Cloning and characterization of PEP4 gene in a Thai-isolated thermotolerant yeast *Pichia thermomethanolica* for efficient recombinant protein secretion

Daryong Kim¹, Takao Ohashi¹, Niran Roongsawang², Sutipa Tanapongpipat², Kazuhito Fujiyama¹

¹International Center for Biotechnology, Osaka University, Japan
²National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand

**Contents**

- Introduction
- Methods & Results
- Conclusion
- Acknowledgements

**Methylotrophic Yeast**

As a recombinant protein secretion host

- **Major species**
  - *Pichia pastoris*
  - *Hansenula polymorpha*
  - *Pichia methanolica*
  - *Ogataea minuta*

- **Advantages**
  - The strong promoters for expression of heterologous gene (Methanol-inducible promoter)
  - The ability to grow to high-cell density (200 gL⁻¹ dry weight for *P. pastoris* , (Heyland et al., 2010))
  - The simple purification of secreted proteins due to the relatively low levels of secretion of endogenous proteins

**Pichia thermomethanolica**

- *Pichia thermomethanolica* BCC16875 – New methylotrophic yeast

- **Properties**
  - Isolated from soil in Thailand (Limtong et al., 2005)
  - Utilize methanol as a sole carbon source
  - Able to tolerate comparatively high temperature (up to 40°C)

- **Additional advantage**
  - Reduction of cooling cost in large-scale production
  - Lower risk of contamination

Potential good host for recombinant protein production

**PEP4 gene**

- **PEP4 gene – Encoding vacuolar protease, proteinase A.**

- **Function of proteinase A**
  - Playing a key role in maturation and activation of other proteases such as proteinase B and carboxypeptidase Y

- **Structure of proteinase A**

- **Deletion**

  The degradation of recombinant protein decreased by 5-fold in pep4Δ strain

**Methods & Results**
3’RACE

1. Extracted mRNA from the yeast and reverse-transcribed to cDNA using oligo-dT adapter primer
2. Conducted PCR using adapter primer and specific primers

**Obtained the sequence which had high identity with H. polymorpha, including 3’ UTR and poly A tail**

**Inverse PCR**

**Method of inverse PCR**

1. Genomic DNA is cut by restriction enzyme
2. The fragments are circularized by DNA ligase

**Inverse PCR**

3. Circularized DNA are used as template in PCR

**Conclusions**

➢ Obtained the full sequence of *P. thermomethanolica* PEP4 gene by a series of PCR methods
➢ Revealed that Amino acid sequence of the *PtPep4p* were closest to that of *HpPep4p*
➢ Found that *PtPEP4* gene complemented *S. cerevisiae pep4Δ* strain, indicating that *PtPEP4* gene function as proteinase A

**Acknowledgements**

➢ *S. cerevisiae* strain and vectors was provided by the National Bio-Resource Project (NBRP) of the MEXT, Japan
Functional analysis of the genes involved in the biosynthesis of the circular bacteriocin, enterocin NKR-5-3B (Ent53B)

Rodney H. Perez¹, Tomoko Inoue¹, Kohei Himeno¹, Naoki Ishibashi¹, Takeshi Zendo¹, Jiro Nakayama¹, Kenji Sonomoto¹,²

¹Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
²Department of Functional Metabolic Design, Bio-Architecture Center, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

Bacteriocins are antimicrobial peptides that possess inhibitory activity against closely related strains. Among these bacteriocins, a group of circular bacteriocins is the most promising due to its high resistance to thermal stress as well as resistance to proteolytic enzyme degradation. The sturdiness of this group of bacteriocins is attributed to its circular structure. These bacteriocins undergo dehydration reaction between the N- and C-terminal residues during maturation thereby resulting in the covalent binding of the terminal ends. However, detailed mechanism of the cyclization process still remains a mystery. Understanding the detailed mechanism by which these circular bacteriocins are synthesized will materialize its full application potential such as biological delivery agents and as scaffolds in drug design through genetic engineering.

Enterococcus faecium NKR-5-3, isolated from a Thai fermented fish, Pla-ra, produces multiple bacteriocins. One of the bacteriocins, named enterocin NKR-5-3B (Ent53B), is a novel circular bacteriocin. The putative structural gene and 9 putative orfs were identified after sequencing a region of 8 kb in the producer strain’s genome through a fosmid library. In an effort to understand the functions of these putative Orfs, plasmids encoding different combinations of the Ent53B gene cluster using pMG36c vector were constructed and cloned into E. faecalis JH2-2 as a host strain. The production of Ent53B was only observed from the mutant expressing ent53B-orf1234, suggesting that its production and secretion require a cooperative function of these Orfs. Moreover, self-immunity assay of these transformants revealed that strain NKR-5-3 has two self-immunity systems. Transformants expressing orf4 and/or combination of orfI and orf3 were immune to the inhibitory action of Ent53B.

Keywords: bacteriocin, circular bacteriocin, heterologous bacteriocin expression, bacteriocin biosynthesis, bacteriocin immunity
Elucidation of regulatory mechanisms in multiple bacteriocin production by *Enterococcus faecium* NKR-5-3

**Naho Matsumoto**, Naoki Ishibashi, Rodney Honrada Perez, Kohei Himeno, Tomoko Inoue, Takeshi Zendo, Jiro Nakayama, Kenji Sonomoto

*Bacteriocins are ribosomally synthesized antimicrobial peptides. Among them, bacteriocins from lactic acid bacteria are expected to be used as safety food preservatives. *Enterococcus faecium* NKR-5-3 isolated from Thai fermented fish, Pla-ra, produces 5 bacteriocins, enterocins NKR-5-3A, Z, B, C, and D (PepA, Z, B, C, and D), which have varied spectra of activity against gram-positive bacteria (1). This bacteriocin production is regulated by several factors, such as media, temperature, and an inducing peptide (PepD). Especially, bacteriocin production is increased by addition of PepD even in TSB-YE medium, which is an unfavorable medium for bacteriocin production (2). Therefore, the aim of this study is elucidation of regulatory mechanisms of multiple bacteriocin production in *E. faecium* NKR-5-3.

Transcriptions of the biosynthetic genes for the multiple bacteriocins were analyzed to evaluate the effects of PepD on the regulatory mechanism. Strain NKR-5-3 was cultivated in TSB-YE medium, and the total RNA was extracted at logarithmic and stationary phases. Transcriptional analysis was performed by real-time RT-PCR using the cDNAs from the RNA. The result showed that the transcriptional levels of structural genes of PepA, Z, C, and D (ent53A, Z, C, and D) increased more than 1.5 times by addition of PepD while that of PepB (ent53B) did not increase. In addition, the transcriptional levels of regulatory genes (ent53R, K) and a transporter gene (ent53T) also increased. Thus, PepD has been shown to enhance the bacteriocin production except PepB through increase of the transcriptional levels of the structural genes and the biosynthetic genes.

To clarify the molecular mechanisms of pepD induction, Δent53D mutant strain was constructed from strain NKR-5-3. Δent53D mutant strain showed no antimicrobial activities against *Listeria innocua* ATCC 33090 T and *E. faecalis* JCM 5803 T, but showed antimicrobial activity against *Bacillus subtilis* JCM 1465 T (sensitive only to PepB). The result also suggested that PepB production is not under the regulation of PepD. Moreover, in Δent53D mutant strain, the transcriptional levels of each gene except ent53B were much lower than those of strain NKR-5-3. Therefore, it was shown that PepD is necessary to trigger bacteriocin production except PepB in strain NKR-5-3.

**Keywords:** multiple bacteriocin, lactic acid bacteria, regulatory mechanism, inducing peptide

**References:**
Characterization of regulatory genes involved in secondary metabolism in *Streptomyces lavendulae* FRI-5

**Yohanes Novi Kurniawan, Shigeru Kitani, Asa Maeda, Takuya Nihira**

*International Center for Biotechnology, Osaka University, Suita, Osaka 565-0871, Japan*

*Streptomyces* species are soil-dwelling Gram-positive bacteria renowned as a rich source of secondary metabolites with a range of biological activities. The chemistry of these secondary metabolites is structurally diverse and based on a number of different backbone structures. The secondary metabolites are products of complex biosynthetic pathways that include biosynthetic gene cluster and regulatory genes. One of the most interesting features of this microorganism is the control of secondary metabolism by small signaling molecules called γ-butyrolactones (GB). GB autoregulator-receptor systems are widely employed by *Streptomyces* species and well known to regulate antibiotic production and/or morphological differentiation. *Streptomyces lavendulae* FRI-5 produces blue pigment (indigoidine), D-cycloserine, and nucleoside antibiotics as secondary metabolites. These productions are tightly regulated by the excellent signaling system with GB autoregulator IM-2 and its cognate receptor FarA. Notably among the known GB, IM-2/FarA system possesses unique characteristics regarding the regulation of secondary metabolism. In contrary to the solely positive effects exerted by other autoregulators, IM-2 is capable not only of inducing the production of nucleoside antibiotics and indigoidine but also of turning off the production of the antituberculosis antibiotic D-cycloserine. In the farA-flanking regions, a regulatory island consists of five more putative regulatory genes (farR1-farR5) and one biosynthetic gene for autoregulator (farX) was identified (1). They belong to the family of SARPs (*Streptomyces* Antibiotic Regulatory Protein) (farR3 and farR4), two-component response regulator (farR1), and pseudo-receptor family protein (farR2). Previously we demonstrated that FarA acts as a negative regulator of the biosynthesis of nucleoside antibiotics and indigoidine in the absence of IM-2 (2). It also directly controls the transcription of both the farR1 and farR2 genes in an IM-2 dependent manner by binding to the specific sequence in the promoter region of both genes (1). Moreover, we also found that farR3 and farR4 are transcribed in an IM-2 dependent manner, suggesting that the four genes (farR1 to far4) belong to the IM-2 regulon. Recently, we demonstrated that FarR3 acts as a positive regulator of indigoidine production, while FarR4 negatively regulates the expression of farX (unpublished). Further clarification of the IM-2/FarA system will provide greater insights into the γ-butyrolactone autoregulator signaling cascade in streptomycetes.

