mutant did not increase CHOP production in cells expressing SmP4H, whereas *N*-acetyl CHOP was slightly increased by coexpression of SmP4H and Mpr1 in Δ*putA* cells. It was suggested that the growth and *N*-acetyl CHOP production in cells were relevant to the limited activity of SmP4H. Our hypothesis is that CHOP could be incorporated into the newly synthesized proteins competitively with L-proline, leading to growth inhibition or cell death caused by the accumulation of abnormal proteins. Thus, it is difficult to describe material balance among L-proline, CHOP, and *N*-acetyl CHOP without understanding of CHOP metabolism and CHOP-tolerant mechanism in *E. coli*. The addition of L-proline to the medium increased intracellular L-proline level, not *N*-acetyl CHOP content (Fig. 11C). However, the cell growth is inhibited in the absence of L-proline under the osmotic stress condition, leading to a decrease in the yield of *N*-acetyl CHOP. We think that L-proline plays an important role as an osmoprotectant and inhibits the incorporation of CHOP into the nascent proteins in *E. coli* cells.

However, *in vivo* CHOP synthesis may be crucial to improve the productivity of *N*-acetyl CHOP. The purified SmP4H from *E. coli* had the specific activity of 607 nmol min^{-1} mg^{-1} of protein (Supplementary Table 1). The conversion rate from L-proline (5 mM) to CHOP was approximately 10% *in vitro*. Since SmP4H described here is a member of the 2-oxoglutarate-dependent dioxygenase family and requires

ferrous ion, 2-oxoglutarate, and L-ascorbate for full activity, adding ferrous ion to the medium may be effective for *N*-acetyl CHOP production, and/or to enhance the 2-oxoglutarate synthesis in *E. coli* cells. The addition of L-ascorbate has been explored for the optimization for bacterial prolyl hydroxylase reactions (Myllyla et al., 1978). In the present study, the addition of both L-ascorbate and NaCl resulted in a 2.2-folds increase in *N*-acetyl CHOP productivity (Fig. 11B). However, the combination of higher concentrations of NaCl and L-ascorbate caused a reduction in *N*-acetyl CHOP productivity (Supplementary Fig. 2). We observed an *in vitro* degradation of *N*-acetyl CHOP (Chapter III, Fig. 18 and 19), suggesting that knockout of the genes involved in the degradation improves the *N*-acetyl CHOP productivity.

We examined the degradation of *N*-acetyl CHOP with *E. coli* crude extract. Approximately 30% of *N*-acetyl CHOP was degraded after 10 h incubation (Chapter III, Fig. 19A). Some compounds were detected by this decomposition on TLC (Chapter III, Fig. 18). *E. coli* cells cannot utilize CHOP and *N*-acetyl CHOP as the carbon and nitrogen sources (Supplementary Fig. 4), but the cells expressing SmP4H or coexpressing SmP4H and Mpr1 produced some unknown compounds detected by HPLC (Fig. 9). It is interesting to see the relationship between the unknown compounds and *N*-acetyl CHOP metabolism. This study is the first to report the microbial production of *N*-acetyl CHOP. Further improvement in its production is therefore expected to weaken its degradation pathway.

The recombinant cells expressing only SmP4H were used to examine CHOP production in the optimized medium containing L-ascorbate. However, the cells showed slow growth in the medium and the pigment formation occurred in recombinant cells after 40 hours cultivation (Supplementary Fig. 6). Furthermore,

the intracellular CHOP did not increase in a time dependent manner (data not shown). These results suggested that Δ*putA* strain expressing SmP4H did not only produced cytotoxic compounds but also suffered from osmotic and oxidative stress in the optimized medium. *E. coli* cells may degrade CHOP to avoid the toxicity. Rational production of CHOP in *E. coli* cells should be examined for further improvement of the *N*-acetyl CHOP production.

At this time, there are no experimental data or references regarding *N*-acetyl CHOP, to our knowledge, in part because *N*-acetyl CHOP has not yet been commercialized. Cohen and Diegelmann (1978) reported the effect of *N*-acetyl CHOP on collagen synthesis, but those authors mistook oxaceprol (*N*-acetyl THOP) for *N*-acetyl CHOP. Oxaceprol is currently well known as an atypical inhibitor of inflammation, and is useful for the treatment of diseases affecting the connective tissues. In general, the toxicity of CHOP is dependent on the efficiency of transport into cells (Edward et al., 2011; Tristram and Neale, 1968), incorporation into newly synthesized protein (Penny and Karin 2000), and also collagen types in mammalian cells (Tan and Ryhane, 1983). *N*-Acetylation of CHOP will provide a soluble drug that is useful for drug delivery across epithelial barriers via physiological transport systems. Importantly, Poiani et al., (1994) proved that the modification of the amino group in CHOP by conjugating with poly(PEG-Lys) has antifibrotic effects *in vivo* and *in vitro*. The *N*-acetylation of CHOP might be a novel approach as a prodrug instead of the conjugation of poly(PEG-Lys). Moreover, the substrate specificity of carriers, the design of prodrug substrates, and the pharmacogenetics relevant to drug transporters have gained enormous interest in recent years.

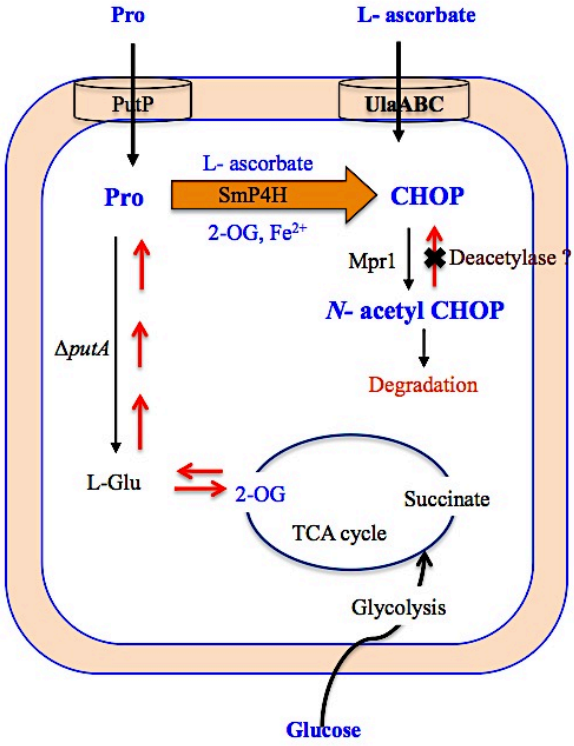


Fig. 16. Summary of *N*-acetyl CHOP biosynthesis in *E. coli* cells that overexpress SmP4H and Mpr1. L-Proline is transported via proline permease (PutP) into *E. coli* cells. The Rhizobium SmP4H catalyzes the reaction of L-proline *cis*-4-hydroxylas, which hydroxylates L-proline to CHOP requiring cosubstrates, such as oxoglutarate, ferrous, and L-ascorbate. CHOP can be then acetylated into *N*-acetyl CHOP by the yeast *N*-acetyltransferase (Mpr1). Proline oxidase (PutA) mutant strain accumulates L-proline in the cells. Glucose enters the glycolytic pathway and the TCA cycle to form 2-oxoglutarate (2-OG). 2-OG can be converted into L-glutamate (Glu) that is importannt for L-proline biosynthesis.

Chapter III

Metabolism of *N*-acetyl *cis*-4-hydroxy-L-proline in *Escherichia coli*

3.1. Introduction

Acetylation widely occurred in α-amino group of protein amino-terminal residues or ε-amino group of protein lysine residue. Acetylation of histones has a crucial role in transcriptional regulation and of non-histone proteins involves in control cell cycle progression in eukaryote and prokaryote, respectively (Kouzarides, 2000). While, majority functions of histone deacetylation are transcriptional repressors. There are two families of deacetylase enzymes: the polysaccharide deactylase and the histone deacetylases (HDACs) or the Sir2 (silent information regulator)-like family of NAD-dependent deacetylases (Gray and Ekstrom, 2001). HDACs and Sir2 have been conserved in prokaryotes and eukaryotes, and both proteins have more specific functions in the regulation of key downstream genes and transcription. Moreover, the deacetylation process is dynamically and temporally regulated by histone deacetylases. There is no comparison of deacetylase activity for free amino acids and protein amino acid residues.

L-Hydroxyproline differs from L-proline by the presence of a hydroxyl group attached to the γ carbon atom. The hydroxylation of L-proline is done mainly by the enzyme prolyl 4, 3-hydroxylase as a post-translational modification protein in mammals or by proline *cis/trans* 3, 4-hydroxylase in bacteria. The appearance of free L-hydroxyproline in mammals is caused by degradation of collagen protein. Thus, the decrease or increase in excretion of L-hydroxyproline in patients has been used as a signal diagnosis for different diseases involved in collagen metabolism,

such as disorders of bone metabolism and prostatic carcinoma. In contrast, the synthesis of L-hydroxyproline has not yet been clarified by bacterial studies. It has been suggested that proline hydroxylase converts free L-proline into L-hydroxyproline (Katz et al., 1978; Shibaki et al., 2000). In animals, L-Hydroxyproline catabolism has been clarified that produces 4-hydroxyl-2-ketoglutarate (Wu et al., 2011) (Fig. 17A). In bacteria, *Pseudomonas* strain isolated from soil that can utilize THOP as a carbon-nitrogen source. L-Hydroxyproline catabolism of bacteria differs from the catabolism of mammals (Adams and Leonard, 1980). This pathway includes the conversion of THOP to CHOP (D) and then to α-ketoglutaric acid (Fig. 17B). Preliminary studies showed that the *Pseudomonas* pathway is present in other bacterial strains, including other *Pseudomonas* isolates (Chang and Adams, 1977; Thacker, 1969), *Achromobacter* (Jayaraman and Radhakrishnan, 1965) and *Aerobacter aerogenes* (Gryder and Adams, 1978). These researches suggested that catabolic pathway for THOP may be present in a wide variety of bacteria. The detection of pyrrole-2-carboxylic acid (P2C) is a signal of the degradation of THOP.

