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Genes, enzymes and secondary metabolites in industrial microorganisms
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T Beppu

Department of Applied Biological Science, College of Veterinary Medicine, Nihon University, Kameino 1866, Fujisawashi, Kanagawa 252, Japan

Apparently contrasting approaches, ie genetic engineering and screening of new microorganisms, play essential complementary roles to develop current industrial microbiology. Three topics, production and modification of milk-clotting proteinases by genetic engineering, hormonal control of secondary metabolism in streptomycetes, and screening of bioactive metabolites, are introduced as cases of such a hybrid approach, while symbiotic microorganisms are discussed as an example of the vast *terra incognita* still remaining for the future microbiology.

Keywords: chymosin; *Rhizomucor* pepsin; A-factor; hormonal control in prokaryotes; trichostatin; eukaryotic cell cycle inhibitors; symbiotic thermophiles

Prokaryotes, eukaryotes, archaea and even viruses are referred to as microorganisms, yet they represent several different phytogenic groups. One way to define microorganisms is by the use of methods that examine the diverse structure and function of microorganisms [13]. Even though this vast diversity has created taxonomic confusion in microbiology, it serves as the primary basis for screening to discover novel secondary metabolites as well as enzymes for fermentation industries. In addition, recombinant DNA techniques have enabled rational creation of strains in a variety of microbial species as well as the design of protein functions based on structural information. Combined efforts of screening and rational design are being conducted to develop new industrial processes and provide important information for better elucidation of the amazingly diverse microbial world. A few examples are described below.

Genetic and protein engineering of milk-clotting aspartic proteinases

Milk-clotting, the first step of cheese production processes, is conducted by using several characteristic proteinases, all belonging to the group of aspartic proteinases. Chymosin obtained from the fourth stomach of calves had been used primarily, but its shortage in 1950 prompted the development of several similar enzymes from microorganisms such as *Rhyzomucor* pepsin. Then recombinant DNA technology enabled the production of original chymosin by using genetically engineered microorganisms. Our early work on cloning and expression of prochymosin cDNA contributed to its production in *E. coli* [1].

The chromosomal gene of *Rhizomucor pusillus* pepsin was also cloned and expressed in *Saccharomyces cerevisiae*, which secreted the correctly processed enzyme with N-glycosylation with a yield of about 200 mg L^{-1} [7]. The expression system permitted site-directed mutagenesis based on the three-dimensional structure of the enzyme. Among a number of the mutant enzymes generated, a set of mutants at the 75th Tyr residue gave new aspects on the structure-function relationship in this proteinase [3]. Exchange of this Tyr to other amino acids resulted in almost complete loss of activity, indicating the essential role of the Tyr residue in the catalytic function of this enzyme. Among them, the Tyr75Asn mutant caused not only a distinct increase in the relative milk-clotting activity but also decreased heat stability, which is convenient for rapid killing of the residual enzyme activity after the clotting process. Thus, site-directed mutagenesis of *Rhizomucor* pepsin has succeeded in improving practical applications of this enzyme as a milk coagulant. The research will also provide information on the structure-function relationship common in other members of the aspartic proteinases such as renin and HIV proteinase possessing similar three-dimensional structures.

Hormonal control of secondary metabolism in streptomycetes

Streptomycetes are Gram-positive bacteria characterized by a marked ability to produce diverse secondary metabolites and highly differentiated morphology as in eukaryotic fungi. Many observations have indicated close relationships between secondary metabolism and morphological differentiation in these organisms. One of the best examples is observed in *Streptomyces griseus*, which produces a diffusible self-regulatory factor named A-factor to induce both streptomycin biosynthesis and sporulation at extremely low concentrations [2]. A-factor was first discovered by Khokhlov in 1967 and rediscovered in 1982. Since then, involvement of similar autoregulatory factors with a common γ-butyrolactone structure in the secondary metabolism of