**Keywords:** γ-butyrolactones, IM-2/FarA, secondary metabolism

**References:**
Characterization of regulatory genes involved in secondary metabolism of *Streptomyces lavendulae* FRI-5

Yohanes Novi KURNIAWAN, Asa MAEDA, Shigeru KITANI, and Takuya NIHIRA

International Center for Biotechnology, Osaka University

Characteristics of *Streptomyces*

![Life cycle diagram](image)

Autoregulatory mechanism in *Streptomyces*

![Autoregulator mechanism diagram](image)

IM-2, an autoregulator of *Streptomyces lavendulae* FRI-5

![IM-2 structure](image)

*S. lavendulae* FRI-5 far-regulatory island and putative biosynthetic gene clusters of secondary metabolites

![far-regulatory island](image)
Hypothetical Model

FarA
Target: SARP (FarR4), pseudo autoregulator receptor (FarR2), etc
FarX
IM-2
FARE
(*FARE: FarA Responsive Element)
FarA
Hypothetical Model
Secondary metabolites

Functional analysis of FarA

FarR1 FarR2 FarR3 FarR4 FarX FarA FarR5
FARE1 FARE2 FARE3 FARE4 FARE5

Autoregulator signaling cascade in S. lavendulae FRI-5

Transcription profile of farR3 and farR4

farR3-farR4 belong to the IM-2 regulon

Functional analysis of FarR3 and FarR4

What are roles of FarR3 and FarR4 in secondary metabolism?
Summary and future work

![Diagram showing relationships between farR1, farR2, farR3, farR4, farX, farA, and farK5, with notes on unknown function, Indigoidine, and Nucleoside antibiotics]

Thank you for your kind attention
Characterization of regulatory genes involved in the production of avermectin, an anthelmintic agent

Suandi Pratama Sultan¹, Kiyoko Miyamoto², Shigeru Kitani¹, Haruo Ikeda², Takuya Nihira¹³

¹International Center for Biotechnology, Osaka University, Suita, Osaka 565-0871, Japan
²Kitasato Institute for Life Sciences, Kitasato University, 1-15-1, Kitasato, Sagamihara, Kanagawa 252-0373, Japan
³Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Rd, Bangkok 10400, Thailand

Streptomyces hormone - also known as autoregulator- controls the production of various secondary metabolites among Streptomyces species. When the amount of extracellular autoregulator reaches threshold level, an autoregulator binds to an autoregulator-receptor, and activates transcription of biosynthetic gene cluster for particular antibiotic. Previously, we discovered a new type of autoregulator -called avenolide- in the broth culture of Streptomyces avermitilis. Avenolide is essential to elicit production of avermectin, an important anthelmintic agent. However the molecular mechanism governing this process remains unknown. Here we show the function of regulatory genes involved in the production of avermectin.

Initially, we examined the in-vitro and in-vivo function of AvaR1, avenolide-receptor protein. Gel shift assay revealed that AvaR1 bound to a 30 base pairs of palindromic DNA motif -called autoregulatory element (ARE)-, located in the promoter of aco, an avenolide biosynthetic gene and this interaction was inhibited by presence of avenolide. Disruption of avaR1 increased the transcription level of aco with the production of avenolide, indicating that AvaR1 is a transcriptional regulator that represses avenolide production.

Next, the function of avaR3, a homologue of avaR1, was investigated by gene disruption. An avaR3 disruptant showed decreased production of avermectin compared to the wild-type strain with delayed expression of a pathway-specific activator for avermectin production. Furthermore, the avaR3 disruptant also displayed fragmented growth in liquid culture and conditional morphological defects on solid medium. These findings demonstrated that AvaR3 acts as a global regulator that controls antibiotic production and cell morphology.

In this study, we revealed multiple roles of AvaR1 and its homolog AvaR3 in the regulatory mechanism for avermectin production controlled by avenolide. This genetic information will lead not only to higher yields in antibiotic production but also to the ability to awaken silent gene clusters for the discovery of novel natural compounds.

Keywords: Streptomyces avermitilis, secondary metabolism, avermectin, avenolide

References:
Characterization of regulatory genes involved in the production of avermectin, an anthelmintic agent

Suandi Pratama Sultan, Kiyoko Miyamoto, Shigeru Kitani, Haruo Ikeda1, and Takuya Nihira

International Center for Biotechnology, Osaka University
1Kitasato Institute for Life Sciences, Kitasato University

Streptomyces avermitilis – producer of various secondary metabolites


Avermectin – a powerful anthelmintic agent

Avermectin biosynthetic gene cluster
Ikeda et al. PNAS. 1999

Avermectin B1b

Bioactivity

Nematode
Arthropod

Global impact of avermectin to human life

Avermectin B1b

River blind
Product of pharmaceutical industry
US$ 850 million/year

Regulation of secondary metabolism by Streptomyces-hormone (autoregulator)

Streptomyces-hormone (autoregulator)
RNA Polymerase
Receptor

Autoregulator gene
Receptor gene
Regulatory gene

Regulation of secondary metabolism by Streptomyces-hormone (autoregulator)
Regulation of secondary metabolism by *Streptomyces*-hormone (autoregulator)

Discovery of a new type of *Streptomyces*-hormone in *Streptomyces avermitilis*

Genes encoding Acyl CoA Oxidase and Cytochrome P450, located in the avaR cluster, are involved in avenolide production

There are three *Streptomyces*-hormone receptors homolog, which belong to the TetR family, in the avaR cluster

DNA-binding activity of AvaR1 in vitro

Role of AvaR1 in avenolide biosynthesis
Transcriptional regulation by AvaR3

Role of AvaR3 in the avermectin production

Role of AvaR3 in morphological differentiation

Hypothetical model of avenolide biosynthesis
Oral presentation 22 (O-22)

Oligosaccharide (DFAIII) production from dahlia tuber with actinomycete Nonomuraea sp.

Nanami Takano¹, Sri Pudjirahatri², Midori Ohtani¹, Keisuke Sugimoto³, Teruo Sone¹, Michiko Tanaka¹, Kozo Asano¹

¹Laboratory of Applied Microbiology, Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, Japan
²Research Center for Chemistry, Indonesian Institute of Sciences, Jl. Sangkuriang, Bandung 40135, Indonesia
³Department of Materials Chemistry, Asahikawa National College of Technology, Asahikawa, Hokkaido 071-8142, Japan.

DFAIII is one of non-digestible oligosaccharides, which promotes absorption of minerals in the small and large intestines and improves intestinal flora in rats and humans. This oligosaccharide is made from inulin by inulin fructotransferase (IFT). Most dahlia tubers are wasted at farm but rich in inulin. This suggests that they may be available as an inulin source for DFAIII production. On the other hand, there was a report that dahlia tuber contained atropine, which disrupts the parasympathetic nervous system’s ability. So we estimated an availability of dahlia tuber as inulin source for DFAIII production from the viewpoints of inulin content and existence of atropine and its related substance, scopolamine.

Dahlia tuber cultured in Indonesia was sliced, blanched for prevention of oxidation by polyphenol oxidase and degradation of inulin, and dried. Dahlia tuber chips were milled into powder. The mixture of powder, citric acid / NaOH buffer (pH 5.5) and culture broth of Nonomuraea sp. AHU 1850 isolated as IFT producer from soil in Indonesia was incubated at 65°C then DFAIII was produced. Inulin content was calculated to be 63.3% (w/w) by comparing the concentration of DFAIII produced from dried dahlia tuber with one from pure inulin.

The dahlia tuber powder was mixed with methanol which atropine and scopolamine were extremely soluble in and this extract was analyzed with HPLC. Atropine and scopolamine were not detected from the extract exceeding safety level.

Estimated IFT gene (nsp-ift gene) in Nonomurae sp. AHU1850 was amplified by inverse PCR based on the genetic data of Arthrobacter sp. H65-7. Base length of nsp-ift gene was 1,326bp. Amino-acid sequence homology between IFT of Nonomurae sp. AHU1850 and Arthrobacter sp. H65-7 was 54%. Cloned nsp-ift gene was expressed in E. coli and cell-free extract was purified by affinity chromatography. Expressed protein had a size of 45kDa and IFT activity with optimum pH 5.5 and optimum temperature 65 to 70°C. It was confirmed that Nonomurae sp. AHU1850 had a unique IFT.

Keywords: difructose anhydride III (DFAIII), Nonomurae sp., inulin fructotransferase
Oligosaccharide (DFAIII) production from dahlia tuber with actinomycete Nonomuraea sp. AHU1850

Nanami Takano¹, Sri Pudjiraharti², Midori Ohtani¹, Keisuke Sugimoto¹
Teruo Sone¹, Michiko Tanaka¹, Kozo Asano³

¹Laboratory of Applied Microbiology, Graduate School of Agriculture, Hokkaido University
²Research Center for Chemistry, Indonesian Institute of Sciences
³Department of Materials Chemistry, Asahikawa National College of Technology

Physiological functions of DFAIII
1. Alter the intestinal microflora toward a healthier composition in rats and humans
2. Decrease the formation of secondary bile acid, which enhance liver and colon carcinogenesis
3. Promote Ca absorption

DFA III is produced by inulin fructotransferase (IFT)

+ Inulin
+ FOS
→ DFA III

Inulin
Fructotransferase

Dahlia (Dahlia sp.)
Jerusalem artichoke (Helianthus tuberosus)
Family Compositae

Selection of inulin source and IFT producer

- Candidate of inulin source
  Dahlia
    - Cultivated for cut flowers
    - Tubers are dumped as wastes
    - Rich inulin in tubers
  However, there was a report that dahlia tuber contained atropine

- IFT producer
  - Screened from actinomycetes isolated from soil in Indonesia

  Genus Nonomuraea: First report as IFT producer
  Nonomuraea sp. AHU 1850
    - Actinomycete in Streptosporangiaceae family
    - Produces IFT in culture supernatant

Today’s topics
1. Estimation of the availability of dahlia tuber as a material for making DFAIII
  i) Inulin content
  ii) Existence of atropine
2. Characterization of IFT gene
Dahlias tuber as a material for making DFAIII

How to measure the inulin content in dahlias tuber

- Dahlia tuber powder 800 mg or pure inulin 400 mg
- 10 mM CA-NaOH buffer (pH 5.5) 24 ml
- Culture broth of Nonomuraea sp. AHU 1850

Production yield from dahlias tubers: 16.8 % ± 3.4
Water content: 8.8 % ± 0.6

Estimated inulin content in dahlias tuber powder

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Yield of DFAIII (% w/w)</th>
<th>Estimated inulin content (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure inulin (BENE Orafti)</td>
<td>90.3</td>
<td>100</td>
</tr>
<tr>
<td>Dahlia tuber powder run1</td>
<td>57.2</td>
<td>63.3 (57.2/90.3 x 100%)</td>
</tr>
<tr>
<td>Dahlia tuber powder run2</td>
<td>57.2</td>
<td></td>
</tr>
</tbody>
</table>

Toxicity of atropine

- Toxic effect: Inhibition of acetylcholine esterase
- Symptoms of poisoning: Thirst, Convulsive seizure, Hallucination, Impaired eyesight, Rapid Pulse
- Toxic dose (atropine sulfate): 5 mg-10 mg orally; over 100 mg intravenously
- Estimated lethal dose orally in humans: 5 mg-10 mg
- Child: over 10 mg
- LD₅₀ orally in mice: 548 mg/kg

Detection of atropine and Scopolamine in dahlias tuber

- Methanol extract of dahlias tuber powder
- Methanol extract of dahlias tuber powder + atropine, scopolamine
- Reference atropine and Scopolamine (0.5 mg/mL)

Mightysil RP-18 GP Aqua 250-4.6 (5 µm), 40°C, UV=215 nm, phosphate buffer (pH 3.0): acetonitrile =9:1, 1.0 mL/min
Characterization of IFT gene from *Nonomuraea* sp. AHU 1850

**Partial IFT gene fragment**

- *Genomic DNA*  
  - Digestion-Kpn I
  - Primer designing
  - *Ligation*  
  - Inverse PCR
  - **IFT gene**  
    - Sequencing & Analysis
    - *Cloning and Expression with E. coli*
    - **IFT protein**  
      - Activity measurement (TLC, HPLC)

IFT from *Nonomuraea* sp. AHU 1850 was distinct from known IFTs:

- *Actinomycetes*  
  - *Frankia* sp. EAN1pec (YP_001511283.1)
  - *Arthrobacter globiformis* (BAB20662.1)