The initial step in the degradation pathways of L-proline and L-hydroxyproline is catalyzed by proline oxidase and hydroxyproline oxidase, respectively, yielding L-Δ^1-pyrroline-5-carboxylic acid (P5C) and L-Δ^1-pyrroline-3-hydroxy-5-carboxylic acid (3-OH-P5C), respectively. Hydroxyproline oxidase differs from proline oxidase with regard to its substrate recognition. In mammalian cells, L-amino acid oxidase acts mainly on the L-isomer of THOP. However, in *Pseudomonas* sp., D-amino acid oxidase is able to oxidize the D-isomer of CHOP to P2C after converting the L-isomer of THOP into the D-isomer of CHOP (Chang and Adams, 1977; Yoneya and Adams, 1961; Manoharan, 1980). Moreover, oxidization of the D-isomer of CHOP to P2C was

found in rat kidney, which is similar to the hydroxyl catabolism in bacteria (Letellier and Bouthillier, 1956; Radhakrishnan and Meister, 1957). There may be distinct pathways of catabolism for *cis* and *trans* L-hydroxyproline, but studies on catabolism are lacking for the L-form of CHOP. The second step is the oxidation of P5C to L-glutamic acid and of 3-OH-P5C to γ-hydroxy-glutamic acid. These products are somehow recycled for growing of bacteria and mammals cells. However, there is no information to clarify physiological roles of P2C in prokaryotic and eukaryotic cells

In my previous experiments (Chapters I and II), *N*-acetyl CHOP was produced and purified by coexpressing SmP4H and Mpr1 in *E. coli* cells. Next, to optimize of the *N*-acetyl CHOP production, I examined the cellular metabolism of *N*-acetyl CHOP. To improve *N*-acetyl CHOP productivity, one of the candidate strategies is to weaken its degradation pathway. Therefore, I attempted to identify enzymes involved in the degradation of *N*-acetyl CHOP using the *E. coli* mutant library. I also tried to isolate the *E. coli* proteins into which CHOP is incorporated, to predict essential proteins required for CHOP and *N*-acetyl CHOP catabolism using proteome analysis. The third endeavor was to engineer enhanced *cis*-4-hydroxylase (SmP4H) activity for the efficient conversion of L-proline into *N*-acetyl CHOP.

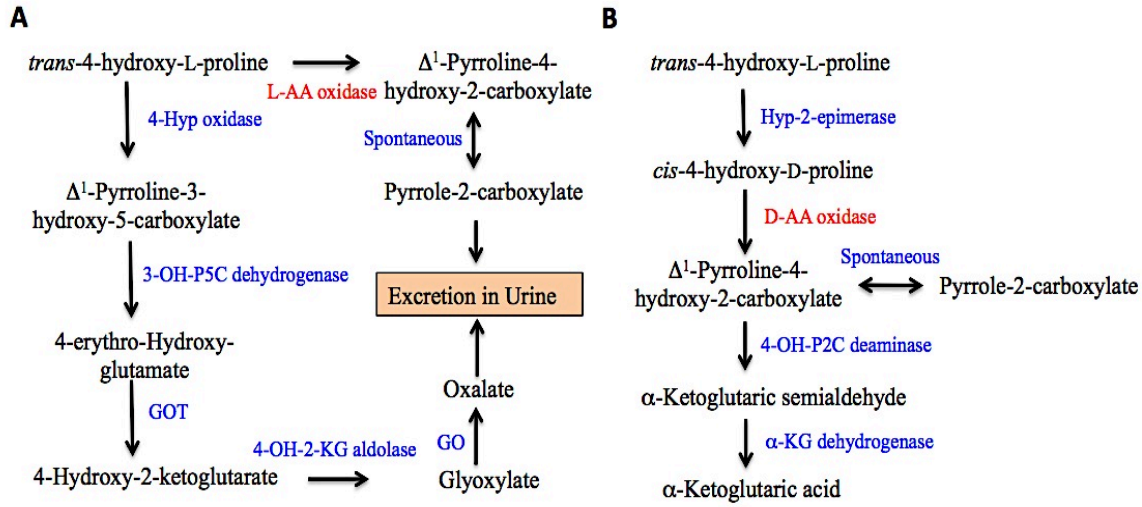


Fig. 17. Metabolism of L-hydroxyproline in animals (**A**) or bacteria (**B**). (**A**) 4-Hyp oxidase, trans-4-hydroxy-L-proline oxidase; L-AA oxidase, L-amino acid oxidase; 3-OH-P5C dehydrogenase, Δ1-pyrroline-3-hydroxy-5-carboxylate; GOT, glutamate oxaloacetate transaminase; GO, glycolate oxidase; 4-OH-2-KG aldolase, 4-hydroxy-2-ketoglutarate aldolase;, Δ1-pyrroline-3-hydroxy-5-carboxylate;. (**B**) Hyp-2-epimerase, hydroxy-proline-2-epimerase; D-AA oxidase, D-amino acid oxidase; 4-OH-P5C dehydrogenase, Δ1-pyrroline-4-hydroxy-5-carboxylate dehydrogenase; 4-OH-P5C deaminase, Δ1-pyrroline-4-hydroxy-2-carboxylate deaminase; 4, α-KG dehydrogenase, α-ketoglutaric semialdehyde dehydrogenase.

3.2. Materials and Methods

3.2.1. Strains and plasmids

E. coli strain BW25113 (Δ*(araD-araB)567* Δ*lacZ4787*(::rrnB-3) *λ*⁻ *rph-1* Δ*(rhaD-rhaB)568 hsdR514),* a wild type and deacetylase mutants: such as Δ*nagA* (BW25113 Δ*nagA*::kan), Δ*agaA* (BW25113 Δ*agaA*::kan), Δ*cobB* (BW25113 Δ*cobB*::kan), Δ*yadE* (BW25113 Δ*yadE*::kan), Δ*yibQ* (BW25113 Δ*yibQ*::kan), Δ*pgaB* (BW25113 Δ*pgaB*::kan), Δ*ygeY* (BW25113 Δ*ygeY*::kan), and Δ*argE* (BW25113 Δ*argE*::kan) (provided by Dr. Hirotada Mori, Nara Institute of Science and Technology) were used for screening *E. coli* genes involved in deactylation of

N-acetyl CHOP.

3.2.2. Amino acid derivatization

L-Proline and hydroxyl-L-proline analogs were analyzed by the pre-column derivatization with an advanced Marfey's method, reported previously (Fujii et al., 1997). Samples containing L-proline and hydroxyl-L-proline were derivatized as follows. Five microliters of 2 M NaOH was added to 200 μl of sample, and 50 μl of 15 mM fluorodinitrophenyl-5-L-leucine amide (FDLA) dissolved in acetone was added. This solution was mixed and incubated at 40°C for 1 h. After the derivatization was quenched by the addition of 100 μl of 1 M HCl, this mixture was diluted with 700 μl of methanol and then 10 μl was subjected to HPLC analysis. The reaction mixtures containing hydroxyl-L-proline analogues were analyzed using HPLC (L-2000 series; Hitachi, Tokyo, Japan). Chromatographic conditions were as follows. Separations were carried out on a Hydrosphere C18 column (YMC, Kyoto, Japan) maintained at 40°C. Methanol-formate (pH 2.5) was used as the mobile phase under a linear gradient (30-70%, 20 min). The flow rate was 0.5 ml/min, and derivatized hydroxyl-L-proline analogs were detected by their UV absorption at 331 nm.

3.2.3. Two-dimensional isoelectric focusing (2D IEF)/SDS-PAGE analysis

E. coli cells of strain BL21(DE3) were grown overnight on 15 ml of 1.5% NaCl-supplemented LB medium with or without 5 mM CHOP. The cell culture was centrifuged at 13,000 g for 10 min, and the pellet was suspended in distilled water. The washed cells were collected and lysed in 1 ml of Protein Extraction Reagent (2-D Clean-up kit, Amersham Biosciences, Buckinghamshire, England) using a

2-min sonication. The cell debris was removed by centrifugation, and the total protein concentration in the cell lysate was estimated by the Bradford method (Bradford, 1976). The *E. coli* lysate was diluted to 200 mg proteins/ml. An aliquot volume (150 μl) of lysate solution was loaded onto a sample paper and put onto a gel strip pI 7-4 (GE Healthcare, Buckinghamshire, UK). Electrophoresis was performed in Cool Phore Star IPG-IEF Type P (Anatech, San Diego, CA) with 3500 V and 160 mA (http://proteome.tmig.or.jp/2D/2DE_method.html). After the first-dimension run, the gel strip was incubated with SDS-treatment solution for 30 min at room temperature. The gel strip was drained briefly and placed on top of a 12 cm x 14 cm x 3 mm 12% acrylamide gel. The second-dimension separation was performed under the conditions of Tokyo Metropolitan Institute of Gerontology standard methods in proteomics (Toda and Kimura, 1997). Gels were stained with SYPRO™ Ruby protein gel stain kit (Bio-Rad, Hercules, CA).

