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Proteomics for Streptomyces: "Industrial Proteomics" for Antibiotics

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The recent trends in explaining various biological events in cells and tissues as dynamics of protein networks and metabolic systems require large scale omics (i.e. genomics, proteomics, and metabolomics) analysis and interpretation. The genomics data often generated by DNA micro-array provide the information of the expression level of mRNA, indirectly indicating the level of protein expression. Whereas proteomics data generated by mass spectrometry give direct information of expression level of proteins, and additionally provide somewhat unique information of protein-protein interaction, secreted proteins and peptides, post translational modification (PTM) of proteins, complex protein structures and the structures of other biomolecules such as lipid, carbohydrates and metabolites which are present in very small quantities in biological samples. Most common topics in functional proteomics are identifying the differences in protein expression level, presence of biomarker proteins, and degree of protein modification, i.e. PTM, between the two samples for control and treatment, such as normal and cancer patients, different tissues and organelles, such as kidney and heart and Golgi body and endoplasmic reticulum, and the samples from different points in time. More often the issues of the analysis are qualitative and quantitative PTM analyses of specific proteins from minute amounts of the samples from the treatment tissues and body fluids. In contrast, the proteomics study for industrial microorganisms, so called "industrial proteomics", emphasizes different aspects of the proteomics. Microbial samples are relatively easy to harvest, so that the access of the samples is not the limitation, but due to their short doubling time and rapid fluctuation of metabolite and protein concentrations, rapid sampling and getting a snap shot of the changes in the protein concentration and cell metabolites in cell are critical to acquire a right information of cell physiology and determining a rate limiting step. Since most of the final products from the industrial microorganisms are recombinant proteins, cell metabolites, and secondary metabolites, understanding detailed gene regulations of biosynthetic pathway, transporters, antibiotics resistance and secretion machinery are the primary concerns of the research.

The most popular industrial host strains are *E. coli*, *Bacillus*, *Yeast*, *Corynebacteria*, *Streptomyces*, *Aspergillus*, and lactic acid bacteria, and their major products are recombinant proteins [3] and primary metabolites [70, 76], extracellular enzymes [30, 105] and vitamins [1, 109], ethanol [65, 72], amino acids [74, 119], antibiotics [46, 83, 94], extracellular enzymes [99, 108], and lactic acid and live vaccines [138]. Since those industrial products are in demand of large quantity, large scale fermentations with high cell density, high yield and productivity are recommended, and the development of good host systems with high yield is a key for the success of the production of each product. As each host is different and has its own specific issues to enhance the yield and productivity of the products, we want to confine our scope of this review article into secondary metabolite production in *Streptomyces*.

Key words: Proteomics, streptomyces, industrial microorganism, industrial proteomics

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Streptomyces

Streptomyces are Gram-positive soil bacteria that produce various secondary metabolites and undergo unique morphological and physiological differentiation [6]. Since genome sequencing of *Haemophillus influenza* was completed

in 1995 [35], the number of published complete genome sequence has already reached 356 up to now. Among them, two *Streptomyces* genome are found. *Streptomyces coelicolor* A3(2) is the best genetically characterized streptomycete and its genome sequence was completed [8]. This strain is known to have 20 gene clusters of secondary metabolites, but produce at least four distinctive antibiotics, two of which are pigmented; red tripyrolle undecylprodigiosin (Red) and blue polyketide actinorhodin (Act). In 2003, the genome project of *S. avermitilis* producing insecticidal antibiotics, i.e. avermectin, for ornamental plants was completed by H. Ikeda group in Kitasato University [57], and 30 gene clusters related to secondary metabolites are found in the strain. Although American biotech company called "Diversa Inc." has been known to finish the genome project of *S. diversa*, the data is not yet published. Recently, Korean company called "GenChem Inc." has also completed the genome project of *S. peucetius* producing anticancer drug, i.e. doxorubicin, and found about 15–20 secondary metabolite gene clusters in the strain (personal communication). Although current research is mainly focused on the improvement of yields of the commercially available antibiotics/anticancer drugs, induction of other cryptic gene clusters still undetected or unexpressed might give some opportunities to find new antibiotic compounds never known before [148]. Then the newly-found gene clusters can be used to further develop new hybrid antibiotics in the future.