- *Gram (+)*  
  - *Bacillus* sp. snu-7 (AAZ66341.1) 56%  
  - *Arthrobacter* sp. H65-7 (BAAB18967.1) 57%  
  - *Nocardioides* sp. JS614 (YP_921301.1)
  - *Lactobacillus jensenii* SJ-7A-US (ZP_05864909.1)

Protein with identified IFT enzymatic activity

Expressed protein had IFT activity

**IFT activity of expressed protein**

<table>
<thead>
<tr>
<th>Sample</th>
<th>IFT sample</th>
<th>Protein activity concentration (U/ml)</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty vector</td>
<td>nd</td>
<td>2.2</td>
<td>nd</td>
</tr>
<tr>
<td>Extracted protein</td>
<td>543</td>
<td>0.6</td>
<td>961</td>
</tr>
<tr>
<td>Fraction 1</td>
<td>799</td>
<td>0.4</td>
<td>1991</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>2216</td>
<td>1.7</td>
<td>1322</td>
</tr>
</tbody>
</table>

1U = 1 μmol DFAIII /min

**Characteristics of expressed IFT protein**

- **Crude IFT protein produced by *Nonomuraea* sp. AHU1850**
  - Optimum temperature: 65°C  
  - Optimum pH: pH 5.5  
  - Heat stability: 70°C

**Characteristics of expressed IFT**

- Optimum temperature
- Optimum pH
- Heat stability

**Conclusion**

- IFT protein of *Nonomuraea* sp. AHU1850 was unique.
- IFT protein was confirmed its activity, which was optimum at pH 5.5 and 65°C.
- Dahlia tuber is a good source for DFAIII production.
- Concentration of atropine and scopolamine was estimated to be in safety level.
Heterologous production and characterization of *Arabidopsis* polygalacturonase produced in the fission yeast

Nabilah Sari Mustafa, Takao Ohashi, Kazuhito Fujiyama

*International Center for Biotechnology, Osaka University, Suita, Osaka 565-0871, Japan*

Polygalacturonase (PG) is an enzymatic protein that hydrolyze $\alpha$-1,4 glycosidic linkages in polygalacturonic acid producing D-galacturonic acid. PGs form the most abundant glycosylhydrolase family in *Arabidopsis thaliana* and they are considered to be involved in fruit ripening, organ abscission and pollen grain development (Torki, M. et al. 2000). Indeed, the genome database of *A. thaliana* predicts 64 putative PG genes, which are then phylogenetically divided into 6 clades (clade A, B, C, D, E and F). Some of the genes from clade A, B, C and D have been partially studied but those from clade E and F have never been identified and characterized. Furthermore the physiological function and even the enzyme activity of PGs from clade E and F remained elusive. In this study, we tried to identify and characterize Arabidopsis polygalacturonase in clade F (AtPGF) as the first step for the elucidation of the PG functions.

First, we cloned AtPGF genes from *A. thaliana* cDNA and constructed the yeast expression vector. Four AtPGF proteins (AtPGF7, AtPGF8, AtPGF11 and AtPGF15) were heterologously produced in the fission yeast *Schizosaccharomyces pombe*. Among these, polygalacturonic acid hydrolyzing activity of AtPGF11 was successfully detected using fluorophore-labeled oligogalacturonic acids as the substrate. The substrate preference and other enzyme characteristics will be described.

**Keywords:** *Arabidopsis thaliana*, heterologous protein production, *Schizosaccharomyces pombe*, polygalacturonase

**Reference:**
Heterologous production and characterization of Arabidopsis polygalacturonase produced in the fission yeast

Nabilah Sari Mustafa¹, Takao Ohashi¹, Kazuhito Fujiyama¹

¹International Center for Biotechnology, Osaka University, Japan

Introduction

Plant Cell Wall
- The primary cell wall is thin and flexible. The major polysaccharides are cellulose, hemicellulose and pectin.
- The secondary cell wall is thicker and stronger, which consists mainly of cellulose, lignin and glycoproteins.

Functions:
- Determine cell shape
- Support the plant cells
- Store the carbohydrate

Polygalacturonase
- Polygalacturonase hydrolyzes α-1,4-glycosidic linkages in polygalacturonic acid.

Arabidopsis thaliana
- A model organism in plant biology which has a small genome, a large collection of mutant lines, and rapid life cycle.

Strategy

Cloning and heterologous expression of AtPGF gene in the fission yeast
- to examine putative gene of AtPGF activities
  - Isolation of AtPGF genes from A. thaliana cDNA
  - Construction of yeast expression vector
  - Transformation into fission yeast
  - Expression of AtPGF protein in the fission yeast
  - Enzymatic analysis

Production host

Schizosaccharomyces pombe
- a unicellular eukaryote
- It has a similar transcription mechanism with higher organism
- It has unique post-translational modifications
- Promoters that work in mammalian cells are also functional in S. pombe
Results

- Cloning and heterologous expression of AtPGF gene in the fission yeast to examine putative gene of AtPGF activities
- Isolation of AtPGF genes from A. thaliana cDNA
- Construction of yeast expression vector
- Transformation into fission yeast
- Expression of AtPGF protein in the fission yeast
- Enzymatic analysis

Enzymatic assay

<table>
<thead>
<tr>
<th>Preparation of permeabilized protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth yeast in MM-Leu medium (48 h)</td>
</tr>
<tr>
<td>Extracted the protein by glass beads</td>
</tr>
<tr>
<td>Centrifuged at 1000 x g, 5 min</td>
</tr>
<tr>
<td>Supernant</td>
</tr>
<tr>
<td>Centrifuged at 100,000 x g, 30 min</td>
</tr>
<tr>
<td>Pellet</td>
</tr>
<tr>
<td>Permeabilized membrane pellet in homogenizing buffer + 0.1 % Triton X-100</td>
</tr>
<tr>
<td>Supernant</td>
</tr>
<tr>
<td>DEAE-HPLC</td>
</tr>
</tbody>
</table>

Enzymatic analysis

- Reaction mixture:
  - Assay buffer
  - PA-GalUA
  - Permeabilized protein
  - Incubated at 37°C, 2 h
  - Heated at 100°C, 5 min
  - Centrifuged at 3,000 x g, 5 min
  - Supernant

Conclusion

- Eleven of AtPGF genes were isolated from A. thaliana cDNA
- Four of AtPGF protein (AtPGF7, AtPGF8, AtPGF11 and AtPGF15) were successfully expressed in the fission yeast
- PA-GalUA_{15} was the best substrate for AtPGF11

Future works

- Enzymatic assays for the remaining AtPGF
- Phenotypic analysis and Identification of T-DNA insertion mutant (to examine physiological function of AtPGF gene)
Development of bioprocess with designed biomass:
High $\beta$-lactic acid production efficacy from cellobiose and xylose mixture by *Enterococcus mundtii* QU 25

**Ying Wang**¹, Mohamed Ali Abdel-Rahman¹,², Yukihiro Tashiro¹, Takeshi Zendo¹, Kenji Sakai¹, Kenji Sonomoto¹,³

¹Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
²Botany and Microbiology Department, Faculty of Science (Boys), Al-Azhar University, PN:11884, Cairo, Egypt
³Department of Functional Metabolic Design, Bio-Architecture Center, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

Production of optically pure lactic acid (LA) from lignocellulose-derived mixed sugars is attractive but challenging because most of microbes exhibit carbon catabolite repression (CCR) in the presence of preferable sugar such as glucose. Utilization of xylose is generally inhibited by glucose, which becomes a major obstacle to economical utilization of mixed sugars derived from lignocellulosic biomass. *Enterococcus mundtii* QU 25, an optically pure $\beta$-LA-producer, which was isolated and characterized in our laboratory, presented a high activity of $\beta$-glucosidase grown in cellobiose as a sole carbon source (1). Furthermore, strain QU 25 was proved to homoferment xylose to $\beta$-LA via pentose phosphate/glycolytic pathway rather than phosphoketolase pathway (2). Therefore, this study aimed at homo-$\beta$-LA production from mixed sugars without CCR by *E. mundtii* QU 25.

Strain QU 25 fermented 100 g/l cellobiose and 60 g/l xylose (C100X60) simultaneously avoiding CCR caused by glucose in glucose/xylose mixture (G100X60). In batch fermentation with C100X60, strain QU 25 exhibited higher xylose consumption rate (1.78 g/l/h), LA concentration (90.2 g/l) and LA yield (0.756 g/g-consumed sugars) than 0.748 g/l/h, 71.2 g/l and 0.603 g/g that obtained using G100X60, respectively. Key enzymes related to xylose metabolism were also investigated, and more than 3 times specific activities of xylose isomerase and xylulokinase were found in C100X60 grown cells than in G100X60 grown cells. The effects of several kinds of nitrogen sources on LA fermentation using cellobiose and xylose were investigated, additional 5 g/l yeast extract resulting in a higher LA yield (0.831 g/g) than with other nitrogen sources. Furthermore, batch fermentation (C100X60) with addition of yeast extract improved cell growth from 3.54 g/l to 5.43 g/l and consequently LA concentration at 122 g/l in jar fermenter. Subsequently, with simulated energycane hydrolysate (C80X40G10), strain QU 25 fermented sugars simultaneously by addition of yeast extract with improved cell growth (6.67 g/l), LA production (115 g/l) and LA yield (0.863 g/g) using ammonium hydroxide as neutralizing agent for pH controlled fermentation compared to 5.33 g/l, 106 g/l, and 0.798 g/g using sodium hydroxide, respectively. Under the optimal conditions obtained, high $\beta$-LA concentration up to 163 g/l at the yield of 0.870 g/g and maximum LA productivity of 7.21 g/l/h were obtained without CCR in fed-batch fermentation. Thus, we established a high $\beta$-lactic acid fermentation system using lignocellulose-derived mixed sugars without CCR.

**Keywords:** $\beta$-lactic acid, carbon catabolite repression, mixed sugar fermentation, cellobiose, xylose

**References:**
Development of an integrating vector in an oleaginous yeast *Rhodosporidium toruloides* DMKU3-TK16 for lipid engineering

**Takenori Kanazawa**¹, **Takao Ohashi**¹, **Savitree Limtong**², **Kazuhito Fujiyama**¹

¹International Center for Biotechnology, Osaka University, Japan
²Faculty of Science, Kasetsart University, Thailand

Oleaginous yeasts, showing capability to accumulate lipids over 20 % of dry cell weight, have received massive attention for sustainable energy source. It is important to use a high lipid productive strain for industrial purpose because lipid productivity in yeasts varies with species and strains. DMKU3-TK16 (TK16) strain, isolated from soil samples of Thailand, was identified as *Rhodosporidium toruloides* by the D1/D2 domain sequence of the large subunit rRNA gene. The TK16 strain showed exceptionally higher lipid production than known representative oleaginous yeasts and accumulated lipids to more than 70% of its dry cell weight under the optimized culture condition (Limtong et al., 2010). To exploit its biotechnological potential, the development of effective genetic manipulation methods is needed. Previously we constructed a host vector system in the TK16 strain using pUXV vectors that included an autonomously replicating sequence and the glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter from *Ustilago maydis*. However, this system was severely limited due to instability of these vectors in the TK16 strain. In this study, we constructed integrating vectors, the pTK1 and the pTK2, which included TK16 *URA3* gene and GAP promoter from *R. toruloides* NBRC 0880 strain and were different in the direction of the *URA3* gene. The pTK1 and pTK2 vectors were integrated into chromosome of the TK16 *URA3* mutant by homologous recombination to develop a stable heterologous protein expression system. Transformation efficiencies of the pTK1 and the pTK2 by electroporation were $4.1 \times 10^4$ and $1.3 \times 10^5$ colony formation unit per μg of plasmid DNA, respectively. Furthermore we expressed enhanced green fluorescent protein using pTK1 and pTK2 vectors. This transformation system would be indispensable for development of lipid engineering in this strain.