3.2.4. Thin-layer chromatography (TLC) for analysis of *N*-acetyl CHOP degradation

The reaction mixture was treated with 80% acetone to remove protein, and concentrated in vacuo using a Concentrator 5301 (Eppendorf, Hamburg, Germany). An aliquot (10 μl) of the sample was spotted onto a 10 x 10 cm plate (Silicagel 60 F_{245}, Merck, Darmstadt, Germany). One-dimensional thin layer chromatography TLC analysis was performed with *n*-butanol: acetic acid: water in a volume ratio of 4:1:1 as the mobile phase. Spots were observed under UV light at 366 nm before spraying with 1% potassium permanganate, 2.4% phosphomolylidic acid, 0.2% ninhydrin, or 0.3% bromocresol green. *N*-Acetyl CHOP and CHOP (2 mM each) were used as the standards.

3.2.5. Hydroxyproline assay by ninhydrin colorimetric method

The assay (Moore, 1968) measures the reaction containing 2 mM isomer hydroxylproline and 7.8 mg/ml active crude extracts or heat-treated crude extracts incubated at 37°C for 10 h. Two hundred microliters of 0.35% ninhydrin was added to 1 ml of 5X diluted reaction mixture, and then reacted at 80°C for 10 min. After cooling to room temperature, absorbance at 440 nm of the sample was measured with a DU-640 spectrophotometer (Beckman Coulter, Fullerton, CA). Amino acid concentration was determined by comparing of the absorbance at 440 nm to the isomer hydroxylproline standard curve.

3.3. Results

3.3.1. *N*-Acetyl CHOP degradation in *E. coli* cells

To improve *N*-acetyl CHOP productivity, I examined the degradation of *N*-acetyl CHOP *in vitro* using crude extracts of *E. coli* Δ*putA* cells. Five degraded compounds (Nos. 1-5 in Fig. 18) with different R_f values were detected in the active crude extracts (Fig. 18A). The conversion rate of *N*-acetyl CHOP into products 4 and 5 was dependent on the amount of active crude extracts (Fig. 18B). To characterize of the degraded compounds, I tried various detection systems for TLC (Table 5). Products 1-4 and CHOP could be visualized by spraying ninhydrin reagent, whereas *N*-acetyl CHOP did not react (Fig. 18C). Bromocresol green could detect the product No. 1, CHOP, and *N*-acetyl CHOP. These results suggest that deacetylation and/or decarboxylation of *N*-acetyl CHOP occurs in *E. coli* cells. Approximately 30% and 20% decreases in *N*-acetyl CHOP content were observed in the intact and dialyzed crude extracts, respectively (Fig. 19A). As a negative control, little degradation of

N-acetyl CHOP occurred in the heat-treated crude extracts. It should be noted that product No. 1 had R_f value and the retention time that were similar to those of a standard CHOP resulted in TLC and HPLC (Fig. 19B and Fig. 21B).

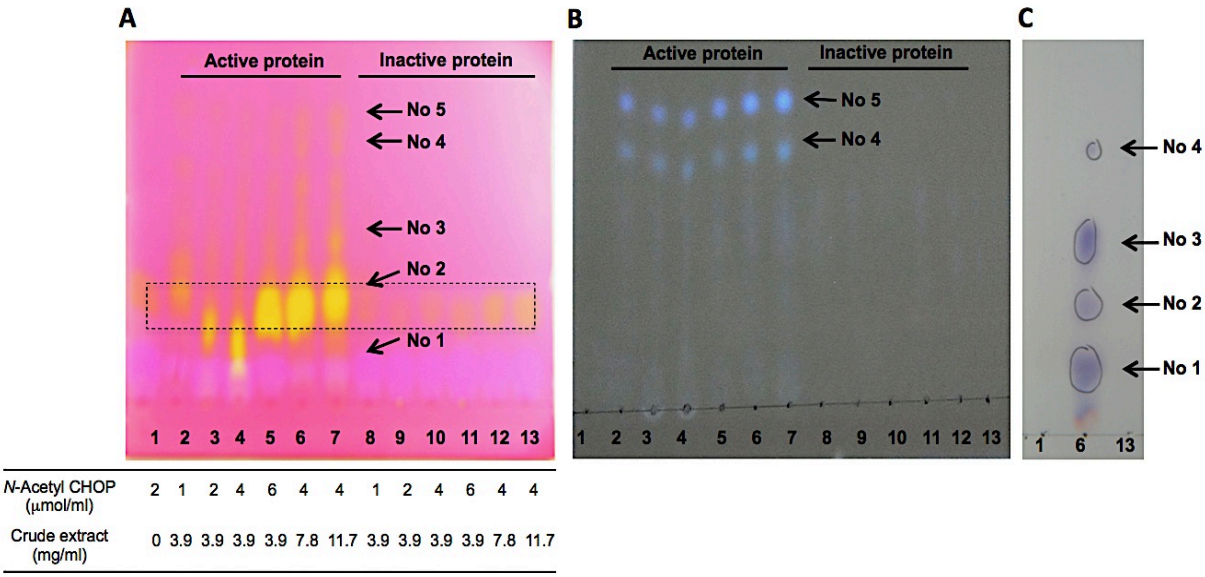


Fig. 18. TLC analysis of *N*-acetyl CHOP with *E. coli* Δ*putA* crude extracts. *N*-acetyl CHOP was incubated at 37°C for overnight in 1 ml of 25 mM potassium phosphate buffer (pH 7.5), with or without dialyzed *E. coli* Δ*putA* crude extracts. The negative control was examined under the same conditions except that dialyzed extract was heated at 100°C for 10 min. Ten ml of each sample was subjected to TLC analysis and was detected with $KMnO_4$ (**A**), UV (**B**), and ninhydrin (**C**). Lane 1, 10 μl of 2 mM *N*-acetyl CHOP; lanes 2-7, crude extract; lanes 8-13, heat-treated crude extract. The concentrations of crude extracts and *N*-acetyl CHOP are shown in Fig. 19A. The R_f values for standard *N*-acetyl CHOP are shown in Table 5.

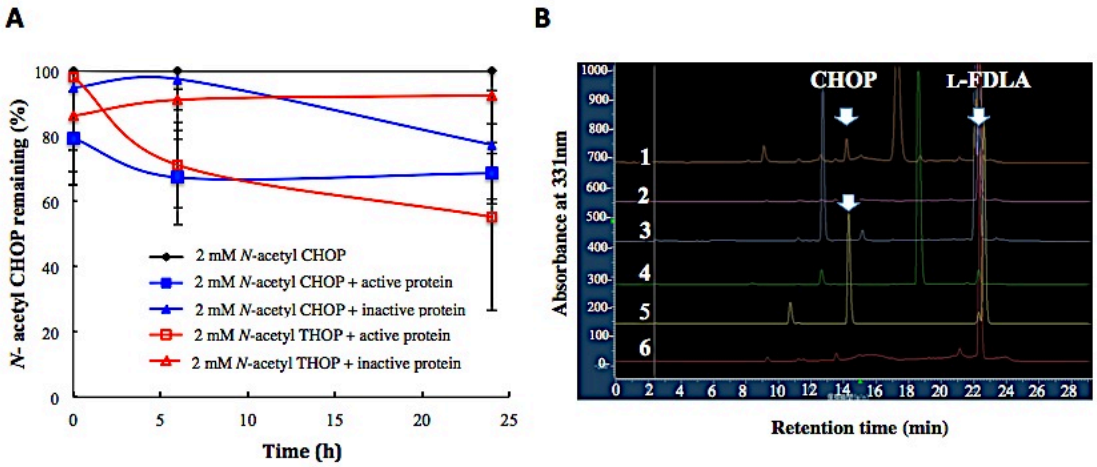


Fig. 19. Analysis and comparison of *N*-acetyl CHOP degradation. (**A**) Comparison of the degradation of *N*-acetyl CHOP with active and inactive crude extracts. *N*-Acetyl CHOP or *N*-acetyl THOP (2 mM) was incubated at 37°C for overnight in 1 ml of 25 mM postassium phosphate buffer (pH 7.5) with or without dialyzed *E. coli* Δ*putA* crude extracts. The negative control was examined under the same conditions except that dialyzed extract was heated at 100°C for 10 min. Samples were derivatized with NPH-HCL and subjected to HPLC. The remaining percentage values were calculated by comparing final and initial concentration of *N*-acetyl CHOP. (**B**) HPLC chromatograms of L-FDLA-derivatized *N*-acetyl CHOP in the reaction mixture with active crude proteins (line 1) or with inactive crude proteins (line 2), THOP (line 3), L-proline (line 4), CHOP (line 5), and a blank reaction mixture with active protein (line 6). CHOP peak (arrow) was observed in line 1 at the same retention time of CHOP standard samples (line 5).

