Searching for Useful Enzymes from Metagenome Library

Sangryeol Ryu, Jiae Yoon, Seowon Kang, and Sulhee Park

Department of Food Science and Technology,
School of Agricultural Biotechnology Seoul National University

The classical approach for isolating enzymes from environmental samples that includes enrichment of microorganisms in pure culture can analyze only subset of the total microbiota in nature since it has been estimated that less than 1% of microorganisms observable in nature can be cultivated by standard techniques. Total DNA was isolated directly from various environmental samples and metagenomic libraries were constructed using both pUC and bacterial artificial chromosome (BAC) vectors to obtain useful microbial enzymes from uncultured microorganisms. We found several clones with amylase activity and sequencing of these genes revealed novel enzymes with low homology to the known amylases. One of the putative amylase genes was overexpressed and purified for characterization. The amylase hydrolyzed soluble starch and β-cyclodextrin to produce high level of maltose and pullulan to produce panose. The enzyme showed high transglycosylation activity to make 1,4-linkages exclusively. Programmed cell lysis system that is induced on glucose depletion by using *ptsG* promoter of *Escherichia coli* is under development to improve screening of metagenomic library by eliminating steps to make *E. coli* cells permeable.

Introduction

Naturally occurring assemblages of microorganisms often encompass a bewildering array of physiological, metabolic, and genetic diversity. However, a large fraction of the diversity in an environment is still unknown due to difficulties in enriching and isolating microorganisms in pure culture. It has been estimated that more than 99% of microorganisms observable in nature typically cannot be cultivated by standard techniques and this was supported by 16S rRNA studies. The traditional approach for isolating biologically active materials from environmental samples is to enrich, isolate, and screen a wide variety of microorganisms for the desired activity. The material is then recovered from the identified organism. An alternative approach is to use the genetic diversity of the microorganisms in a certain environment as a whole to encounter previously unknown genes and gene products for various purposes. *Escherichia coli* is the most widely used host for foreign gene expression or construction of metagenomic library. The main reasons for the popularity of *E. coli* are the high growth rate and expression level as well as the simple and inexpensive growth media, giving it economical advantages. However, one of the major problems in using *E. coli* as an expression host for screening of metagenomic library is its impermeable membrane that makes direct detection of gene products impossible. At present, a variety of techniques to disrupt bacterial cells are available but all these processes require additional steps that make screening more difficult and expensive.

Programmed Cell Lysis System

We tried to develop cell lysis system that can remove extra lysis step by inducing lysis of bacterial cells when it is necessary. The lytic system of bacteriophage T4 was used to lyse *E. coli* cells. Lysis of *E. coli* by T4 phage requires the action of two gene products from gene *e* (Gpe) and *t* (Gpt). Gene *e* encodes a lysozyme,

which degrades the peptidoglycan of *E. coli* cell wall and Gpt is a holin considered to degrade or alter the cytoplasmic membrane, thus allowing Gpe to reach the periplasm and gain access to the peptidoglycan layer. Here, Gpt and T7 lysozyme were used to induce bacterial cell lysis and the *ptsG* P1 promoter was employed to modulate expression of gene *t* by glucose (Fig.1). *ptsG* transcription is regulated through two global systems, positively by CRP·cAMP and negatively by Mlc, a glucose-inducible regulator of carbohydrate metabolism. In addition, Fis assists both Mlc repression and CRP·cAMP activation of *ptsG* P1 through the formation of Fis-CRP-Mlc or Fis-CRP nucleoprotein complex at *ptsG* P1 promoter depending on the availability of glucose.

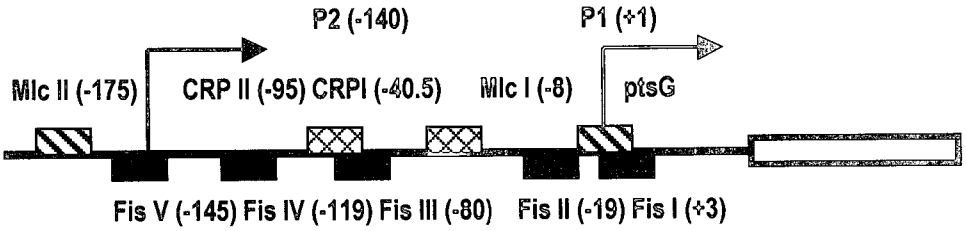

Fig. 1. Regulatory region of *ptsG* gene of *E. coli.* hematic diagram of *ptsG* promoter region. The two transcription initiation sites of *ptsG* are shown with arrows. All numberings of this figure are based on the transcription start point of the P1 promoter. The binding sites of two major regulators of *ptsG* expression, CRP and Mlc, are indicated in boxes.