**Keywords:** biodiesel, lipid production, oleaginous yeasts, *Rhodosporidium toruloides*, transformation

**Reference:**
Development of an integrating vector in an oleaginous yeast *Rhodosporidium toruloides* DMKU3-TK16 for lipid engineering

Takenori Kanazawa¹, Takao Ohashi¹, Savitree Limtong², Kazuhito Fujiyama¹

¹ International Center for Biotechnology, Osaka University, Japan
² Faculty of Science, Kasetsart University, Thailand

Biodiesel: renewable fuel derived from organisms

Fatty acid methyl ester is used as biodiesel

Application examples of FAME

- Fuel for diesel car or airplane
- Raw material for plastics

Oleaginous yeast *Rhodosporidium toruloides* DMKU3-TK16

- Isolated from soil sample in Thailand
- Oleaginous yeasts
  - Ability to accumulate lipid more than 20 % of dry cell weight
  - ex. Yarrowia, Rhodotorula, Cryptococcus, Lipomyces sp.
- High lipid production, 71 % lipid content of dry cell weight

Objective in this study

Development of transformation system in TK16 for lipid engineering by genetic manipulation

Our goal

Biodiesel is mainly produced from edible vegetable oils

- ex. rapeseed, sunflower and soybean

- Competition with food for human
- Long time to grow up

Biodiesel production from oleaginous yeast

- No competition with food for human
- Short life cycle
- More than 20 % of dry cell weight lipid production

Our final target is the development of biodiesel production system using oleaginous yeast isolated in Thailand

3. Transformation

**TK16 ΔURA3 strain**

- Precultivation
  - 5 ml YM medium at 30° C for 6 h
- Cultivation
  - 100 ml YM medium at 30° C for 16 h
- Harvest of cells

**Electroporation**

- Wash of cells by 1 M sorbitol twice
- Suspension of cells in 1 M sorbitol containing of 1 µg of linearized vector
- Electric pulse (5.0 kV/cm, 200 Ω, 25 µF)
- Addition of ice-cold 1 M Sorbitol
- Spread on SD -ura plate

(Kraisintu et al., 2010)
In vitro production of n-butanol with thermophilic enzymes

Borimas Krutsakorn, Kohsuke Honda, Xiaoting Ye, Takashi Imagawa, Xiaoyu Bei, Kenji Okano, Hisao Ohtake

Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan

The heat treatment of recombinant mesophiles having heterologous thermotolerant enzymes results in the one-step preparation of highly selective biocatalytic modules. The rational assembly of these modules enables the construction of an artificial metabolic pathway in vitro. In this work, we constructed a non-natural, cofactor-balanced, and oxygen-insensitive pathway for n-butanol production using 16 thermotolerant enzymes (Figure 1). To achieve an endogenous regeneration of ATP and ADP, the non-ATP-forming chimeric glycolytic pathway (1) was employed. In the metabolic pathway of a natural butanol producing strain, Clostridium acetobutylicum, the butyryl-CoA dehydrogenase (BCDH) catalyzes the reduction C=C double bond of an intermediate, crotonyl-CoA, to form butyryl-CoA. However, BCDH requires specific redox partner proteins for electron transfer and is highly sensitive to oxygen stress; therefore the enzyme cannot be used in vitro. In order to avoid the use of BCDH, the downstream enzyme, the aldehyde dehydrogenase (TtADDH) of Thermus thermophilus was directly used for the reductive removal of CoA residue of crotonyl-CoA. The reduction of C=C double bond of the resulting intermediate, croton aldehyde, could be catalyzed by the thermophilic NADH-dependent flavinoxidoreductase (TiNFO) from T. thermophilus. Through the artificial pathway, n-butanol could be produced from glucose with a molar yield of 82% at a rate of 8.2 μmol l⁻¹ min⁻¹.

Figure 1 The in vitro metabolic pathway for conversion of glucose to n-butanol.

Key words: n-butanol, thermophilic enzyme, in vitro metabolic pathway

Reference:
In vitro production of n-butanol with thermophilic enzymes

5 August 2010
JSPS-NRCT Young Scientist Seminar 2013 Osaka

Bacterial/Eukaryotic EM pathway


gene encoding thermophilic enzyme into
Transformation of an expression vector for the
Expression
Heating at 70°C for 20 minutes

Whole cells as biocatalysts

- Increase cell membrane
- Permeability
- The side reaction

No side reaction

- Heating at 70°C

Heating at 70°C

- Heat treatment:
- Increased cell membrane

permeability

Other uses of heated

in vitro

G0' = -282 kJ/mol

\Delta G = -282 kJ/mol

n-Butanol

C₆H₁₂O₆ \rightarrow C₄H₉OH + 2CO₂ + H₂O; \Delta G° = -282 kJ/mol

n-Butanol

C₆H₁₂O₆ \rightarrow C₄H₉OH + 2CO₂ + H₂O; \Delta G° = -282 kJ/mol
**Bypassed Pyruvate Decarboxylation Pathway**

Pyruvate dehydrogenase complex (PDHC)

Pyruvate: ferredoxin oxidoreductase (PFOR)

**Artificial butanol pathway**

Glucose  \( \rightarrow \) 3-Hydroxypropionaldehyde-CoA

Acetobacter pasteurianus

Pyruvate decarboxylase

Substrate specificity of ADDH from Thermus thermophilus

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (U/mg)</th>
<th>[Km] (mM)</th>
<th>[Vmax] (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA</td>
<td>3.6 \pm 0.8</td>
<td>11.3</td>
<td>18.3</td>
</tr>
<tr>
<td>Acetoacetyl-CoA</td>
<td>3.0 \pm 0.1</td>
<td>3.8</td>
<td>17.4</td>
</tr>
<tr>
<td>Butyryl-CoA</td>
<td>3.7 \pm 0.8</td>
<td>3.9</td>
<td>19.0</td>
</tr>
</tbody>
</table>

**Reordering of the butanol pathway**

Native pathway

Artificial pathway

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Butanol production</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4 mM</td>
<td>3.5 mM</td>
</tr>
</tbody>
</table>

**Flux optimization**

**In vitro butanol production**

Reaction Mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>1.0</td>
</tr>
<tr>
<td>NAD+</td>
<td>1.0</td>
</tr>
<tr>
<td>ADP</td>
<td>0.2</td>
</tr>
<tr>
<td>ATP</td>
<td>2.0</td>
</tr>
<tr>
<td>ATP</td>
<td>2.0</td>
</tr>
<tr>
<td>ADP</td>
<td>0.2</td>
</tr>
<tr>
<td>ATP</td>
<td>0.2</td>
</tr>
<tr>
<td>ADP</td>
<td>0.2</td>
</tr>
<tr>
<td>ATP</td>
<td>0.2</td>
</tr>
<tr>
<td>ADP</td>
<td>0.2</td>
</tr>
<tr>
<td>ATP</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Thermal stability of ApPDC (TtADDH)**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Residual Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>90</td>
</tr>
<tr>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>80</td>
<td>60</td>
</tr>
<tr>
<td>90</td>
<td>50</td>
</tr>
</tbody>
</table>

**In vitro butanol production**

Feeding rate 0.01 mM per minute

C\(_6\)H\(_{12}\)O\(_6\) \( \rightarrow \) C\(_4\)H\(_9\)OH + 2CO\(_2\) + H\(_2\)O; \( \Delta G^\circ \) = -282 kJ/mol
Development of bioprocess with designed biomass: Butanol production without catabolite repression from mixed sugars and elucidation of the mechanism of xylose metabolism

Takuya Noguchi¹, Yukihiro Tashiro¹, Kenji Sakai¹, Kenji Sonomoto¹,²

¹Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
²Department of Functional Metabolic Design, Bio-Architecture Center, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

Butanol produced by acetone-butanol-ethanol (ABE) fermentation draws much attention due to its promising characteristics. Lignocellulosic biomasses are considered to be a feasible substrate for ABE fermentation due to their abundance. These biomasses consist of different ratios of cellulose and hemicellulose as resulting available sugars. For efficient use of these biomasses, chemical and enzymatic pretreatment are generally performed to yield fermentable sugars. In pretreatment process, mixtures of hexose and pentose can be obtained by hydrolysis of lignocellulosic biomass. However, glucose derived from cellulose is known to inhibit the activity of cellulolytic enzymes such as cellulase, β-glucosidase, and to suppress the utilization of unfavorable other sugars such as xylose and arabinose, called carbon catabolite repression (CCR). Therefore, in this study, we aimed to study butanol production from mixed sugars of cellobiose and xylose without CCR, and to investigate the mechanisms for reducing CCR.

Clostridium saccharoperbutylacetonicum N1-4 was used in this study. ABE fermentation was conducted anaerobically for 72 h at 30°C without agitation in TY medium containing CaCO₃. Firstly, we performed batch culture with several mixed sugars. Thirty gram per litre glucose or cellobiose was used together with 30 g/l xylose (G30X30 and C30X30, respectively) as substrates. As a result, mixed sugars of cellobiose and xylose achieved efficient butanol production without CCR, and C30X30 precultured with xylose exhibited the highest butanol production, productivity and yield (16 g/l, 0.92 g/l/h, and 0.40 C-mol/C-mol, respectively). Moreover, we found that increase in initial xylose concentration (10-30 g/l) promoted butanol production and yield (60% and 12%, respectively). Lastly, fed-batch culture with cellobiose and xylose mixed sugars were performed. The initial concentrations of each sugar were 10 g/l to shorten the lag phase of sugar consumption. Through the 2-rounds fed-batch cultures, butanol production was increased in 150% compared to control (5.1 g/l). Thus, these results suggested that the mixed sugars can be applied to continuous culture¹).

We presumed that the use of cellobiose as a hexose component might contribute to the elimination of CCR in terms of xylose consumption. Thus, we investigated the xylose metabolism in G30X30 and C30X30 by analyzing activities of upstream enzymes, xylose isomerase and xylulose kinase. As a result, xylose isomerase in C30X30 showed ca. 3-fold higher activities (1.6 U/mg-protein) than that in G30X30 (0.52 U/mg-protein) while few differences of xylulose kinase were observed between C30X30 and G30X30. For further understanding of xylose metabolism, it is important to investigate other enzymes including transketolase and transaldolase in pentose phosphate pathway (PPP). Moreover, we will reveal the metabolic flux of ABE fermentation with mixed sugars.

Keywords: butanol, cellobiose, xylose, carbon catabolite repression, xylose isomerase

Butanol production without carbon catabolite repression from mixed sugars and elucidation of the mechanism of xylose metabolism

Takuya Noguchi1, Yukihiro Tashiro1, Kenji Sakai1, Kenji Sonomoto1, 2

2 Dept. Funct. Metab. Design, Bio-Arch. Ctr., Kyushu University

Butanol as a prospective biofuel

- 30-40% Higher calorific value than ethanol
- Safer to handle due to its lower vapor pressure
- No need for modification of combustion engines

Lignocellulosic biomass and pretreatment

Pretreatment (Physical & chemical)

Major problem in the utilization of mixed sugars
- Saccharification process
- Fermentation process

Carbon Catabolite Repression (CCR) suppresses the utilization of other sugars such as xylose and arabinose in mixed sugars fermentation.