Table 5. R_f values of degradation products from the active protein reactions.

Substrate	R_f	UV	$KMnO_4$	Ninhydrin	Bromocresol green	PMA
N-acetyl CHOP	0.28 ± 0.02	ND	+	ND	+	+
Product No. 1	0.16 ± 0.01	ND	+	+	+	ND
Product No. 2	0.30 ± 0.01	ND	+	+	ND	ND
Product No. 3	0.43 ± 0.02	ND	+	+	ND	ND
Product No. 4	0.47 ± 0.02	+	+	+	ND	+
Product No. 5	0.56 ± 0.02	+	+	ND	ND	ND

ND: Not detected.

3.3.2. Screening of *E. coli* genes involved in deacetylation of *N*- acetyl CHOP

On the basis of the above results (Fig. 18 and 19), deacetylation may be the first

step in the degradation of *N*-acetyl CHOP (Fig. 20). Based on profiling of the *E. coli* chromosome (PEC) (http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp), nine genes appear to encode deacetylases: three polysaccharide deacetylases (YadE, PgaB, and YibQ), four glucosamine deacetylase (LpxC, NagA, AgaA), Sir2-like deacetylases CobB, and two other deacetylases (ygeY and ArgG) (Table 6). Among these, I focused on eight genes which are non-essential for *E. coli* growth, and analyzed the deacetylation of *N*-acetyl CHOP in deletion mutants lacking these genes (Fig. 21).

NagA, AgaA, and CobB belong to the Sir2 family whose members are NAD^+-dependent deacetylase enzymes that modulate gene silencing (Rusche et al., 2003; Li et al., 2010), cell cycle regulation (Dryden et al., 2003), fatty acid metabolism (Starai et al., 2003), lifespan extension (Rogina and Helfand, 2004), and apoptosis (Langley et al., 2002). Although a few substrates have been identified, functions of the bacterial Sir2-like proteins are still largely unknown. YadE, PgaB, and YibQ are the members of the carbohydrate esterase family. These enzymes catalyze the de-*N*-acetylation or de-*O*-acetylation of *N*-acetylglucosamine residues or *O*-acetylxylose residues, respectively. Acetylornithine deacetylase (ArgE and ygeY) plays a role in recycling the acetyl group in L-arginine biosynthetic pathways of microorganisms (Caldovic and Tuchman, 2003).

To test whether the above deacetylases participate in deacetylation of *N*-acetyl CHOP in *E. coli*, dialyzed crude cell extracts of the wild-type strain and eight mutants were incubated with *N*-acetyl CHOP, and I then detected the deacetylation products with $KMnO_4$ and ninhydrin by TLC (Fig. 21). The level of *N*-acetyl CHOP was reduced approximately 30% in all mutants, which is similar to the wild-type strain. CHOP was identified in the crude extract reaction with ninhydrin reagent, but was not detected in the negative control (Fig. 21B). These results suggest that

N-acetyl CHOP is not a substrate for the predicted deacetylases tested in this study.

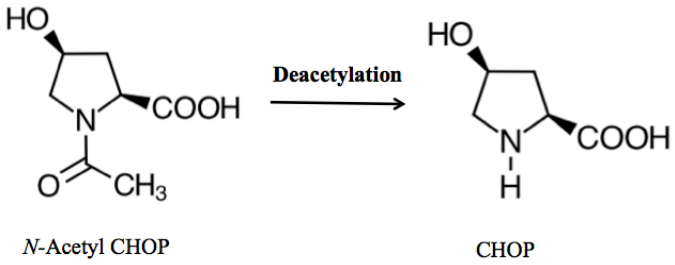


Fig. 20. Proposed *N*-acetyl CHOP deacetylation reaction.

Table 6. List of deacetylase genes in the *E. coli* genome.

Gene	Protein name	Class
lpxC	UDP-3-O-acyl *N*-acetylglucosamine deacetylase	Essential
nagA	*N*-Acetylglucosamine-6-phosphate deacetylase	Non-essential
agaA	Putative *N*-acetylgalactosamine-6-phosphate deacetylase	Non-essential
cobB	NAD-dependent deacetylase	Non-essential
yadE	Predicted polysaccharide deacetylase lipoprotein	Non-essential
yibQ	Predicted polysaccharide deacetylase	Non-essential
pgaB	Polysaccharide deacetylase	Non-essential
ygeY	Acetylornithine deacetylase	Non-essential
argE	Acetylornithine deacetylase	Non-essential

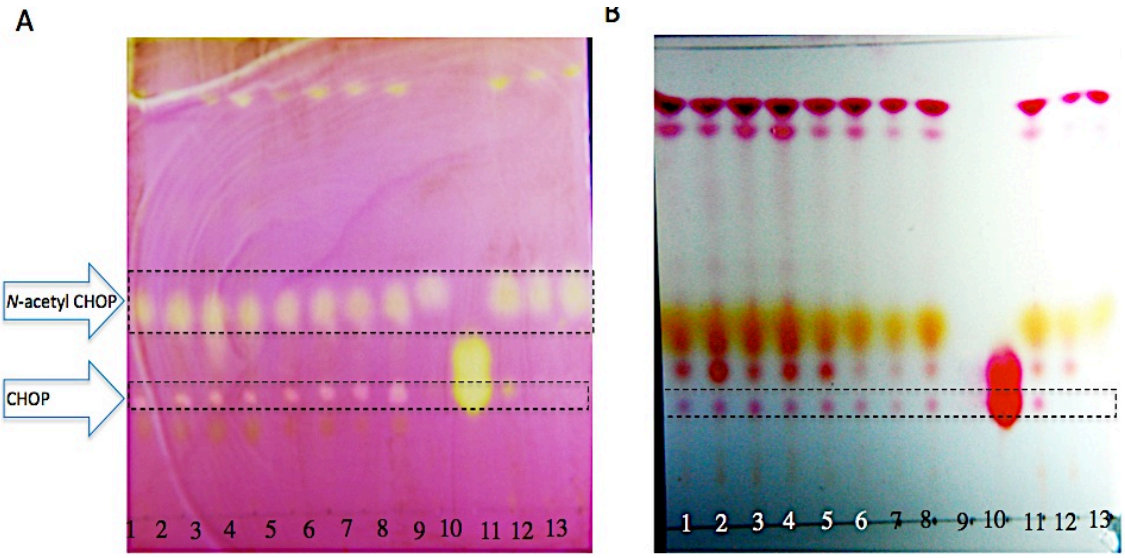


Fig. 21. TLC analysis of *N*-acetyl CHOP degradation with deacetylase mutant crude extracts. *N*-acetyl CHOP was incubated at 37°C for overnight in 1 ml of 25 mM postassium phosphate buffer (pH 7.5) with or without dialyzed *E. coli* crude extract. The negative control was examined under the same conditions except that dialyzed extract was heated at 100°C for 10 min. Ten ml of each sample was subjected to TLC analysis and was detected with $KMnO_4$ (**A**) and ninhydrin (**B**). Lanes 1-8: crude extracts of Δ*yadE*, Δ*pgaB*, Δ*yibQ*, Δ*nagA*, Δ*agaA,* Δ*cobB*, Δ*ygeY*, and Δ*argE* strains; lanes 9 and 10: 10 μl of 2 mM *N*-acetyl CHOP and 0.1 M CHOP, respectively; lanes 11: crude extract of wild-type (BW25113); lane 12: heat-treated crude extract; lane 13: crude extract without *N*-acetyl CHOP. Crude extract reaction conditions are described in Material and Methods.

3.3.3. Pigment formation in *E. coli* cells under culture conditions containing CHOP

Interestingly, I noticed that the color of *E. coli* cells and medium became brownish when the cells were cultured on CHOP/NaCl-containing medium. To date, there has been no report on pigment production by *E. coli* cells or other bacterial cells under culture conditions containing hydroxyproline, besides an extracellular pyrroles-derived pigment in culture medium of *Chromobacterium* and *Aerobacter* (William, 1962). To understand the relation of pigment formation from imino acids, I examined the effect of CHOP or THOP on pigment production during cultivation of *E. coli* cells (Fig. 22).

E. coli BL21(DE3) cells were tested for their of pigment-forming ability in CHOP-, THOP-, and L-proline-containing medium. Fig. 22A indicates that high concentrations of CHOP triggered pigment formation in *E. coli* cells, but this phenotype could not be observed in cells overexpressing Mpr1. The addition of L-proline slightly decreased pigment production in medium containing high concentrations of CHOP (Fig. 22B). In contrast, no pigment production occurred in THOP-containing medium (Fig. 22C).

Extracellular and intracellular pigment was extracted, and the absorption

spectra of this pigment were recorded at 320-360 nm. The pigment color was stable even at high temperature (data not shown).