To develop *Streptomyces* strain as a host system for production of secondary metabolites, many different strategies can be exploited. In general, we can summarize them into seven different approaches (Fig. 1) : 1) insertion or deletion of global and pathway specific genes, 2) control of primary metabolism to enhance the concentration of precursors or cofactors, 3) insertion and amplification of biosynthetic genes and clusters for target molecules, 4) replacement of the related promoters, 5) introduction of antibiotics resistance gene, 6) increase the transport rate of antibiotics by mutation of transporter, 7) removal of biproducts or branched pathway. The problem solving of the above issues are not simply resolved by only using proteomics approach, but rather looking at the clues and clear path with more integrative approaches such as transcriptome analysis [14, 63, 81], metabolite analysis [62, 79], flux analysis [5, 68, 88], bioinformatic analysis [75, 120, 133] and modeling of regulatory networks [20, 22, 55]. Here, selected proteomics approaches and studies are introduced and reviewed with related cellular mechanisms.

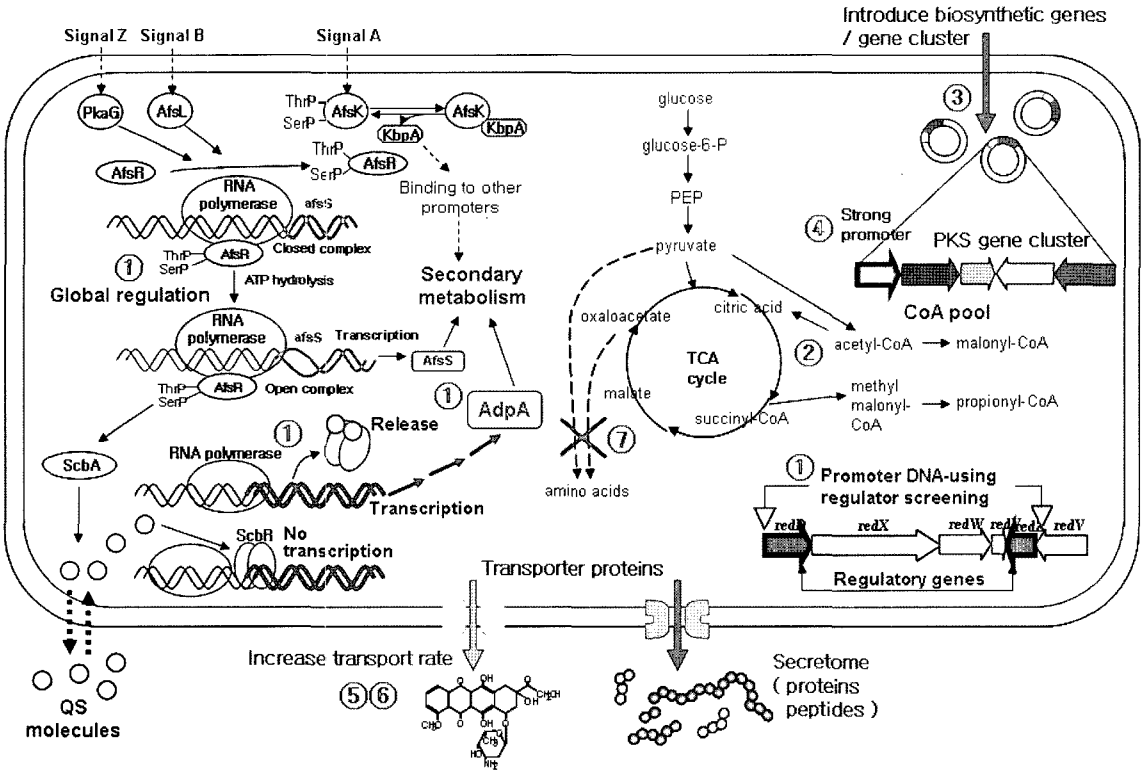

Fig. 1. Several different strategies to develop host systems for production.
1) insertion or deletion of global and pathway specific genes, 2) control of primary metabolism to enhance the concentration of precursors or cofactors, 3) insertion and amplification of biosynthetic genes and clusters for target molecules, 4) replacement of the related promoters, 5) introduction of antibiotics resistance gene, 6) increase the transport rate of antibiotics by mutation of transporter, 7) removal of biproducts or branched pathway.