Schematic figure of CCR in mixed sugars fermentation

- Genetic engineering to avoid CCR

- Improved xylose utilization but decreased butanol yield
- Improved xylose utilization
- Improved xylose utilization but remained CCR

No report of successful process with wild type.

Major problem in the utilization of mixed sugars

1. Butanol production without CCR using mixed sugars
   - Batch culture with several mixed sugars
   - The effect of initial xylose concentration on butanol production
   - Fed-batch culture with mixed sugars

2. Elucidation of the mechanism of the xylose metabolism
   - Comparison of several enzyme activities between different mixed sugars

Contents
Mixed sugars of cellobiose and xylose showed no CCR.

1. Butanol production with various mixed sugars

Mixed sugars of cellobiose and xylose were consumed simultaneously regardless of the sugars in preculture.

---

1. Butanol production with various mixed sugars

Table 1. Butanol production with mixed sugars

<table>
<thead>
<tr>
<th>Strain</th>
<th>Culture mode</th>
<th>Sugar concentration (g/l)</th>
<th>CCR</th>
<th>Butanol Yield (C-mol/C-mol)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium acetobutylicum</em></td>
<td>Batch</td>
<td>Glc 15</td>
<td>2</td>
<td>0.28</td>
<td>Fu et al. [1993]</td>
</tr>
<tr>
<td><em>Clostridium acetobutylicum</em></td>
<td>Batch</td>
<td>Xyl 15</td>
<td>2</td>
<td>0.30</td>
<td>Ounine et al. [1983]</td>
</tr>
<tr>
<td><em>Clostridium acetobutylicum</em></td>
<td>Batch</td>
<td>Glc 15</td>
<td>2</td>
<td>0.31</td>
<td>El Kanouni et al. [1994]</td>
</tr>
<tr>
<td><em>Clostridium saccharoperbutylacetonicum</em> DSM 782</td>
<td>Batch</td>
<td>Glc 15</td>
<td>2</td>
<td>0.30</td>
<td>Survase et al. [2002]</td>
</tr>
<tr>
<td><em>Clostridium saccharoperbutylacetonicum</em> N-1-4</td>
<td>Batch</td>
<td>Cell 16</td>
<td>2</td>
<td>0.40</td>
<td>This study</td>
</tr>
</tbody>
</table>

Mixed sugars of CX have a potential to achieve efficient butanol production without CCR and to reduce β-glucosidase dosages during saccharification process.

---

2. The effect of initial xylose concentration on butanol production

Table 2. Kinetic parameters of ABE fermentation using cellobiose and xylose

<table>
<thead>
<tr>
<th>Substrate (g/l)</th>
<th>Product (g/l)</th>
<th>Max. productivity (g/l/h)</th>
<th>Max. consumption (g/l)</th>
<th>Yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellobiose 16</td>
<td>Butanol 4.2</td>
<td>0.40</td>
<td>0.47</td>
<td>0.030</td>
</tr>
<tr>
<td>Xylose 16</td>
<td>Butanol 0.3</td>
<td>0.57</td>
<td>0.57</td>
<td>0.057</td>
</tr>
</tbody>
</table>

---

1. Butanol production without CCR using mixed sugars

1. Batch culture with several mixed sugars
2. The effect of initial xylose concentration on butanol production
3. Fed-batch culture with mixed sugars

---

2. Elucidation of the mechanism of the xylose metabolism

*Comparison of several enzyme activities between different mixed sugars

---

**Materials & Method**

- **Strain**
  *Clostridium saccharoperbutylacetonicum* N-1-4 (N1-4)

- **Media**
  - Batch culture: PG medium.
  - Preculture: TYA medium.
  - Temperature: 30°C
  - Volume: 300 ml

- **Culture condition**
  - Anaerobic batch culture

- **Substrates**
  - Procedure: 20 g/l Glucose (G) or Xylose (X)
  - Main culture: 30 g/l Glucose or Cellobiose (C) together with 30 g/l Xylose (G30X30, C30X30)

- Butanol yield (C-mol/C-mol): how many carbons were utilized to produce butanol

\[ \text{Butanol yield} = \frac{C_{\text{buta}}}{C_{\text{pen}} + C_{\text{chex}}} \]
3. Fed-batch cultures with mixed sugars of C10X10

- Butanol conc., cellobiose and xylose consumption rate increased by 150%, 33% and 55% as compared control.

13

0 2 4 6 8 10 12 14 16
OD562, Sugars (g/l)

0 2 4 6 8 10 12 14 16 18
Batch culture

2-rounds fed-batch culture

Butanol (g/l)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>G30X30</th>
<th>C30X30</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conclusion & Further works

- Mixed sugars including cellobiose resulted in simultaneous sugars consumption.
- Increase in xylose concentration improved all the kinetic parameters except for maximum cellobiose consumption rate.
- Fed-batch culture achieved improvement of both maximum sugars consumption rates.

Noguchi et al., J. Biosci. Bioeng., 2013, in press

Further works

- Investigation of the effect of glucose concentration on mixed sugars of CX.
- Investigation of application to continuous fermentation.

Contents

1. Butanol production without CCR from mixed sugars
   - Batch culture with several mixed sugars
   - The effect of initial xylose concentration on butanol production
   - Fed-batch culture with mixed sugars

2. Elucidation of the mechanism of the xylose metabolism
   - Comparison of several enzyme activities between different mixed sugars

Enzymes involved pentose phosphate (PP) pathway

Experimental design

- Condition
  - Refer to chapter 1.
- Main culture
  - TY + CaCO₃ medium (G30X30 vs C30X30).
- Harvesting
  - Cells were collected at the time when exhibiting CCR or not.

<table>
<thead>
<tr>
<th>Batch culture</th>
<th>2-rounds fed-batch culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellobiose</td>
<td>Butanol</td>
</tr>
<tr>
<td>OD₅₆₂</td>
<td></td>
</tr>
</tbody>
</table>

Experimentation

<table>
<thead>
<tr>
<th>Contents</th>
<th>Fin. Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES (pH8.0)</td>
<td>100 mM</td>
</tr>
<tr>
<td>Xylose</td>
<td>500 mM</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>5 mM</td>
</tr>
<tr>
<td>β-NADH</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>LDH</td>
<td>5 U</td>
</tr>
<tr>
<td>ATP</td>
<td>1.5 mM</td>
</tr>
</tbody>
</table>

enzyme activity

<table>
<thead>
<tr>
<th>U/mg-protein=A<em>V/(ε</em>d<em>e</em>p)</th>
</tr>
</thead>
</table>
| A; △ABS/min., V; volume [ml], \(\varepsilon\); millimolar extinction coefficient of NADH [L/mmol/cm], d; light path [cm], p; protein concentration [mg/ml].

167
Enzyme activities between G30X30 and C30X30

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Time [h]</th>
<th>Run</th>
<th>AbsX/min</th>
<th>Protein [mg/ml]</th>
<th>U/mg-protein</th>
<th>Ave U/mg-protein</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>G30X30</td>
<td>20</td>
<td>I</td>
<td>1.104</td>
<td>74</td>
<td>0.48</td>
<td>0.52</td>
<td>0.088</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>1.48</td>
<td>74</td>
<td>0.44</td>
<td>0.46</td>
<td>0.062</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>1.02</td>
<td>50</td>
<td>1.5</td>
<td>1.6</td>
<td>0.062</td>
</tr>
<tr>
<td>C30X30</td>
<td>20</td>
<td>I</td>
<td>2.364</td>
<td>50</td>
<td>1.5</td>
<td>1.5</td>
<td>0.062</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>2.364</td>
<td>50</td>
<td>1.5</td>
<td>1.5</td>
<td>0.062</td>
</tr>
</tbody>
</table>

## Conclusion & Further works

### Conclusion
- Xylose isomerase in C30X30 exhibited 3-fold greater activities than that in G30X30.
- There were few differences in xylulose kinase activities between G30X30 and C30X30.

### Further works
- Transketolase and transaldolase assay
- Transcriptional analysis of xylose transporter
- Elucidation of kinetic mechanism of ABE fermentation with mixed sugars

### Molecular mechanism of CCR in gram positive bacteria

- Xylose isomerase was suppressed under the presence of glucose, while cellobiose did not influence the enzyme activity.
- Few significant differences of xylulose kinase activity between both mixed sugars were obtained.

### PP pathway and gene expression

- Xylose transporter (CAC1339, CAC1345)
- Catabolic repression element (CRE) binding site
- Xylose kinase (CAC1344)
- Xylose-5-P and Ribose-5-P

### Intelligent Fermentation Technology (iFermenTech)

- Excellent strain library → Matching with designed biomass
- Adaptive process → Application and screening of potential feedstock
- Purposeful genetic manipulation → Construction of smart strains

### The concept of designed biomass

- Renewable resources
- Designed biomass study
- Targeted substrates
- Existing excellent strain
- High efficient process
- Mutagenesis
- Molecular breeding
- Searching substrate
- Modifying substrate
- Isolated strain
- Breeding strain
- Establishing efficient process
- Design strain
- Establishing adaptive process
- Useful substance biomaterials (e.g. Lactate)

### Plant thremmatology

- Creation of designed biomass
- Screening of designed biomass
- Plant breeder
- Molecular microbiologist
- Environmental engineer

### Molecular microbiology

- Mutagenesis
- Metabolic analysis
- Expression analysis

---

**Table 3. Xylose isomerase**

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Time [h]</th>
<th>Run</th>
<th>AbsX/min</th>
<th>Protein [mg/ml]</th>
<th>U/mg-protein</th>
<th>Ave U/mg-protein</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>G30X30</td>
<td>20</td>
<td>I</td>
<td>1.104</td>
<td>74</td>
<td>0.48</td>
<td>0.52</td>
<td>0.088</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>1.48</td>
<td>74</td>
<td>0.44</td>
<td>0.46</td>
<td>0.062</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>1.02</td>
<td>50</td>
<td>1.5</td>
<td>1.6</td>
<td>0.062</td>
</tr>
<tr>
<td>C30X30</td>
<td>20</td>
<td>I</td>
<td>2.364</td>
<td>50</td>
<td>1.5</td>
<td>1.5</td>
<td>0.062</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>2.364</td>
<td>50</td>
<td>1.5</td>
<td>1.5</td>
<td>0.062</td>
</tr>
</tbody>
</table>

---

**Intelligent Fermentation Technology (iFermenTech)**

- Excellent strain library → Matching with designed biomass
- Adaptive process → Application and screening of potential feedstock
- Purposeful genetic manipulation → Construction of smart strains

### Conclusion & Further works

**Conclusion**
- Xylose isomerase in C30X30 exhibited 3-fold greater activities than that in G30X30.
- There were few differences in xylulose kinase activities between G30X30 and C30X30.