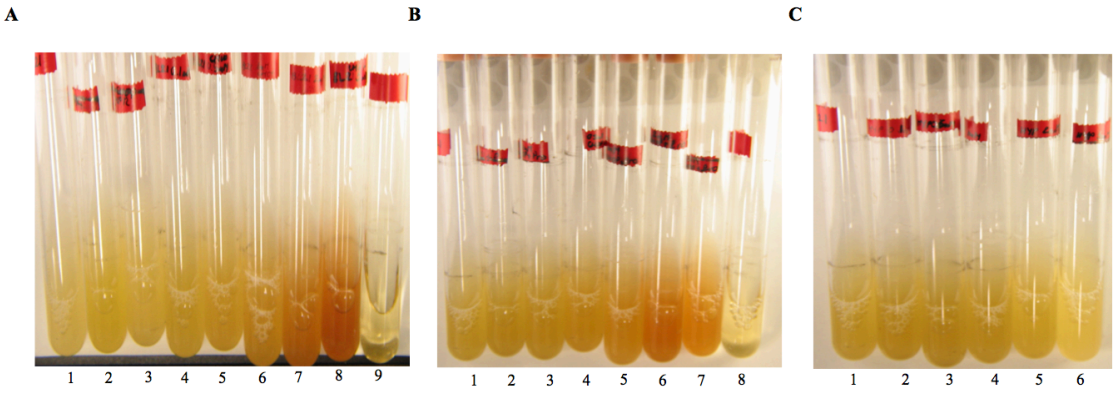


Fig. 22. CHOP induces pigment formation in *E. coli* Δ*putA* cells cultured in LB medium at 37°C for 24 h. (**A**) CHOP-dependent pigment formation. *E. coli* Δ*putA* cells harboring pRSF(Duet)-Mpr1 in LB medium with and without 5 mM CHOP (tube #2 and 3, respectively). *E. coli* Δ*putA* cells harboring pRSF(Duet)-1 in LB medium containing 0, 0.1, 0.5, 1, 2, and 5 mM CHOP (tube #1, 4, 5, 6, 7, and 8, respectively) and LB medium without cells (tube #9). (**B**) Effect of L-proline on pigment formation in the CHOP-containing LB medium. *E. coli* Δ*putA* cells harboring pRSF(Duet)-Mpr1 in LB medium containing 5mM L-proline and 5 mM CHOP (tube #2). *E. coli* cells harboring pRSF(Duet)-1 in LB medium containing 5 mM L-proline plus 0, 0.1, 0.5, 1, 2, and 5 mM CHOP (tubes #1, 3, 4, 5, 6, and 7, respectively) and LB medium without cells (tube #8). (**C**) Effect of THOP on pigment formation. *E. coli* Δ*putA* cells harboring pRSF(Duet)-1 in LB medium containing 0, 0.1, 0.5, 1, 2, and 5 mM THOP (tubes #1, 2, 3, 4, 5, and 6, respectively).

3.3.4. CHOP metabolism in *E. coli* cells

Since the pigment formation occurred in the culture medium with CHOP, it was suggested that *E. coli* has hydroxyproline metabolisms including CHOP. Thus, I further compared the degradation of isomer *cis*- and *trans*- hydroxy L-proline in crude extracts of *E. coli* Δ*putA* with that of *N*-acetyl CHOP and L-proline by detecting the remaining amino acids, using ninhydrin colorimetric methods and HPLC. As shown in Table 7, degradation was observed with CHOP in L-form, but

not with either CHOP in D-form or THOP. Approximately 6 % of CHOP was degraded in the active crude extract. Degradation of L-proline could not be observed, showing that *putA* was completely deleted in the cells. These results suggest that oxidation occurs in the CHOP metabolism pathway.

Table 7. Substrate specificity of degradation activity in crude extract from *E. coli*

Substrate	Degradation activity	Relative activity
	μmol /mg protein/min	%
N-acetyl CHOP	$0.30\ 10^{-3} \pm 0.45\ 10^{4}$	100
THOP	0	0
CHOP (L-form)	$0.56\ 10^{-4} \pm 0.8\ 10^{-5}$	18.5
CHOP (D-form)	0	0
Pro	0	0

3.4. Discussion

The free amino group of the *N*-terminal residue in a protein is often blocked with an acetyl group. This acetylation plays an essential role in post-translational modification that conserved in prokaryotes and eukaryotes. For example lysine acetylation of histone, it involved in a variety of biological processes: such as gene expression regulation, transcriptional activity and key component of chromatin structure (Yang and Seto, 2008). While biochemical function of histone deacetylation is inhibitor of transcription (Haberland et al., 2009). However, the nature of acylamino acids, which are present in the free form, and the deacetylation of free acylamino acid are still poorly understood. The results shown in Fig. 18C and 19B revealed that the conversion of *N*-acetyl CHOP into CHOP occurred in cell-free extracts of *E. coli*. Above results (Fig. 18C and 19B) suggest that the deacetylation of *N*-acetyl CHOP is a major obstacle to optimizing microbial production of *N*-acetyl

CHOP. This degradation is not dependent on co-factors such as NAD and is not inhibited by metal ions (Supplementary Table 1). Mutaiton of eight predicted deacetylases could not reduce the deacetylation of *N*-acetyl CHOP (Fig. 21). These results indicate that cell extracts may have an acetylesterase activity that acts on the carboxylic ester bond. If acetylesterase can degrade *N*-acetyl CHOP *in vivo*, it would be interesting to identify the specific esterase for *N*-acetyl CHOP.

Other degradation products were detected in cell-free extracts of *E. coli* (Fig. 19). CHOP degradation may be involved in the hydroxyproline metabolic pathway. CHOP could be metabolized in cell-free extracts of *E. coli* (Table 7), but *E. coli* cannot utilize CHOP as the sole nitrogen or carbon source (Supplementary Fig. 4). Moreover, CHOP is incorporated into proteins competitively with L-proline, leading to an acceleration of protein synthesis (Supplementary Fig. 6). Ninhydrin and the pre-column derivatization HPLC methods were not suitable for detection of amino acid degradation products. They could not distinguish degraded amino acids from free amino acids because the degraded products contained an amine group. Table 5 shows that there are 4 degraded products from *N*-acetyl CHOP which did not react with bromocresol green, which is used for visualizing a carboxyl group. It means that 4 degraded products do not contain the carboxylic group. These results suggest that CHOP is metabolized to some other amino acids prior to degradation into smaller products in *E. coli*.

Pyrrole has a partial structure of L-proline and L-hydroxyproline. Pyrrole compounds are found in the green leaf pigment, chlorophyll, in the red blood pigment, hemoglobin, and in the blue dye, indigo. Pigment formation could be observed when CHOP, but not THOP, was added (Fig. 22A and 22C). Biochemical studies on the THOP degradation pathway in *Pseudomonas* sp. indicate that the

first conversion of THOP into CHOP in D-form (*cis*-4-hydroxy-D-proline; CHOP (D)) was catalyzed by epimerase (Adams and Leonard, 1980). CHOP (D) is then broken down to 2 and 3- carbon compounds by CHOP (D) oxidase. The resultant products would be a source of intermediates in the TCA cycle (Fig. 17). However, Wild et al. (1978) suggested that the CHOP (D) oxidation step does not exist in *E. coli* cells (Table 7). The results of Fig. 22 and Table 7 suggest that degradation of CHOP in L-form is involved in the biosynthesis of pyrrole-derived pigment in *E. coli* cells.

It was previously reported that the incorporation of CHOP into proteins is probably due to the mis-recognition of proline-tRNA synthetase (Penny and Karin, 2000). Under salt stress conditions, intracellular CHOP accumulation resulted in immediate cessation of growth (Fig. 6B). When a high concentration of CHOP was added, approximately 60% of L-proline and L-hydroxyproline residues in collagen were replaced by CHOP (Rosenbloom and Prockop, 1971). My hypothesis is that pyrrole compounds may be produced from CHOP by some enzymes in *E. coli* and the pyrroles will be easy to be polymerized non-enzymatically to form pigment under alkaline conditions (Mathewson and Corwin, 1960).

The *N*-Acetyltransferase Mpr1 protects of yeast cells from various oxidative stresses by regulating the level of intracellular reactive oxygen species (Nishimura et al., 2010). It appears that *E. coli* cells expressing Mpr1 can grow rapidly in M9 medium and reach higher OD than those carrying empty vector (Fig. 6 and Supplementary Fig. 1). In addition, Mpr1 overexpression reduces the total protein levels of *E. coli* cells (Supplementary Fig. 5), suggesting that *N*-acetylation of CHOP inhibits random incorporation of CHOP into proteins. The overwhelming evidence is that *N*-acetyl CHOP inhibits pigment formation, while pigment production correlates with the CHOP concentration (Fig. 22).

For improvement of *N*-acetyl CHOP productivity, blockage or inhibition of *N*-acetyl CHOP (or CHOP) degradation is an effective method, but another strategy is to enhance CHOP synthesis (Fig 23). I think that *cis*-4-L-proline hydroxylase does not express its full activity in *E. coli* cells, because the specific activity of the purified recombinant SmP4H was about 600 nmol/min/mg of protein (Hara and Kino 2009). Authors also reported that only approximately 10% of L-proline was converted into CHOP *in vitro*. Therefore, error-prone PCR is currently being used to introduce random mutations into the SmP4H gene. Based on the relationship between CHOP accumulation and pigment formation, I am trying to isolate clones producing more pigments, because such clones should express the mutant SmP4H with enhanced activity, leading to accumulation of CHOP, which is highly converted into *N*-acetyl CHOP by Mpr1. However, I have not yet obtained SmP4H variants that can accelerate pigment formation in *E. coli* cells.