TOOLS OF PROTEOMICS: SEPARATION AND MASS ANALYSIS

The main theme of proteomics is understanding complete panoramic pictures of protein composition, dynamics and connections of total proteins (proteome) in the cell under specified conditions. Choices of suitable equipments, especially mass spectrometer, and protein purification/separation techniques for your goal determine or change the levels of the understanding of the theme. Mass spectrometry is a tool to analyze molecules through its ionization with electron energy and subsequent separation of ions. It has been used for small molecules but recently developed soft-ionization methods have spurred its application to biomolecules. Matrix-assisted laser desorption and ionization (MALDI) [124] and electrospray ionization (ESI) [32] are the two well known ionization methods. In terms of types of mass analyzers, quadrupole and time-of-flight (TOF) are two popular instruments. In MALDI-TOF, time delayed extraction technique was a breakthrough to improve mass accuracy and peak resolution. Quadrupole ion trap in ESI was another beakthrough in developing mass analyzer, in that ions with various masses are stored at once during ionization in ion trap quadrupole, and gradually selected ions are extracted and analyzed along dynamic mass ranges. One thing to note is that original quadrapole ion trap was further developed into linear ion trap (LT), which gives high scanning speed and trapping efficiency, resulting almost 100-fold increase in sensitivity [9]. Fourier Transform Ion Cyclotron Resonance (FTICR) is a high resolution mass spectrometer, which gives below 0.5 ppm of mass accuracy in differentiating precursor ions, so that the most sensitive analytical tool in modern analytical chemistry [115].

Several combinations of the above ionization source and the mass analyzer are possible, but MALDI-TOF/TOF and ESI-MS/MS (e.g. LTQ) or ESI-Quadrapole-TOF (e.g. QTOF) are the most popular instruments for proteomics, whereas Triple Quadrapole-MS/MS is more commonly used in metabolomics study, because it has very good performance for quantitative analysis. MALDI-TOF/TOF is good for high-throughput (HT) analysis with chip format, whereas ESI is more time consuming than MALDI, but softer ionization method, and multiple series fragmentation are possible to identify structure determination of target compound when equipped with ion trap. They have become indispensable tools in proteomics and its utility is still growing. Its most important applications are peptide mass fingerprinting by MALDI-TOF (time of flight) and peptide sequencing by ESI-MS/MS (tandem mass spectrometry). While all the above methods show "bottom-up" protein analysis approach using trypsin digestion of whole protein [41], however ESI-FTICR allowed us to perform *de novo* sequencing, called "top-down" approach, due to its high resolving power [12, 149].

In terms of separation tools, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the most widely used method for protein resolution and identification. A proteome is separated by two orthogonal properties of molecular weight and isoelectric point and then, each spot on 2D-gel is individually extracted, digested with trypsin and analyzed by mass spectrometry. However, this procedure is laborious, tedious and time-consuming. So, the need for robust, automated and sensitive high throughput separation technologies is tremendous.

ESI is suitable to combine with liquid chromatography (LC) due to its ionization principle. Among developed technologies for the combination of LC with MS, multidimensional protein identification technology (MudPIT) is the most versatile and distinguished method for rapid and large-scale proteome analysis by multidimensional liquid chromatography, tandem mass spectrometry and database searching by the SEQUEST algorithm [82, 136, 140]. At MudPIT, a

Table 1. Common separation technologies and their applications.

	Technology	PTM analysis	Separation efficiency	Sample preparation	Separation of protein (PR) /peptides (PEP)	Protein abundance
Gel-based	1D electrophoresis	×	Low	Simple	PR/PEP	Med-High
	2D electrophoresis	○	Med/High	Moderate	PR	Med-High
	2DE-DIGE	○	-	Simple	PR	Med-High
Chromatography -based	RP-HPLC	×	Med	Simple	PR/PEP	Low-High
	Cation-exchange	×	Med	Simple	PR	Med-High
	Affinity	×	High	Simple	PR/PEP	Low-High
	CE	×	High	Simple	PEP	Low-High
	2D chromatography	○	High	Simple	PR/PEP	Low-High
	MUDPIT	○	HIgh	Moderate	PR/PEP	Low-High
Application Tech. for quantitative MS analysis	ICAT, iTRAQ™, IDBEST™, AQUA, SILAC	○	-	Moderate (Strict)	PEP	Low-High