Correspondence: T Beppu, Department of Applied Biological Science, College of Veterinary Medicine, Nihon University, Kameino 1866, Fujisawashi, Kanagawa 252, Japan
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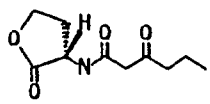
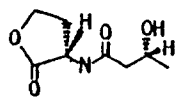
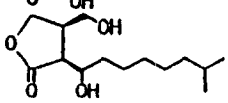
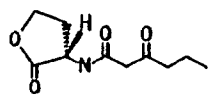
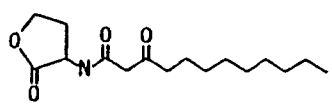
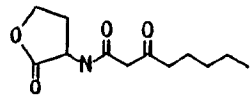
various *Streptomyces* species have been reported (Table 1). Another series of γ-butyrolactones generally called homoserine lactones are also recognized as regulators of a variety of cellular functions in the Gram-negative bacteria, such as bioluminescence in *Vibrio* and conjugation in *Agrobacterium* [4]. These results suggest that the hormonal control is not limited to eukaryotes, but widely distributed even in prokaryotes.

The hormonal feature of A-factor was confirmed by identifying the receptor in *S. griseus* cells. The detection was first carried out by binding analysis with the ^{3}H-labeled A-factor, while its biological role to transduce the A-factor signal was confirmed by isolating the binding protein-deficient mutant. The mutant showed marked overproduction of streptomycin (almost 10-times higher than the wild strain as well as much-enhanced sporulation), suggesting a negative regulatory role of the binding protein as a repressor. Recent purification of the A-factor receptor protein and cloning of its gene have revealed some homology with the binding protein of virginia butanolides, an A-factor analog controlling virginiamycin production in *S. virginiae* [9,10].

One of the targets of the intracellular signal triggered by A-factor was found in the upstream region of a positive regulatory gene for streptomycin biosynthesis, *strR*, in the streptomycin biosynthetic gene cluster. Subsequent experiments narrowed down the region responsible for the A-factor response to a short region and a DNA-binding protein specifically recognizing a promoter sequence in this region was detected by gel-shift assays. The DNA-binding protein was found in *S. griseus* cells grown in the presence of A-factor but not present in cells grown without A-factor [16]. Another regulatory gene, *amfR*, involved in A-factor signaling to control sporulation was also cloned, which showed homology with the response regulators of the so-called prokaryotic two-component protein phosphorylating systems [15]. Again a DNA-binding protein specifically recognizing a promoter sequence in the upstream of *amfR* was detected, but in this case, the binding protein existed in the cells grown without A-factor but was absent in the cells with A-factor. The reciprocal responses of *strR* and *amfR* indicated that the A-factor-signaling cascade was composed of both positive and negative regulators, which may be necessary for controlling a variety of cellular functions with harmony.

Another regulatory system for secondary metabolism in streptomycetes has been recognized through analyses of a global regulatory gene, *afsR*, which was cloned from *S. coelicolor* A-3(2) as a gene inducing production of large amounts of actinorhodin in *S. lividans*. The sequence of *agsR* contains the consensus sequences for ATP-binding as well as the DNA-binding helix-turn-helix motifs and its phosphorylation was confirmed in cell-free extracts of *S. coelicolor* and *S. lividans*. A protein kinase gene responsible for phosphorylation of the AfsR protein was found downstream of *agsR* and named *AfsK*, which exhibited distinct homology with the eukaryotic Ser/Thr-protein kinases [8]. Disruption of *agsK* caused marked reduction of actinorhodin production in *S. coelicolor* indicating its regulatory role. This was the first demonstration of the functionally active Ser/Thr-kinases in the prokaryotes.

Table 1 γ-Butyrolactones as bacterial hormones

A-factor analogues		Biological activity[a]	Homoserine lactones		Biological activity[a]
Factor	Producer		Factor	Producer	
A-factor	*Streptomyces griseus*	Streptomycin sporulation		*Vibrio fischeri*	Bioluminescence
VB-A	*S. virginiae*	Virginiamycin		*V. harveyi*	Bioluminescence
	*S. bikiniensis* *S. cyanofuscatus*	Anthracycline		*Erwinia carotovora* *Pseudomonas aeruginosa* *E. herbicola* *Serratia marcescens*	Carbapenem
Factor I	*S. viridochromogenes*	Anthracycline			
IM-2	*Streptomyces* sp FRI-5	Blue pigment		*P. aeruginosa*	Virulence
Butalactin	*Streptomyces* sp Y-86,36923	Antibiotic activity		*Agrobacterium tumefaciens*	Conjugation

[a]Biological activities refer to the ability to induce production of secondary metabolites or the cellular functions indicated.