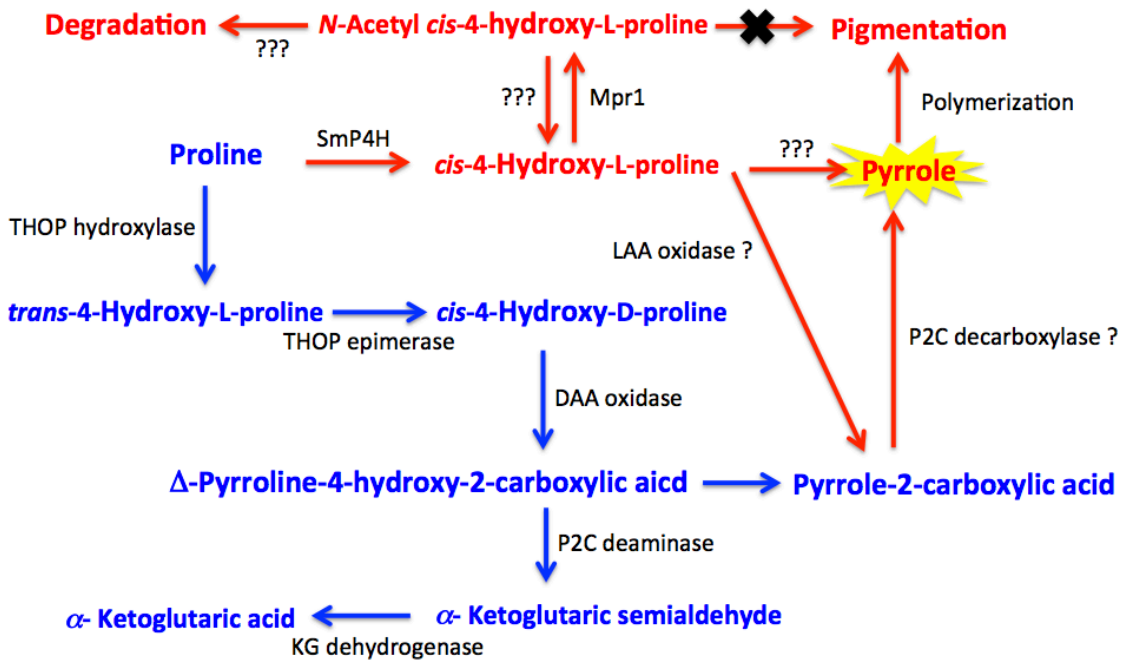


Fig. 23. Proposed scheme of metabolism of THOP in *Pseudomonas* sp. (Blue letter) and

hypothetical *N*-acetyl CHOP in *E. coli* cells expressing SmP4H and Mpr1 (Red letter). The exact degradation pathways of *N*-acetyl CHOP (or CHOP) are still unknown. Pyrrole is a major component involved in colored products in nature, and is expected to be the metabolite in *N*-acetyl CHOP degradation. It could be polymerized under alkaline condition to form pigment.

Conclusions

The objective of this study was to produce *N*-acetyl CHOP directly from free L-proline in *E. coli*. CHOP is a useful compound for several pharmaceutical drugs and is generally manufactured by chemical synthesis. However, its poor water solubility, low molecular weight, and high toxicity limit its therapeutic potential. Regarding *N*-acetyl CHOP, the major obstacles to microbial production are the toxicity of CHOP and our poor understanding of CHOP metabolism. I showed here that *N*-acetyl CHOP could be produced in *E. coli* Δ*putA* cells, in which the L-proline degradation pathway is blocked, by simultaneous overexpression of the Rhizobium L-proline *cis*-4-hydroxylase SmP4H and the yeast *N*-acetyltransferase Mpr1. My results also revealed that the overexpression of Mpr1 could both protect *E. coli* cells from the toxicity of CHOP and enhance their growth. To improve productivity, I focused on the degradation of *N*-acetyl CHOP. After incubation with *E. coli* cell-free extracts, several compounds were detected by TLC. Analysis of an *E. coli* knockout library suggested that non-essential deacetylase genes was not involved in the degradation of *N*-acetyl CHOP. It will be interesting to see reveal the relationship between the unknown compounds and CHOP metabolism. CHOP is incorporated into nascent proteins competitively with L-proline, leading to growth inhibition and pigment formation. It also appears that the low enzymatic activity of SmP4H is one of the major reasons for limited *N*-acetyl CHOP production in *E. coli* cells. Therefore, an engineered SmP4H with higher activity or stability may be promising for improvements in *N*-acetyl CHOP production.

Perspectives

1. CHOP metabolic pathway in *E. coli*

A survey of CHOP metabolism and the associated enzymes in *E. coli* should be undertaken. While the metabolism of *trans*-4-hydroxy-L-proline has been studied in depth in *Pseudomonas* sp., there is little comparable information about the enzymes involved in CHOP metabolism in *E. coli*. An improved understanding of CHOP metabolism could, for example, enhance the yield of *N*-acetyl CHOP by inhibiting the CHOP degradation pathway.

2. Development of a screening method for *cis*-4-hydroxylase mutants in *E. coli*

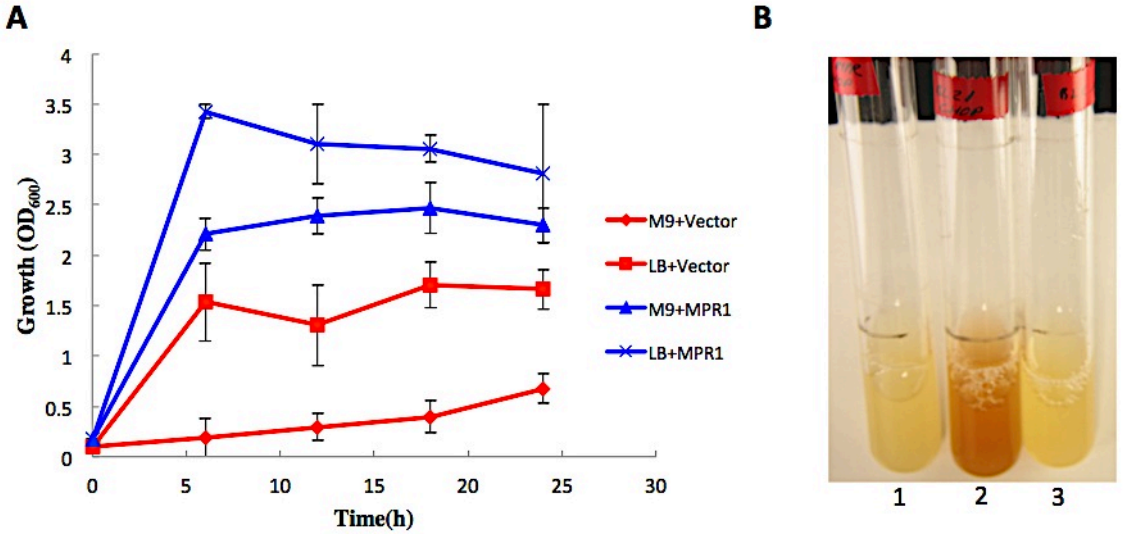
Since the activity of the *cis*-4-hydroxylase SmP4H and MiP4H was low, increased CHOP content may induce pigment formation. A screening method should be developed for *cis*-4-hydroxylase mutants with enhanced activity or stability. Enhancement of *cis*-4-hydroxylase activity may lead to efficient conversion of CHOP into *N*-acetyl CHOP.

3. Evaluation of *N*-acetyl CHOP therapies in cancer cells

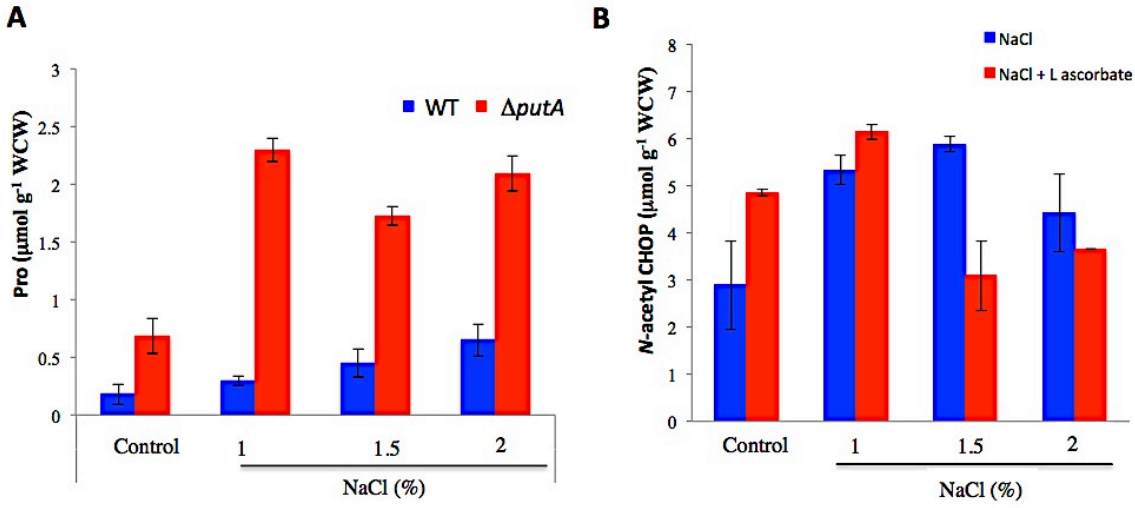
To prove the benefit of *N*-acetyl CHOP as a prodrug, evidence for its therapeutic potential (i.e., lack of toxicity to normal cells but toxicity to target cancer cells) should be obtained. Thus, cancer cell lines which synthesize collagen type I, III and IV, should be examined. It will be particularly interesting to see whether liver, bladder, prostate and renal pelvic cancers are inhibited by CHOP. Evidence that *N*-acetyl CHOP is specifically transported to target cells will also be necessary. To provide such evidence, the uptake of hydroxyproline analogues by the

proton-coupled amino acid transporters, such as PAT1, PAT2, PAT3 and PAT4, should be examined (Edwards et al., 2011). The results of these experiments may suggest which cell lines can take up *N*-acetyl CHOP.