(modified from ref. 40)

denatured and reduced proteome is digested to generate peptide mixture and then this peptide mixture is applied to multidimensional LC composed of a strong cation exchange (SCX) chromatography column and a reversed-phase (RP) chromatography column [39]. The combination of these two columns generates an alternative and efficient two-dimensional separation system like 2D-PAGE. Another important feature of the combination of LC and MS is the considerable reduction of time required for the analysis of complex protein mixture (Table 1).

NEW TECHNOLOGIES IN PROTEOMICS

The newly developed technologies in proteomics are focused on easy preparation of sample, easy comparison, and quantification of mass spectrometric analysis. For more efficient detection of differentially expressed proteins on 2D-gel when comparing gel images, DIGE (differential in-gel electrophoresis) was developed as convenient method, which is used two spectrally different fluorophores a decade ago [132]. In 2D-DIGE, each sample for comparison is covalently labeled with different fluorescent dyes, Cy3 and Cy5 with emission wavelength of 569 nm (orange color) and 645 nm (red color) respectively. Labeled protein samples are mixed with equivalent amount, and electrophoretically run on the same gel. A comparison of the resulting images can be able to quantify the protein expression levels based on the total fluorescence intensity of each protein spot. Even though the high cost of fluorescent dyes, this method is still very useful to comparative 2D-gel analysis.

Another important theme of technological development for proteomics is to improve the quantification of mass spectrometric analysis, whose method uses stable isotope labels (Table 2). SILAC (stable isotope labeling by amino acids in cell culture) is a simple and straightforward method to incorporate stable isotopes such as deuterium, ^{15}N, ^{13}C etc. into proteins metabolically to give heavy or light form of the amino acid [31, 96]. ICAT (isotope-coded affinity tags) uses different mass tags, and affinity purification to quantify the relative abundance of peptide fragments [42, 44]. iTRAQ (isobaric tag relative absolute protein quantitation) uses mass tags of the same molecular weight, but produces different MS/MS patterns which result in higher protein identification than ICAT and SILAC [112]. IDBEST (isotope-differentiated binding energy shift tags) combines the mass defect with stable isotope-paired reagents to create a differential display platform for biomarker discovery and validation with high sequence coverage for isoform detection as well as multiplexed (high-throughput) toxicology and efficacy screening with internal controls [43].

AQUA (absolute quantitation) allows absolute quantitation of the amount of protein and the stoichiometry of protein phosphorylation, which relies on internal standards comprised of synthetic peptides containing an isotopically-labelled amino acids.

REGULATORY NETWORK AND THEIR CONTROL MECHANISMS

Streptomyces has very complex regulatory systems. At least, about 70 sigma factors and 1,000 regulatory proteins exist among about 7,000–8,000 proteins [8], but their regulatory functions still remained unknown. Therefore, understanding detailed mechanism of all the regulatory proteins are the most interesting and difficult issues in *Streptomyces* proteomics. The regulatory proteins can be roughly categorized into two kinds, i.e. global regulatory protein and pathway specific regulatory protein. Global regulatory proteins govern primary metabolism, stress response, house keeping genes, and via signal transduction eventually control morphological changes and physiological differentiation of cell at higher level. On the other hand, pathway specific regulatory proteins are mainly involved

Table 2. Comparison of quantitative technologies for proteomics.

Technology	Quantitative	Key technique	Advantage	Disadvantage
ICAT	Relative	Cysteine-linkable reagent	Easy automation coincident identification & quantification	Available to only cysteine-containing proteins (80% of total)
iTRAQ™	Absolute/ Relative	Amine-linkable reagent with balance moiety	Improved from ICAT, higher efficiency of protein identification	Require more MS time, strict sample preparation
IDBEST™	Relative	Amine/Cysteine-linkable reagent containing elements with high mass defect	Discrimination of labeled species from mixture of unlabeled species	Require the mass spectrometer having higher resolution (>10,000)
AQUA	Absolute	Internal standard for absolute quantification	Absolute quantification, Universal applicable to any proteins	Must have synthesize the standard peptide
SILAC	Relative	*In vivo* amino acids labeling in cell culture	Global quantification simple procedure	Available to only cultivable samples

in local biosynthetic pathway at lower level occurring at specific conditions, such as sporulation or antibiotic production. The global regulatory proteins are somewhat connected to the pathway-specific regulators in related to the expression of biosynthetic genes via one or two links of involved proteins.