The presence of the hormonal control system as well as the Ser/Thr-kinases to control secondary metabolism and morphological differentiation in the streptomycetes suggest the eukaryotic features of these characteristic prokaryotes, but we may expect that similar features will be found in other types of prokaryotes in the future.

Screening of microbial metabolites that specifically inhibit eukaryotic cell cycle

The eukaryotic cell cycle is composed of the four phases, M, G1, S and G2, and the principle regulatory mechanisms to control the cycle are active during the apparently silent phases G1 and G2. The agents interfering with the progression of G1 and G2 are expected to have specific effects on cellular proliferation or differentiation possibly with some anticancer activities.

Friend's erythroleukemia cell line is a stem cell whose differentiation to erythrocytes is blocked by infection of Friend's virus, but differentiation is induced by adding high concentrations of DMSO. This system serves as a model to screen inducers of redifferentiation of cancer cells. Trichostatin, produced by a streptomycetes strain, was first discovered as a potent differentiation inducer and later its characteristic inhibitory effects on the cell cycle were noticed. It causes specific arrest of rat fibroblast cell proliferation at G1 and G2 phases [17]. Upon release from G2 arrest by removal of the agent, cells were converted to proliferative tetraploid cells almost quantitatively. Another interesting feature of the agent is very selective killing of the transformed cells suggesting the possibility that the G1- and G2-specific inhibitors may become selective antitumor agents.

Trichostatin causes hyperacetylation of nuclear histones due to inhibition of histone deacetylase in the cells. The inhibitory activity was confirmed with the partially purified preparation of histone deacetylase. In addition, the similar enzyme preparation from a trichostatin-resistant cell line exhibited increased resistance of the same extent *in vitro* as that of the cells [18]. These results strongly indicate that the molecular target of this agent *in vivo* is histone deacetylase. Nuclear histones exist as the acetylated forms, and the extent of acetylation is controlled by the balance of acetyltransferase and deacetylase. Potent inhibition of deacetylase by trichostatin causes hyperacetylation of nuclear histones, resulting in changes of chromatin structures and in turn changes of various cellular functions.

Although trichostatin is currently not successful as a practical antitumor agent, it has become a powerful tool to analyze roles of histone acetylation [19].

Methodical implication of symbiotic thermophiles

Undoubtedly microorganisms may not only use antibiosis but also symbiosis as a strategy to establish their niches in the ecosystem. We know of many examples of symbiosis in which microorganisms are intimately integrated in the life of higher organisms as their hosts, such as *Rhizobia* in leguminous plants and cellulose-producing anaerobes in termites. If we focus on the symbiosis between two or multiple free-living microbial strains, however, we find that only a few cases have been well-defined. It is due to a simple methodological defect, ie a single colony isolation technique is not an effective method to isolate a member of the symbiosis whose growth is absolutely dependent on the presence of its counterpart. Selective conditions are almost essentially required to isolate such dependent symbionts from the environment. One such case is the symbiotic assimilation system of polyvinylalcohol (PVA). A *Pseudomonas* strain with the ability to produce the extracellular PVA oxidase is fed with its essential nutrient pyroloquinolinequinone by an associating second pseudomonad [11]. It is obvious that selective pressure for the ability to utilize PVA as a single carbon source was effective to enrich this symbiotic pair from the environment.

Another example is the isolation of the parasitic symbiont *Ralobacter* of *Saccharomyces cerevisiae*. This unusual bacterium requires catalase or hemin to degrade H_2O_2 in an aerobic environment. It produces a potent lytic enzyme which attacks living yeast cells [12]. In this case, *Ralobacter*, whose isolation was achieved by using yeast as a specific host, is considered an absolute symbiont of *S. cerevisiae*. Screening of symbiotic microorganisms without such selective conditions becomes extremely difficult as experienced with the screening of thermophiles exhibiting heat-stable tryptophanase activity as described below [14].