Supplementary

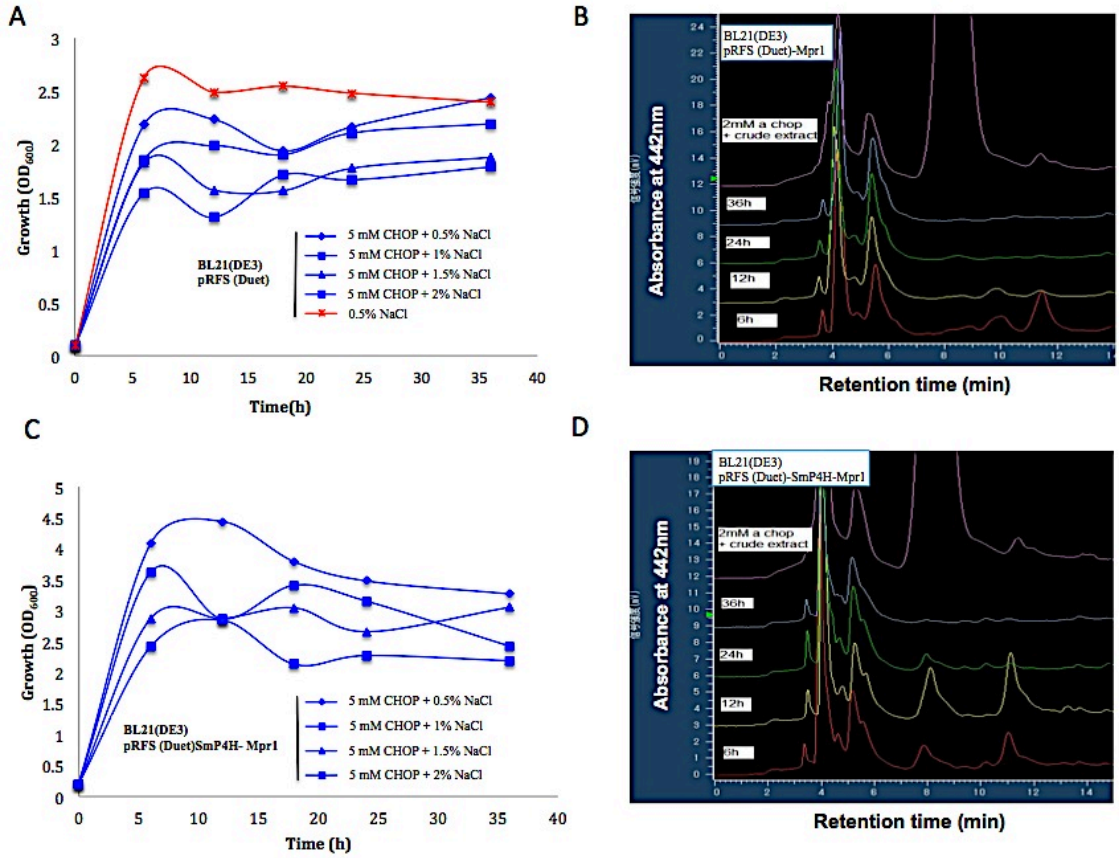


Supplementary Fig.1. (**A**) Growth curve of *E. coli* cells carrying pRSF(Duet)-1 (vector) and pRSF(Duet)-Mpr1 (MPR1) in LB or M9 medium containing 5 mM CHOP and 2% NaCl. (**B**) Pigment formation observed in *E. coli* cells when cultured in the CHOP-containing LB medium. *E. coli* cells harboring pRSF(Duet)-Mpr1 and pRSF(Duet)-1 in LB medium containing 2% NaCl and 5mM 5mM CHOP (tubes # 1 and 2). *E. coli* cells harboring pRSF(Duet)-1 in LB medium containing 2% NaCl (tube # 3).

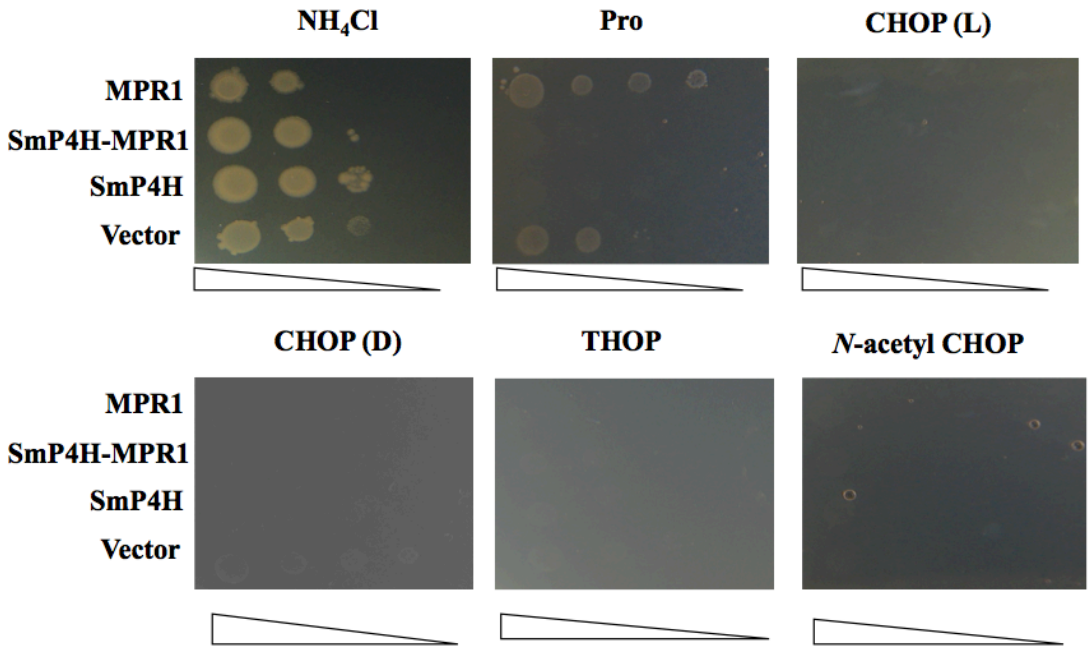


Supplementary Fig. 2. Changes in L-proline and *N*-acetyl CHOP contents under various

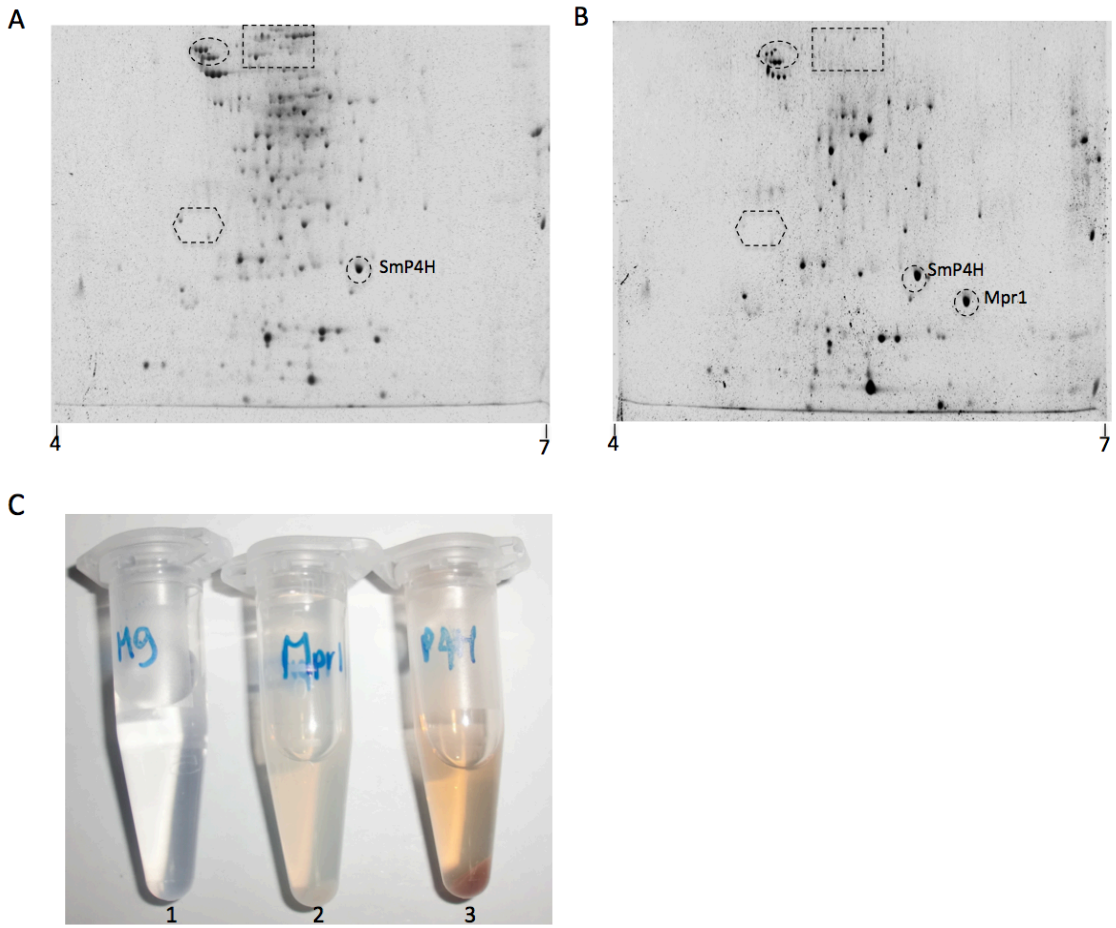
conditions. (**A**) Effect of *putA* disruption and NaCl addition on intracellular L-proline contents. (**B**) Effect of NaCl and L-ascorbate on *N*-acetyl CHOP production. Wild-type and Δ*putA* strains harboring the empty vector pRSF(Duet)-1 (**A**), and Δ*putA* strain harboring pRSF(Duet)-SmP4H-Mpr1 (**B**) were cultivated in 15 ml of M9 medium at 37°C for 24 h under various conditions indicated. L-Proline (**A**) and *N*-acetyl CHOP (**B**) contents were measured as described in Materials and methods.