Global Regulatory Network

Good cell growth usually leads to high cell density, resulting a high productivity of secondary metabolites. As transport and primary metabolism of carbon and nitrogen sources in the cell determine the cell growth rate, the cell growth is mainly controlled by optimizing media components in the broth and feed solution. Therefore, screening of N and C sources, and optimization of growth conditions such as pH, oxygen supply, temperature, phosphate concentration, etc. become the most important procedures to achieve high cell growth rate. Different types of N/C sources give direct effect on global regulatory genes, so that correct understanding the changes in the expression of specific regulons and their modifications under catabolite repression, diauxic growth, stringent response, cell growths at nutrient and oxygen limitations become our interest. In Streptomyces, there are four well-known global regulatory networks are present: 1) ppGpp and c-AMP for N and C source regulation [19, 52, 84], 2) AfsR two component system [36, 48, 77, 131], 3) A factor (g-butyrolactone), its receptor (e.g. scbA-scbR, afsA-arpA) [16, 51, 64, 95, 121, 146] and the receptor-dependent AdpA regulator [92, 145], 4) sigma factors for cell differentiation [26, 40, 101, 111].

AfsR and AdpA. Although the presence of cAMP, ppGpp and an adenylate cyclase equivalent gene is known in *Streptomyces* [107], their functions and roles are still in debate. However, catabolite repression and stringent response mechanisms are playing a role in the cell like other bacteria. Instead, molecular mechanisms of AfsR and A factor are well elucidated. AfsR is a pleiotropic regulator working as a two-component (AfsR-AfsK) system resulting changes in morphology and physiology of *Streptomyces* species. The AfsR system is consisted of four components, KbpA-AfsK-AfsR-AfsS: AfsK is a Ser/Thr kinase phosphorylating AfsR, Kbp is the repressor of AfsK, and afsS is a target binding site of AfsR, which is another pleiotropic regulator and strain specific (e.g. AfsR2 was found in *S. lividans* [134]) (Fig. 2). Although the role of AfsS is not completely revealed, multi copy of afsS also increases the production of antibiotics in *S. coelicolor*. Although AfsR shows no significant homology with typical regulators of the two-component systems in other prokaryotes, such as OmpR and PhoB of *Escherichia coli*, it shows considerable homology with regulatory proteins in antibiotic biosynthetic gene clusters of *Streptomyces* spp., such as actII-ORF4, dnrI and redD. At the lower site of AfsR cascade, AdpA is found as another main pleiotropic regulator, a representative of a large subfamily of AraC/XylS family [91]. AdpA, first found in *S. griseus* [91], controls the morphological