A screen was conducted to detect indol formation from L-tryptophan in liquid medium inoculated with compost samples at 60°C. A few composts contained cultures that produced L-tryptophan, but isolation of the responsible organism as a single colony failed. Seven years later it was discovered that the organism was an absolute symbiont named *Symbiobacterium thermophiulum* whose growth was absolutely dependent on the coculture of a thermophilic *Bacillus* strain *S*. Enrichment of this pair was only possible by repeated sequential dilution and subcultivation with detection of indole-formation (Figure 1). Such series of experiments could occasionally reduce some contaminant strains from the co-culture of *S. thermophilus* and *Bacillus* strain *S* and thus partial enrichment of the pair was achieved step-by-step. Finally, addition of bacitracin to one of the enriched subcultures was effective in removing most of the contaminating strains. Plating this highly enriched culture produced mostly tryptophanase-negative colonies of a *Bacillus* strain with a small percent of tryptophanase-positive colonies which were found to be mixed colonies of a small rod named *S. thermophilum* with the *Bacillus* strain. *S. thermophilum* is Gram-negative and could not grow independently in any medium examined. It seems to require some protein(s) from *Bacillus* strain *S* as an essential growth factor. Two heat-stable tryptophanase genes [5] and a heat-stable β-tyrosinase [6] gene were cloned and expressed in *E. coli* which can be used as biocatalysts for production of L-tryptophan and L-dihydroxyphenylalanine, respectively.

It has been stated that a wide variety of microorganisms exist in ecosystems, but scientists are unable to culture some of them under laboratory conditions. On the other hand, some symbiotic microorganisms, as observed in this case, can be cultured if microbiologists do not insist upon isolating pure cultures. There is no reason to underestimate the population of such symbiotic microorganisms in the

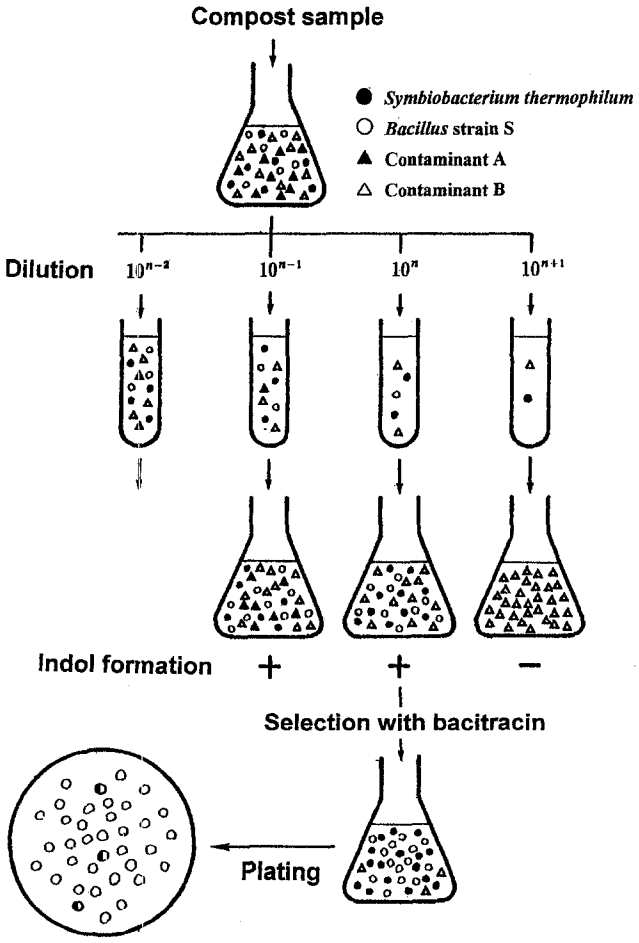


Figure 1 Enrichment process of the symbiotic pair of *Symbiobacterium-Bacillus* through successive dilution and subcultivation. *S. thermophilum* (●) , *Bacillus* strain *S* (○), contaminant A (▲), and contaminant B (Δ). Numbers of each symbol indicate relative abundance of the organisms in the mixed cultures.

ecosystem even though their isolation may be extremely difficult. Therefore, we may assume that microbial symbiosis is a possible fruitful resource of genes with practical usefulness in industrial microbiology.

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