The *ptsG* promoter is induced when cells are grown in the presence of glucose, yet the *ptsG* promoter in the multicopy plasmid is repressed in the presence of glucose and induced in the absence of glucose. Mutations in the CRP binding site of *ptsG* P1 resulted in the promoter with various strength (Fig. 2). Therefore, the lysis gene under the control of *ptsG* P1 in the plasmid is induced only when the colony size increases enough to deplete glucose in the medium. The system is expected to exclude extra steps to lyse the bacteria.

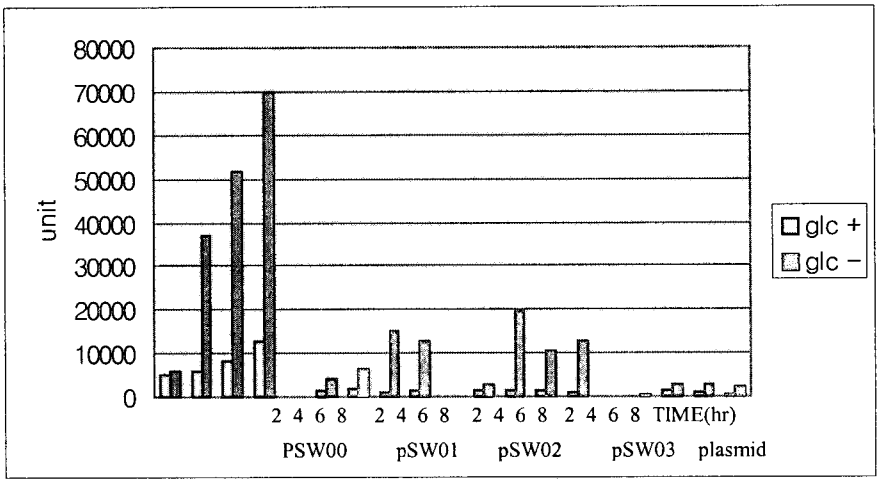

Fig. 2. β-galactosidase assay results of pSW00~03 plasmids in MC4100. Three different mutations in the CRP binding site of *ptsG* P1 promoter resulted in the promoters with lower activity as measured by β-gal fusion.

Few Examples of Novel Genes Found from Metagenomic Library

Metagenome from soil and rumen was screened for various novel enzymes. Both BAC and pUC-based libraries were made and their efficiency was compared. BAC library system is feasible to clone an entire pathway in one BAC clone and very attractive for searching for antibiotics or other complex gene clusters. Moreover, since lager DNA fragment is cloned, the frequency to have positive clones from libraries would be higher than when using the plasmid system. Despite these merits, the possibility to detect gene overexpressed from BAC clones can be low due to low copy number in *E. coli*. Also cloned BAC DNA is fragile for handling. Construction of metagenomic libraries using a high copy number plasmid vector is preferred when searching for novel enzymes because the average size of structural genes of enzymes is around 1-2kb. For example, in 1999 and 2000, Henne *et al.* reported construction of soil DNA libraries using pBluescripot SK^+ and screening for 4-ydroxybutylate utilizing genes and lipases, using small fragments of DNA ranged from 3 to 10 kbp.

Two putative amylase genes were found from our metagenomic library (Fig. 3). The putative amylase gene (*amyM*) was overexpressed and purified for characterization. AmyM had 42° C and pH 9.5 as the optimum temperature and pH conditions. Ca^{2+} was found to be the essential element for enzyme stability. The amylase hydrolyzed soluble starch and β-cyclodextrin to produce high level of maltose and pullulan was also hydrolyzed to produce panose. The enzyme showed high transglycosylation activity to make 1,4-linkages exclusively. These results suggest that amyM is one of the intermediate types of neopullulanases, α-amylases and 4-α-glucanotransferases.