**Further works**
- Transketolase and transaldolase assay
- Transcriptional analysis of xylose transporter
- Elucidation of kinetic mechanism of ABE fermentation with mixed sugars
Metabolic pathway in N1-4

Pentose Phosphate Pathway (PPP) in N1-4

Expression of xylose transporters and metabolic enzymes

Cellobiose transporter is not repressed by xylose

1. Butanol production with various mixed sugars.

Time course of butanol production – sub. consumption
### Acetone and ethanol on batch culture

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Acetone (g/L)</th>
<th>Ethanol (g/L)</th>
<th>Maximum productivity (g/L/h)</th>
<th>Yield (C-mol/C-mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-C30X30</td>
<td>4.96±0.206</td>
<td>0.510±0.066</td>
<td>0.222±0.021</td>
<td>0.227±0.011</td>
</tr>
<tr>
<td>G-C30X30</td>
<td>5.89±0.146</td>
<td>0.613±0.022</td>
<td>0.326±0.034</td>
<td>0.328±0.004</td>
</tr>
<tr>
<td>X-C30X30</td>
<td>6.21±0.265</td>
<td>0.623±0.025</td>
<td>0.382±0.040</td>
<td>0.395±0.007</td>
</tr>
</tbody>
</table>

Glucoseの代わりにセルロースを用いた結果、アセトン、エタノール生産に関わる全てのパラメーターを向上させた。ソルベントの全体的な生産向上がソルベント収率の向上につながったと考えられる。特にX-C30X30はG-C30X30と比較してアセトン生産量が約1.25 g/L上昇した。

特定のソルベントではなくアセトン、ブタノール、エタノール生産量が向上したという結果から、アチル-CoAやアセトアセチル-CoA周辺の酵素の活性や発現が影響を受けたのではないか。

### 3. Fed-batch cultures with mixed culture

- **C10X10 Batch**
  - Cellulose: 16 g/L
  - Xylose: 14 g/L
  - Butanol: 6 g/L
  - DCW: 8 g/L
  - Time: 80 h

- **C10X10 1-round feeding**
  - Cellulose: 14 g/L
  - Xylose: 12 g/L
  - Butanol: 16 g/L
  - DCW: 10 g/L
  - Time: 80 h

1-round feeding exhibited approximately 58.5% higher butanol production (8.02 g/L) than that of batch culture (5.06 g/L).

### How can we get mixed sugars of cellobiose & xylose ??

Traditional process:
- Enzyme saccharification
- Cellulose → Hemicellulose
- Glucose → Xylose

Alternative process:
- Enzyme saccharification
- Cellulose
- Hemicellulose
- Glucose
- Xylose

### Enzyme saccharification by cellulolytic enzymes from Trichoderma reesei


**Endoglucanase**

**Cellulohydrolase**

From β-glucosidase-deficient Trichoderma reesei

**Cellulose**

**Cellobiose**

### How can we get mixed sugars of cellobiose & xylose ??

Traditional process:
- **Pretreatment** (e.g. AFEX)
- **Enzyme saccharification**
- **Cellulose**
- **Hemicellulose**
- **Glucose**
- **Xylose**
the effect of different hexose ratios on xylose consumption

![Graph showing xylose consumption at different hexose ratios](image1)

At least 30 g/l of glucose in mixed sugars prolonged the lag phase of xylose consumption.

Development of butanol separation and purification technology with saved energy (Japan)

![Graph showing butanol production and pH change](image2)

Butanol production using designed mixed sugars. (A); Energy cane (C40G5X20), (B); Hardwood (C50G25X10)

Mixed sugars fermentation by *C. beijerinckii* NCIMB 8052.
Development of bioprocess with designed biomass: Novel butanol fermentation with acetate as substrate by *Clostridium saccharoperbutylacetonicum* N1-4

**Ming Gao**, Yukihiro Tashiro, Tsuyoshi Yoshida, Takuya Noguchi, Jin Zheng, Qunhui Wang, Kenji Sakai, Kenji Sonomoto

1Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan
2Department of Environmental Engineering, School of Civil & Environment Engineering, University of Science and Technology Beijing, Beijing 100083, China
3Department of Functional Metabolic Design, Bio-Architecture Center, Kyushu University, Fukuoka 812-8581, Japan

Butanol is one of the most promising biofuels, which offers several advantages over ethanol for gasoline blending. Considering the high price of petroleum oil and recent reports on the production of butanol from inedible biomass such as agricultural and domestic wastes, it is expected that butanol will become a more economically attractive biofuel. Acetic acid is known to be the most prevalent organic acid accumulated in hydrolysate during hydrolysis of inedible biomass. ABE-producing clostridia are reported to have the utilizing pathways of acetic acid and to metabolize it to produce butanol without carbon losses. However, there are no reports on efficient butanol production from acetic acid as a carbon source. Therefore, this study was conducted whether acetate can be converted to butanol in order to achieve efficient butanol production with acetate as a kind of carbon source.

In order to investigate the effect of acetate on butanol fermentation by *Clostridium saccharoperbutylacetonicum* N1-4, batch culture was performed in TY medium containing various concentrations of acetate and 50 g/l glucose. Particularly, addition of 4 g/l acetate showed 3.36 g/l acetate utilization, and increased butanol concentration from 9.12 g/l to 14.1 g/l and the maximum volumetric production rate from 0.443 g/l/h to 0.755 g/l/h, compared to those without acetate addition. Subsequently, to confirm the conversion of acetic acid to butanol, GC-MS analysis of the batch culture broth with 4 g/l [1,2-13C2] acetate and 50 g/l glucose was performed. 12C2-acetate was detected for 9 h of cultivation, which suggested that acetic acid should be produced from glucose during utilization of additional acetate. In addition, we detected three and four mass spectra of butanol and acetone with different intensities, and we confirmed that additional acetate converted to butanol and acetone with the efficiency of 69.4% and 23.5%, respectively.

On the other hand, acetate cannot be utilized as a sole substrate without the reducing power via glycolysis. As the investigation of the ratio of glucose to acetate (G/A), G/A at 10:1 could gain the maximum acetate consumption. Finally, fed-batch culture by 2 rounds of substrate feeding with G/A at 10:1 was performed, acetate utilization, butanol production and maximum volumetric butanol production rate were improved to 4.16 g/l, 14.9 g/l, and 0.781 g/l/h, compared with 3.30 g/l, 10.5 g/l, and 0.656 g/l/h in batch culture, respectively.

**Keywords:** butanol production, acetate, GC-MS analysis, fed-batch culture
Oral presentation 29 (O-29)

**Direct use of recombinant *Escherichia coli* having thermophilic enzyme as whole cell biocatalyst in continuous bioconversion system**

**Pham Huynh Ninh**, Kohsuke Honda, Yukako Yokohigashi, Kenji Okano, Hisao Ohtake

*Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan*

The direct use of recombinant *Escherichia coli* having heterologous thermophilic enzymes at high temperature results in the heat-induced leakage of the thermophilic enzymes (1). This limits the applicability of thermophilic whole-cell catalysts to continuous and repeated-batch reactions. A potential approach to prevent the heat-induced enzymes leakage is the use of protein cross-linking reagents for the consolidation of the cell membrane as well as the linkage of enzymes to the membrane structure. In present study, *E. coli* cells having a thermophilic fumarase were treated with glutaraldehyde (GA) followed by the heat treatment to inactivate the intrinsic enzymes, and then directly used for the conversion of fumarate to malate in a continuous reactor.

The thermophilic fumarase of *Thermus thermophilus* HB8 (*TtFTA*) was overproduced in *E. coli*. The cells were treated with various concentrations of GA and heated at 70°C. The scanning (SEM) and transmission electron microscope (TEM) analysis were carried out to observe changes of the GA-treated cells. The membrane permeability of GA-treated cells was also assessed by determining the penetration of fluorescent dextrans into the heated cells. Finally, the GA-treated cells were used as whole-cell biocatalysts for the conversion of fumarate to malate at 70°C in a stirred reactor equipped with a PVDF cell-separation filter (2).

The heat-induced leakage of *TtFTA* could be prevented by the pretreatment of the cells with 0.11% or higher concentration of GA. Interestingly, while the SEM images showed no apparent change in the membrane structure of the GA-treated cells, the cell membrane permeability to relatively small molecules (up to at least 3 kDa) was significantly improved by heat treatment. The GA-treated cells retained a sufficient enzyme activity and were applicable to fumarate hydration in the continuous bioreactor over 10 h with a constant product yield of 60%.

**Keywords**: whole-cell catalyst, glutaraldehyde, thermophilic fumarase, continuous reactor

**References**:  
Direct use of recombinant *Escherichia coli* having thermophilic enzyme as whole cell biocatalyst in continuous bioconversion system

Ninh P.H., Honda K., Yokohigashia Y., Okano K., Omasa T. and Ohtake H. Osaka University

05 August 2013

High operational stability
High co-solvent compatibility
Low risk of contamination
Produced in mesophilic host

Limit the application of thermophilic enzymes in repeated and continuous reaction.

Study outline

Enzyme assay

E. coli cells having TtFTA

Enzyme leakage test

Bioconversion efficiency

0.11% (v/v) of Glutaraldehyde can prevent the enzyme leakage in E. coli

Bioconversion condition:
- Cell density: 4 mg wet cells/ml
- Phosphate buffer 0.1 M
- Substrate: 200 mM Sodium fumarate
- Temperature: 70°C

0.11% of GA for the cross-linkage as the best compromise between the prevention of heat-induced leakage and the maintenance of enzyme activity.

Leakage level (%)

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cells, no glutaraldehyde</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>0.03% glutaraldehyde</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>0.07% glutaraldehyde</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>0.11% glutaraldehyde</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>0.15% glutaraldehyde</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
</tbody>
</table>

Bioconversion efficiency

- Free cells
- Cells pretreated with 0.03 % glutaraldehyde
- Cells pretreated with 0.07 % glutaraldehyde
- Cells pretreated with 0.11 % glutaraldehyde
- Cells pretreated with 0.15 % glutaraldehyde

Time course of malate production

Malate concentration (mM)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cells</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Cells pretreated with 0.03 % glutaraldehyde</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Cells pretreated with 0.07 % glutaraldehyde</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Cells pretreated with 0.11 % glutaraldehyde</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Cells pretreated with 0.15 % glutaraldehyde</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
</tbody>
</table>
The permeability of the GA-treated cells

- 0.11% GA-treated cells solution
- Dex 3 or 40 kDal labeled with fluorescent dye
- Shake 30 min, 37°C
- Vortex 70°C 30 min
- Re-suspend in the same fresh buffer
- Fluorescent emission measurement (494-521 nm)

Dex 3 Dex 40

Dextran concentration [µM]

- 0.000
- 0.005
- 0.010

Heat treatment

- Without heat treatment
- GA-treated cells without heat treatment
- GA-treated cells with heat treatment

fluorescent emission measurement (494-521 nm)

SEM (a-d) and TEM (e-h) images of free and GA-treated E. coli cells before and after heat treatment at 70°C for 20 min.