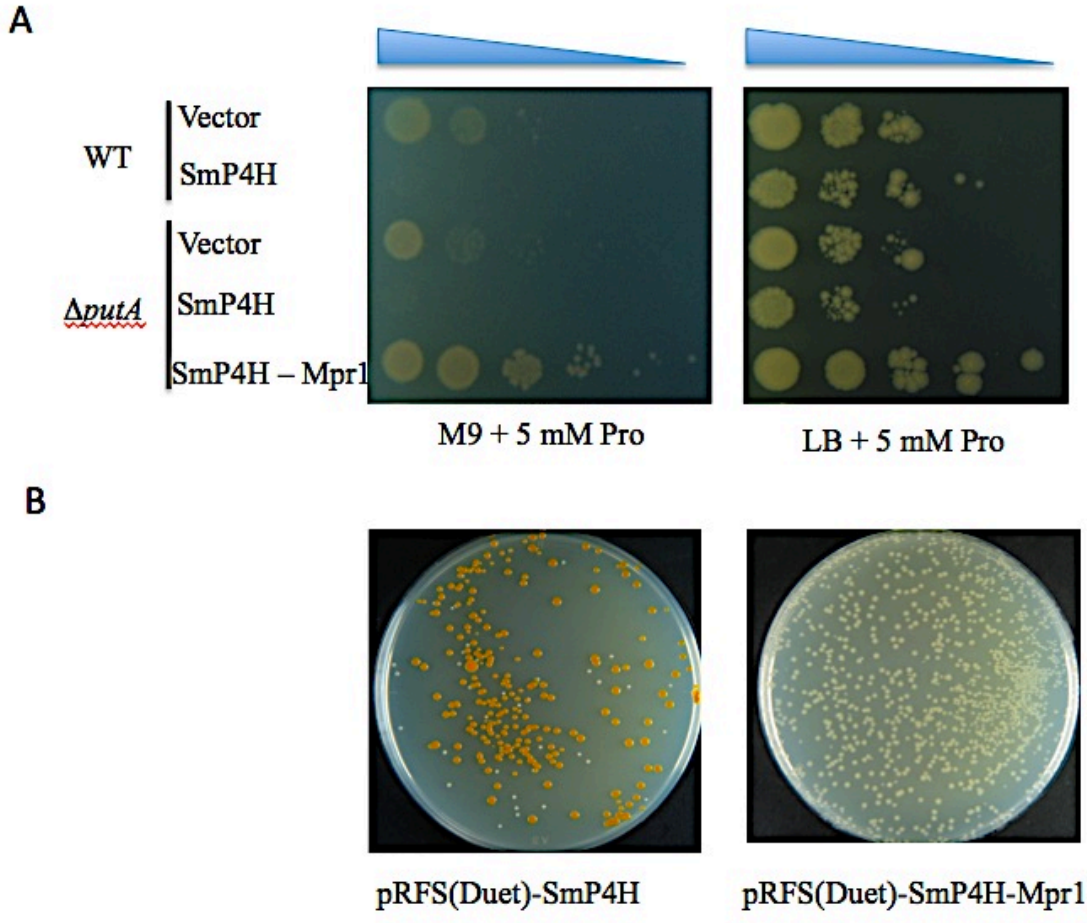
Supplementary Fig. 3. Growth and *N*-acetyl CHOP production of *E. coli* cells that overexpress Mpr1 under various conditions. (**A**) Growth curve of *E. coli* cells harboring pRSF(Duet)-1 in LB medium containing 5 mM CHOP in the presence of 0.5% (diamond), 1% (square), 1.5% (triangle), and 2% (cross) of NaCl. As a control, *E. coli* cells carrying pRSF(Duet)-MPR1 were cultivated in LB medium containing 0.5% NaCl in the absence of CHOP (blue). (**C**) Growth curve of *E. coli* cells harboring pRSF(Duet)-SmP4H-Mpr1 in LB medium containing 5 mM CHOP in the presence of 0.5% (diamond), 1% (square), 1.5% (triangle), and 2% (cross) of NaCl. Time course of *N*-acetyl CHOP production was determined by HPLC. *E. coli* cells carrying (**B**) pRSF(Duet)-1 or (**D**) pRSF(Duet)-SmP4H-Mpr1 in LB medium containing 5 mM CHOP and 2% NaCl. Lines 1-4 are 0, 12, 24, and 36 h cultivation. Line 5 is 2 mM *N*-acetyl CHOP as a standard.



Supplementary Fig.4. Utilization of L-proline and hydroxyl proline analogues as a nitrogen source in *E. coli* cells expressing SmP4H or Mpr1. After cultivation in M9CA medium containing 50 μg/ml kanamycin at 37°C for 18 h, approximately 10^6 cells of *E. coli* BL21(DE3) strains carrying the empty vector or expressing each Mpr1, SmP4H, or SmP4H and Mpr1, and serial dilution of 10^{-1} to 10^{-4} were spotted onto M9 medium containing 0.1 mM IPTG and 50 μg/ml kanamycin in the presence 1 g/l of NH_4Cl, 1 mM L-proline (Pro), CHOP (L), CHOP (D), THOP, and *N*-acetyl CHOP.



Supplementary Fig.5. Partially annotated 2D SDS-PAGE map of up-regulated proteins from *E. coli* BL21 (DE3) Δ*putA* cells harboring pRSF(Duet)-SmP4H (**A**) and pRSF(Duet)-SmP4H-Mpr1 (**B**) cultured in LB medium containing 5 mM L-proline. (**C**) *E. coli* BL21 (DE3) Δ*putA* cells harboring pRSF(Duet)-SmP4H-Mpr1 (eppendorf # 2) and pRSF (Duet)-SmP4H (eppendorf # 3) was cultured in optimized M9 for producing *N*-acetyl CHOP at 28 hours. M9 medium (eppendoff # 1) as blank. Dash box show newly protein synthesis in overexpression SmP4H cells but not in Mpr1 cells.



Supplementary Fig. 6. CHOP-sensitivity of *E. coli* recominant strains.. (A) *E. coli* BL21(DE3) (WT) and the Δ*putA* strains with or without expressing SmP4H and Mpr1 were cultivated in M9CA medium containing 50 μg/ml kanamycin at 37°C for 18 h. Approximately 10^5 of recombinant cells, and serial dilution of 10^{-1} to 10^{-4} were spotted onto M9 and LB media containing 0.1 mM IPTG and 50 μg/ml kanamycin in the presence 5 mM L-proline, 5 mM L-ascorbate, and1% NaCl. (B) Pigment formation of the *E. coli* cells expressing SmP4H and co-expressing SmP4H-Mpr1 in the optimized medium.

Supplementary Table 1. Effect of metal ion on degradation of *N*-acetyl CHOP

	Relative activity (%)
None	100
CuSO4	131.7
CaCl	124.6
MgSO4	89
MnCl	ND
NaSO4	ND
EDTA	ND
FeSO4	143
AgNO3	105
ZnSO4	104

ND: not determine

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In October 2009, I started my Ph.D. study in Laboratory of Applied Stress Microbiology. I still remember how my heart was fluttering with homesick and new life in Japan. Three years have flown like arrow and now time almost comes to the end of my Ph.D. period. This thesis includes my three years' research work and each piece of work has been accomplished with the contribution and support of many people. It is very pleasant that I have the opportunity to express my gratitude for all of them.

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Bach Thi Mai Hoa

September 2012

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