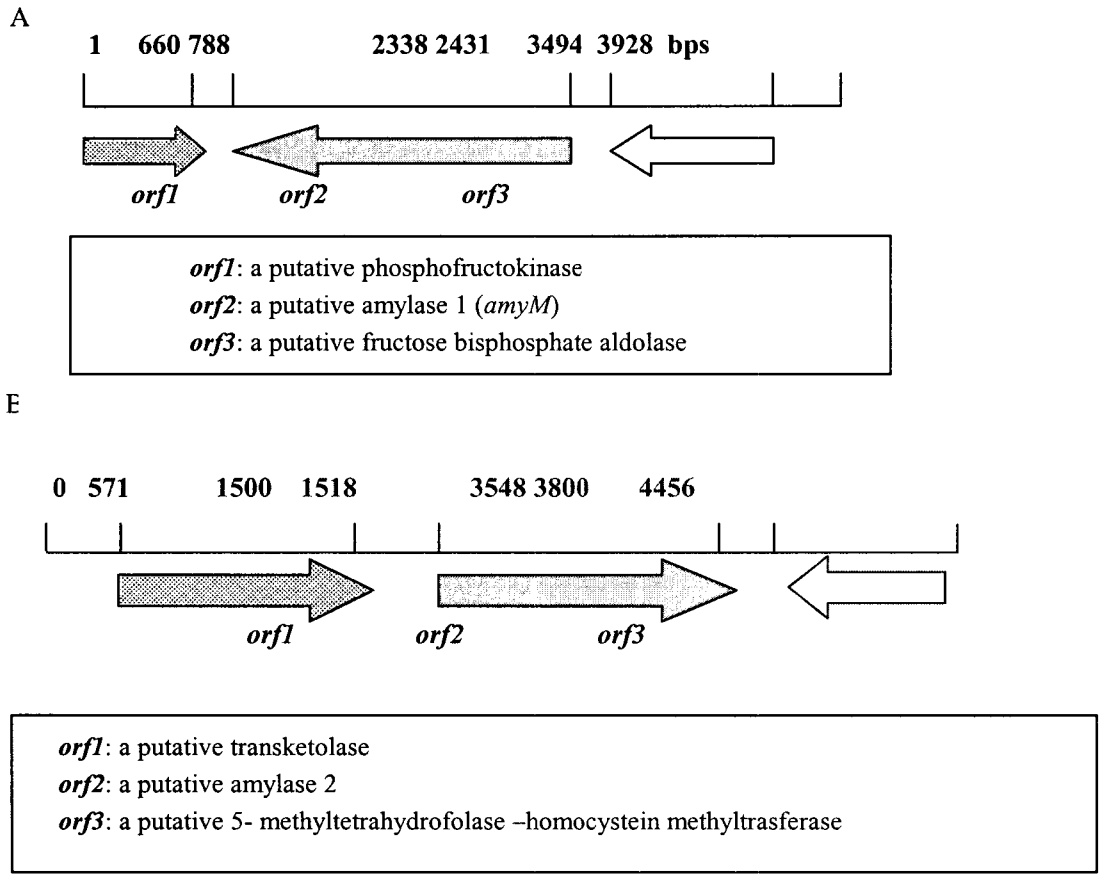

Fig. 3. Schematic diagram of DNA fragments containing putative amylase genes obtained from screening of metagenomic library.

References

1. Courtois, S., Cappellano, C. M., Ball, M., Francou, F. X., Normand, P., Helynck, G., Martinez, A., Kolvek, S. J., Hopke, J., Osburne, M. S., August, P. R., Nalin, R., Guerineau, M., Jeannin, P., Simonet, P. and Pernodet, J. L. (2003). Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. Appl Environ Microbiol 69, 49-55.
2. Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J. and Goodman, R. M. (1998). Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chem Biol 5, R245-249.
3. Henne, A., Daniel, R., Shmitz, R. and Gottschalk, G. (1999) Construction of environmental DNA libraries in Escherichia coli and screening for the presence of genes conferring utilization of 4-hydroxybutyrate, Appl Environ Microbiol 65, 3901-3907.
4. Henne, A., Schmitz, R.A., Beomeke, M., Gottschalk, G.and Daniel, R. (2000) Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on Escherichia coli, Appl Environ Microbiol 66, 3113-3116.
5. Knietsch, A., Waschkowitz, T., Bowien, S., Henne, A., and Daniel, R. (2003). Construction and Screening of Metagenomic Libraries Derived from Enrichment Cultures: Generation of a Gene Bank for Genes Conferring Alcohol Oxidoreductase Activity on Escherichia coli. Appl Environ Microbiol 69, 1408-1416.
6. Lorenz, P., Liebeton, K., Niehaus, F. & Eck, J. (2002). Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space. Curr Opin Biotechnol 13, 572-577
7. Dongwoo Shin, Namwook Cho, Sunggi Heu, S. Ryu (2003) Selective regulation of ptsG expression by Fis: formation of either activating or repressing nucleoprotein complex in response to glucose. J. Biol. Chem. 278, 14776-14781.