Continuous bioconversion system

- Flow rate: 0.25 ml/min
- Total volume: 10 ml
- Residence time: 40 min
- Fumarate cont.: 0.2 M

\[ \Delta [S_{in}] - [S_{out}] = \gamma \times t \]

- \( \Delta [S_{in}] \): change of inlet substrate
- \( [S_{out}] \): change of outlet substrate
- \( \gamma \): conversion rate (mM/min)
- \( t \): operation time (min)

Summary

- The leakage of thermophilic fumarase was prevented by treating the recombinant E. coli cells with Glutaraldehyde (≥ 0.11% v/v).
- The cellular structure of GA-treated E. coli was not apparently changed by the heat treatment, however, their membrane permeability was significantly improved.
- GA-treated E. coli having thermophilic fumarase has been successfully applied to a continuous reactor for the conversion of fumarate to malate in more than 600 min with a molar conversion yield of 60%.
- A fast and simple method for the preparation of highly selective and stable biocatalysts was developed.

Acknowledgments

- Dr. Y. Muranaka, Research Center for Ultra-High Voltage Electron Microscopy, Osaka University.
- Dr. Takashi Mimitsuka, Toray Industries, Inc.

Thank for your attention
Oral presentation 30 (O-30)

**Carbon utilization in a chemolithoautotroph**

*Hydrogenophilus thermoluteolus* TH-1

**Nguyen Huu Tri**\(^1\), Hirofumi Nishihara\(^2\), Hiroyuki Arai\(^3\), Masaharu Ishii\(^1\)

\(^1\)Department of Biotechnology, Graduate school of Agriculture and Life Science, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan
\(^2\)Department of Bioresource Science, Graduate School of Agriculture, Ibaraki University, Mito, Ibaraki 310-0056, Japan
\(^3\)Department of Biology, Faculty of Science, University of Agricultural and Forestry, Ho Chi Minh 70-800, Vietnam

The thermophilic hydrogen-oxidizing bacterium *Hydrogenophilus thermoluteolus* TH-1 belongs to the beta-proteobacteria. *H. thermoluteolus* TH-1 can grow either autotrophically on \( \text{H}_2, \text{O}_2 \) and assimilate \( \text{CO}_2 \) via the Calvin cycle or heterotrophically but the control of carbon metabolism is poorly understood.

Growth experiments of *H. thermoluteolus* TH-1 have been conducted at 50°C autotrophically, heterotrophically on a variety of organic compounds and mixotrophically. The results showed that *H. thermoluteolus* TH-1 cannot utilize glucose, fructose, xylose, glycerol, glyoxylate or formate. In mixotrophic condition with 20 mM malate, the growth was vigorous. Interestingly, *H. thermoluteolus* TH-1 cannot grow mixotrophically with concentration of acetate at 20 mM but at 5 mM acetate *H. thermoluteolus* TH-1 showed ability to grow mixotrophically. RubisCO activity under autotrophic condition was 13.89 ± 0.59 (U/mg) which was higher than that under mixotrophic condition (with malate 4.03 ± 0.62 (U/mg)). RubisCO activity was not detected in the heterotrophic conditions.

*H. thermoluteolus* TH-1 have a potential to produce biopolymer poly-3-hydroxybutyrate (PHB). The initial results showed that after 48 hours cultivation autotrophically, PHB accumulation in induction medium was higher than in mineral salts basic medium. PHB accumulation reached approximately to 44% of the cellular dry weight.

**Keywords**: chemolithoautotrophs, hydrogen-oxidizing bacterium, poly-\( \beta \)-hydroxybutyrate, *Hydrogenophilus thermoluteolus*

**Reference**:
Carbon utilization in a chemolithoautotroph

Hydrogenophilus thermoluteolus TH-1

Nguyen Huu Tri 1, 3, Hirofumi Nishihara 2, Hiroyuki Arai 1, Masaharu Ishii 1

1. Department of Biotechnology, Graduate school of Agriculture and Life Science, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan
2. Department of Bioresource Science, Graduate School of Agriculture, Ibaraki University, Mito, Ibaraki 310-0056, Japan
3. Department of Biology, Faculty of Science, University of Agricultural and Forestry, Ho Chi Minh 70-800, Vietnam

Hydrogenophilus thermoluteolus TH-1

- Thermophilic hydrogen-oxidizing bacterium
- The maximum specific growth rate: 0.68 hr⁻¹
- Facultative chemolithoautotroph
- Gram-negative, non spore-forming
- Temperature: 50 °C
- pH: 7.0

Goto, E., Kodama, T., Minoda, Y. (1977)

Purpose of study

1. Clarifying carbon and energy metabolism of Hydrogenophilus thermoluteolus TH-1 in various culture conditions
2. Making useful products by using Hydrogenophilus thermoluteolus TH-1

Cultivation

The components of mineral salts medium

- Sodium chloride 1 g
- KH₂PO₄ 0.1 g
- MgSO₄•7H₂O 2 g
- FeCl₃•6H₂O 0.005 g
- Trace elements solution: 0.5 ml

pH 7.0

The components of trace elements solution

- FeCl₃•6H₂O 4 mg
- ZnCl₂ 4 mg
- CuCl₂•2H₂O 2 mg
- MnCl₂•4H₂O 4 mg
- CoCl₂•6H₂O 4 mg

In heterotrophic condition, butyrate was shown to be a favourable substrate for strain TH-1.

In mixotrophic condition with butyrate at 10 mM, a long lag time was observed before vigorous growth.
Acetate utilization in *Hydrogenophilus thermoluteolus* TH-1

*H. thermoluteolus* TH-1 showed ability to grow mixotrophically with acetate at 10 mM or 5 mM. Interestingly, at 20 mM acetate, mixotrophic growth was completely inhibited.

**Rubisco assay**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Rubisco activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autotrophy</td>
<td>13.89 ± 0.59</td>
</tr>
<tr>
<td>Mixotrophy (Butyrate)</td>
<td>3.38 ± 0.14</td>
</tr>
<tr>
<td>Mixotrophy (Acetate)</td>
<td>7.95 ± 0.12</td>
</tr>
<tr>
<td>Mixotrophy (Malate)</td>
<td>4.03 ± 0.82</td>
</tr>
<tr>
<td>Heterotrophy</td>
<td>Not Detected</td>
</tr>
</tbody>
</table>

Rubisco activity under autotrophic condition was significantly higher than under mixotrophic conditions.

**Poly-3-Hydroxybutyrate (PHB) production in *Hydrogenophilus thermoluteolus* TH-1**

**HPLC assay: PHB content of biomass was determined by acid hydrolysis of PHB to crotonic acid**

<table>
<thead>
<tr>
<th>Sample</th>
<th>PHB accumulation after 48 hours induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control (Before induction)</td>
<td></td>
</tr>
<tr>
<td>Positive control (Standard PHB)</td>
<td></td>
</tr>
<tr>
<td>Autotrophy</td>
<td>43.9 ± 1.2</td>
</tr>
<tr>
<td>Mixotrophy (Acetate)</td>
<td>63.3 ± 1.9</td>
</tr>
</tbody>
</table>

**Conclusion**

- Strain TH-1 showed ability to grow mixotrophically with butyrate at 10 mM, however, a long lag time was observed. Butyrate was a favourable substrate with strain TH-1 in heterotrophy.
- Rubisco activity was highest in autotrophic condition but it was not detected in heterotrophic conditions.
- Under N-depleted condition, *H. thermoluteolus* TH-1 had a potential to produce biopolymer poly-3-hydroxybutyrate.

**Future study**

- Comprehensive analyses of carbon and energy metabolism of strain TH-1.
- Modification of the metabolic pathway of TH-1 to produce useful compounds.
Identification of protein glycosylation operon from *Campylobacter jejuni* JCM 2013

**Akkaraphol Srichaisupakit, Takao Ohashi, Kazuhito Fujiyama**

*International Center for Biotechnology, Osaka University, Suita, Osaka 565-0871, Japan*

*Campylobacter jejuni* is a human enteropathogenic bacterium possessing a glycosylation system (1, 2). In this work, protein glycosylation (*pgl*) operon conferring prokaryotic glycosylation in *C. jejuni* JCM 2013 was cloned and identified. Fourteen open reading frames (ORFs) were found in the *pgl* operon. The organization of the *pgl* operon was similar to that of *C. jejuni* NCTC 11168 with 98 and 99 % identities in overall nucleotide and amino acid sequence, respectively. The *pgl* operon was heterologously co-expressed with model protein CmeA in *Escherichia coli* BL21 mutant. The immuno- and lectin-blotting analysis indicated the protein glycosylation on the recombinant CmeA. Furthermore, to analyze the glycan composition, the recombinant CmeA was purified and subjected to in-gel trypsin digestion followed by mass spectrometry analysis. The mass spectrometry analysis showed the predicted prokaryotic glycosylation. Further glycan structural study using the conventional fluorophore-labeling method would reveal the detailed glycan structure such as monosaccharide constituent and their linkage. In conclusion, *pgl* operon from *C. jejuni* JCM 2013 successfully functioned, resulting in the observed prokaryotic glycosylation.

**Keywords:** *Campylobacter jejuni, Escherichia coli*, prokaryotic glycosylation, protein expression

**References:**
Identification of protein glycosylation operon from
Campylobacter jejuni JCM 2013

Srichaisupakit Akkaraphol, Takao Ohashi,
and Kazuhito Fujiyama
Lab. of Applied Microbiology
International Center for Biotechnology
Osaka University

Presentation outline

- Introduction
  - General Eukaryotic N-glycosylation
  - Campylobacter jejuni bacterium and its N-glycosylation pathway
- Experiment
  - Characterization of Campylobacter jejuni JCM 2013 protein glycosylation (pgl) operon
  - Heterologous expression of pgl operon in Escherichia coli
  - Analysis of protein glycosylation
- Prospective
  - Glyco-engineering in Escherichia coli

General Eukaryotic N-linked Glycosylation

Campylobacter jejuni

- Gram-negative
- Common cause of bacterial diarrhea
  - acute abdominal pain, inflammatory diarrhea, fever
- Possesses N-glycosylation pathway with components encoding from protein glycosylation operon

Previous studies

- C. jejuni NCTC 81116 and NCTC 11168
  - Both are food-borne pathogens
  - Former strain’s protein glycosylation was studied by Fry et al. 1998
- C. jejuni JCM 2013
  - Japan Collection of Microorganisms deposit
  - The protein glycosylation operon remains elusive

Objective

Characterization of protein glycosylation operon of C. jejuni JCM 2013

Experiment

- Characterization of Campylobacter jejuni JCM 2013 protein glycosylation (pgl) operon
- Heterologous expression of pgl operon in Escherichia coli
- Analysis of protein glycosylation
Summary of *C. jejuni* JCM 2013 pgl operon characterization

- *pgl* operon with sequence of 15,597 nucleotides was determined
- Compared to that of *C. jejuni* NCTC 11168:
  - 98% identities for nucleotide sequence
  - 99% identities for amino acid sequence
  - no insertion, deletion, or change in direction of organization

2. Heterologous expression of *pgl* operon in *Escherichia coli*

To examine the function of the cloned operon, heterologous expression was performed with selected model protein, CmeA

**Features of CmeA**
1. Abundant in *C. jejuni*
2. Modified construct yield soluble protein production in *E. coli*
3. Has 5 N-X-S/T sequon, only special two are recognized by *C. jejuni* PglB

**Conclusion**

- Protein glycosylation (*pgl*) operon of *C. jejuni* JCM 2013 was cloned and characterized
  - Similar organization to that of NCTC 11168 strain
- Protein glycosylation operon was expressed in *E. coli*
  - Putative CmeA model protein glycosylation was observed
- CmeA protein glycosylation was analyzed by Mass Spectrometry
  - Glycan structure of Hex-HexNAc_6_ was detected

**Prospectives**

- Glyco-engineering in *E. coli*
- Glycan analysis by fluorophore tagging
  - Elucidate the monosaccharide constituent and their linkages

**Thank you for your attention**
Characterization of genes involved in the biosynthesis of Virginiamycin M, a streptogramin type A antibiotic

Mario Rovani¹, Shigeru Kitani¹, Nattika Pulsawat¹, Takuya Nihira¹²

¹International Center for Biotechnology, Osaka University, Suita, Osaka 565-0871, Japan
²MU-OU Collaborative Research Center for Bioscience and Biotechnology, Faculty of Science, Mahidol University, Rama VI Rd, Bangkok 10400, Thailand

Streptomyces virginiae produces virginiamycin M (VM) and virginiamycin S (VS), both of which are belonging to the streptogramin family and work synergistically with potent bactericidal activity against Gram-positive bacteria. The VM and VS biosynthetic genes show a unique organization because they are not situated in separate clusters but are scattered in the same cluster. The VM framework is predicted to be assembled by both polyketide synthase and nonribosomal peptide synthetase (NRPS), starting from sequential condensation of isobutyryl-CoA as a primer molecule, followed by incorporation of two malonyl-CoAs, one glycine, four additional malonyl-CoAs, one serine, and one proline. We have already identified four genes (virA, virF, virG and virH) encoding a large multifunctional PKSs and hybrid PKS–NRPS and seven genes (virB, virC, virD, virE, virI, virJ and virK) encoding monofunctional polypeptides. Recently, we also already identified an NRPS gene, orf7 which is located in the left-hand extremity of virginiamycin supercluster. Disruption of orf7 abolished VM production, without affecting VS production, suggesting the possibility that orf7 might be involved in VM production by incorporating proline essential for finalizing the VM structure. Sequencing analysis of a 4.8 kb downstream fragment demonstrated that the region has two flavin-utilizing monooxygenase genes, a flavin-dependent oxidoreductase gene, and S-adenosylmethionine synthetase gene, which are presumably involved in the post modification steps of virginiamycin biosynthesis. Combining the current and previously identified sequence information, we propose that the genes participating in the synchronized biosynthesis of VM and VS are extended over an approximately 105-kb region.

Keywords: virginiamycin M, hybrid polyketide-nonribosomal peptide, Streptomyces virginiae

References:
Characterization of genes involved in the biosynthesis of virginiamycin M, a streptogramin type A antibiotic

Mario Rovani*, Shigeru Kitani, Natikla Pulsawat, and Takuya Nihira

International Center for Biotechnology, Osaka University

Streptogramin antibiotic

Two structurally unrelated antibiotics

Streptomycetes spp.

Streptomyces antibiotic

Streptomyces spp.

Cyclic depsipeptides

Cyclic macroactones

Strong synergistic antibactericidal effect

Virginiamycin biosynthetic genes

Regulation

VirM biosynthesis

Resistance

Virginiae butanolide

Virginiamycin M (VM)

Virginiamycin S (VS)

Synercid®

First drug choice to combat Vancomycin-resistant bacteria

Virginiamycin production profile in S. virginiae

Virginiamycin M is a hybrid polyketide peptide compound

Our identified genes are crucial structural genes for virginiamycin biosynthesis
**Biosynthetic pathway of starter unit, isobutyryl-CoA**

- **Branched-chain α-ketoisovaleric acid**
- **Branched-chain α-ketoacid Dehydrogenase (BCDH)**
- **α-ketoisovaleric acid**
- **NAD+ and CoA**
- **NADH and CO2**

**Branched-chain amino acid transaminase**

**Valine**

**Isobutyryl-CoA**


---

**Gene disruption of bkdA disruption**

- **bkdA disruption abolished VM production**


---

**The effect of bkdA overexpression on VM production**

- **bkdA overexpression enhanced VM production**


---

**Proposed model for VM biosynthetic pathway**

- **38 kb consisting of**
  - 11 genes = virA to virK
  - 9 modules = 7 PKS + 2 NRPS

*Pulsawat et al. Genes (2007)*

---

**The biosynthetic genes for VM structure modification**

<table>
<thead>
<tr>
<th>Protein</th>
<th>AA</th>
<th>Predicted function</th>
<th>Representative homolog</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orf7</td>
<td>349</td>
<td>Flavin-utilizing monoxygenase</td>
<td>SnaA, S. pristinaespiralis</td>
<td>81</td>
</tr>
<tr>
<td>Orf8</td>
<td>371</td>
<td>Flavin-utilizing monoxygenase</td>
<td>SnbB, S. pristinaespiralis</td>
<td>49</td>
</tr>
<tr>
<td>Orf9</td>
<td>261</td>
<td>Flavin reductase</td>
<td>FMN_red, Kitasatospora reitii</td>
<td>46</td>
</tr>
<tr>
<td>Orf10</td>
<td>402</td>
<td>S-adenosylmethionine synthetase</td>
<td>SamB, S. pristinaespiralis</td>
<td>93</td>
</tr>
</tbody>
</table>

*Rovani et al. Unpublished data.*
The biosynthetic genes for VM structure modification

\[ \text{orf7, orf8, orf9} \quad \text{Ca 4.8 kb} \]

- Orf7, Orf8: Flavin-utilizing monoxygenase
- Orf9: Flavin reductase

Virginiamycin M, a hybrid polyketide/peptide compound

\[ \text{SnaD} \]

High homology

ID/SM% (69/92)


VisF

VisG

bkdC

bkdD

orf4 orf6

virP virO

VisE

SnaD

Ser

Gly

VirA

VirG

VirH

Pro

The adenylation domain of VirP

VirP-domain organization

N C A PP E C PP TE C

C: Condensation

A: Adenylation

PP: Peptidyl-carrier-protein

TE: Thio-esterase

E: Epimerization

A D V H R V A Y: no homology

The substrate of VirP-A domain is proline?

The biosynthetic genes for VM structure modification

\[ \text{orf7, orf8, orf9} \quad \text{Ca 4.8 kb} \]

- Orf7, Orf8: Flavin-utilizing monoxygenase
- Orf9: Flavin reductase

Virginiamycin M, a hybrid polyketide/peptide compound

\[ \text{SnaD} \]

High homology

ID/SM% (69/92)


VisF

VisG

bkdC

bkdD

orf4 orf6

virP virO

VisE

SnaD

Ser

Gly

VirA

VirG

VirH

Pro

The adenylation domain of VirP

VirP-domain organization

N C A PP E C PP TE C

C: Condensation

A: Adenylation

PP: Peptidyl-carrier-protein

TE: Thio-esterase

E: Epimerization

A D V H R V A Y: no homology

The substrate of VirP-A domain is proline?

Conclusion / Future work

Manipulation of NRPS genes

A-domain substrate alteration

Novel Virginiamycin M analogs
Characterization of genes involved in the biosynthesis of virginiamycin S, a streptogramin type B antibiotic

Fitria Ningsih, Shigeru Kitani, Eriko Fukushima, Takuya Nihira

International Center for Biotechnology, Osaka University, Suita, Osaka 565-0871, Japan

Streptogramins, a family of antibiotics predominantly produced by Streptomyces species, have gained more interest as potential alternative drugs for the treatment of vancomycin-resistant Enterococcus faecium and other Gram-positive bacteria. Virginiamycin S (VS), produced by Streptomyces virginiae, is a cyclic hexadepsipeptide antibiotic that belongs to the type B compounds in the streptogramin family, together with a macrolide antibiotic virginiamycin M 1 (VM), a streptogramin type A compound, as its synergistic counterparts. VS is synthesized in vivo by stepwise condensation of two proteinogenic and five nonproteinogenic amino acids: 3-hydroxypropionic acid, L-threonine, D-a-aminobutyric acid, L-proline, N-methyl-L-phenylalanine, 4-oxo-L-pipecolic acid, and L-phenylglycine. Previously, we have cloned plausible nonribosomal peptide synthetase (NRPS) genes, visE and visF, necessary for assembling the VS framework. In addition, with respect to the supply of necessary non-proteinogenic amino acids, four VS biosynthetic genes (visA, visB, visC, and visD) were required for creation and activation of the first and the sixth amino acid moiety, in the VS structure. However, other pathways to synthesize aminobutyric acid and PheGly are still needed, and remain to be elucidated. We have identified, in the left-hand extremity of the virginiamycin supercluster, two genes (visG and orf6) that might be involved in VS production by participating in L-phenylglycine biosynthesis. Transcriptional analysis revealed that visG, encoding a protein with homology to a hydroxyphenylacetyl-CoA dioxygenase, and orf6, encoding a protein with homology to a dihydroxyphenylglycine aminotransferase, are under the transcriptional regulation of virginiae butanolide (VB), a small diffusing signalling molecule that governs production of VS and VM. Gene deletion of visG resulted in complete loss of VS production without any changes in VM production, suggesting that visG is required for VS biosynthesis. The abolished VS production in the visG disruptant was fully recovered either by the external addition of L-phenylglycine or by gene complementation, which indicates that VisG is involved in VS biosynthesis as the provider of an L-phenylglycine molecule. orf6 gene is situated downstream of visG, and is presumed to convert the benzoylformate to L-phenylglycine which would eventually be activated and incorporated into VS by the action of VisF as a NRPS. Disruption of orf6 affected not only VS production but also VM production. Decreased production of VM is probably due to a polar effect that blocks the activity of Orf7, putative NRPS for VM biosynthesis, encoded by a gene downstream of orf6. Currently, gene complementation analysis is underway to confirm the possible involvement of the orf6 gene in VS biosynthesis.

Keywords: virginiamycin, streptogramin, phenylglycine.

References:
Characterization of Genes Involved in the Biosynthesis of Virginiamycin S, a Streptogramin Type B Antibiotic

Fitria NINGSIH, Shigeru KITANI, Eriko FUKUSHIMA, and Takuya NIHIRA
International Center for Biotechnology, Osaka University

Virginiamycins

Streptogramin antibiotics produced by Streptomyces virginiae

Type A
Virginiamycin M₁ (VM)

Macrolide

Type B
Virginiamycin S (VS)

Non-ribosomal peptide

Virginiamycins

Streptogramin antibiotics effective against wide-range Gram-positive bacteria

Chemical modification

Dalfopristin
Quinupristin

Novel therapeutic drug for vancomycin-resistant pathogens

The Use of Streptogramin Antibiotics

Virginiamycins Biosynthetic Gene Cluster

Gene Organization of visE-downstream Region

qRT-PCR Analysis of visE-downstream Genes

Chemical modification

Virginiae butanolide (VB)
autoregulator

Novel therapeutic drug for vancomycin-resistant pathogens

Gene Putative function
visF NRPS for VS biosynthesis
visG Enoyl-CoA hydratase
visC E₂ subunit of the BCDH complex
visD E₂ subunit of the BCDH complex
orf4 Thioesterase
orf6 Aminotransferase
virO MbtH-like protein
virP NRPS

Genes (Control)

Virginiamycin S Framework

Amino Acid Alignment of VisG with DpgC

Proposed VisG Function

Phenotypic Analysis of visG-disruptant Strain

Phenotypic Analysis of ΔvisG-complemented Strain

Feeding Experiment with L-Phenylglycine