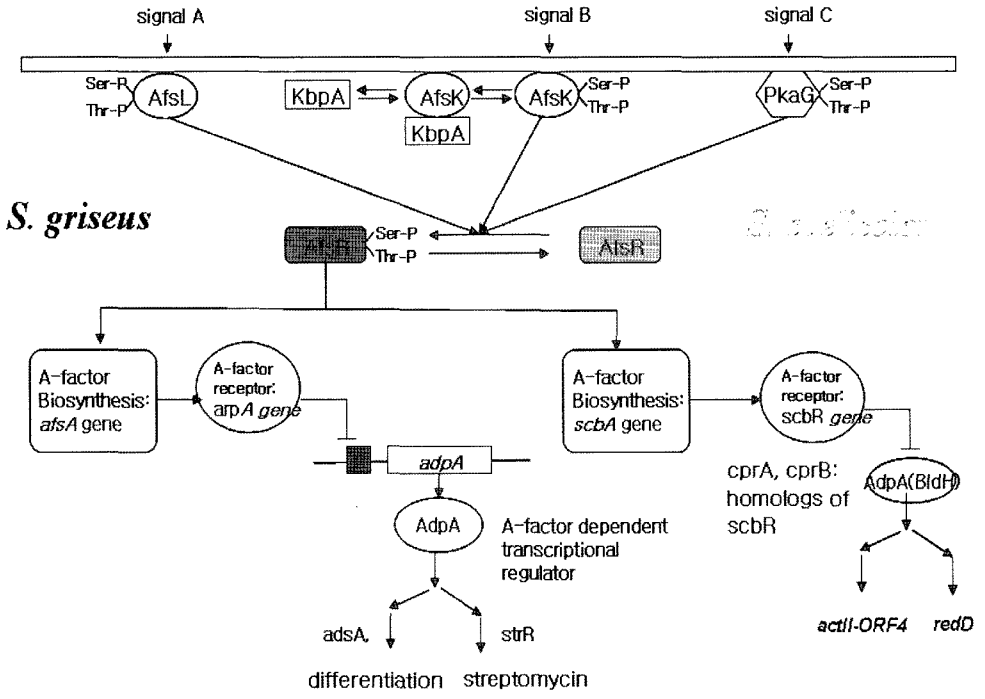

Fig. 2. Hypothetical events of the morphological and physiological differentiation in Streptomyces. See text for details (adapted from ref. 49).

development and secondary metabolism as a positive regulator, and its expression is controlled by ArpA, which is A-factor receptor and transcriptional repressor [145]. Although AdpA negatively autoregulates its own transcription, it binds various positions of starting sites of target genes and help their transcriptional initiation of RNA polymerase in *S. griseus*. All these functions were confirmed by the amplification and deletion of adpA gene in *S. griseus* [64, 67, 123, 128, 143, 144]. On the contrary, the function of AdpA in *S. coelicolor* is a little bit different. Here, AdpA is not in the control of gamma-butanolide A-factor like molecule (i.e. SCB1), but mainly working on morphological change [21]. Recent study showed close relationship with antibiotics production by the fact that AdpA disruption mutant of *S. coelicolor* overproduced undecylprodigiosin, but failed to produce actinorhodin [92], but the role of AdpA in *S. coelicolor* is still not completely reveled in many parts. Other known AdpA regulons are SgmA (secreted metalloprotease), SsgA (septum formation), StrR (streptomycin production), etc. (Fig. 3) [92]. To further confirm other binding partners with AfsR and AdpA, even though they are more likely to be DNA binding proteins, Tandem Affinity Purification (TAP) tag [97] and mass spectrometry can be used. To profile whole Ser/Thr phosphorylated proteins and to compare the fractions of the phosphorylated form, phosphoproteome analysis can be done with 2D-gel and fluorescent dye-tagged Ser-PO_4, or Thr-PO_4 antibody(Ab) solution [7, 106], LC-based phosphoproteomic techniques such as IMAC (Immobilized Metal Affinity Chromatography) with mass spectrometry or conventional 2D-gel with MudPITsystem [34] can be used to the identification and quantification of phosphorylated form of key regulators and total proteome influenced by a specific regulator. All these efforts will not be only for the study of *Streptomyces* cells, but also for the improvement of secondary metabolite production.

A-Factor and γ-Butanolides. γ-Butanolide quorum sensing [51, 52] is another component of global regulatory network. A-factor like quorum sensing molecules are involved in various cellular events, such as cell density control [86], secondary metabolite production [122], cell differentiation [50], biofilm formation [118], and virulence [90]. The key enzyme in the biosynthesis of γ-butanolides is encoded by scbA (equivalent to afsA) involved in the ligation reaction between hydroxyl acetone phosphate and β-ketoacyl CoA [53], and the γ-butyrolactones are recognized by their receptors, such as ScbR and ArpA. The binding of γ-butyrolactone to ScbR subsequently release the repression of ScbR againt the expression of AdpA by the removal of the bound ScbR from the upstream promoter region of adpA. Then as mentioned above, AdpA gives pleiotropic effects to its regulons. Until an optimum level of γ-butyrolactone, it induces early production of antibiotics and other secondary metabolites. However, beyond the threshold level, it conversely inhibits the production of antibiotics production. Early induction of antibiotics production appears to give high yields in the early stage, but final yield of the treated flask did not result the better yield, but more or less similar yield to that of the control flask (data not published). The reason would be that early

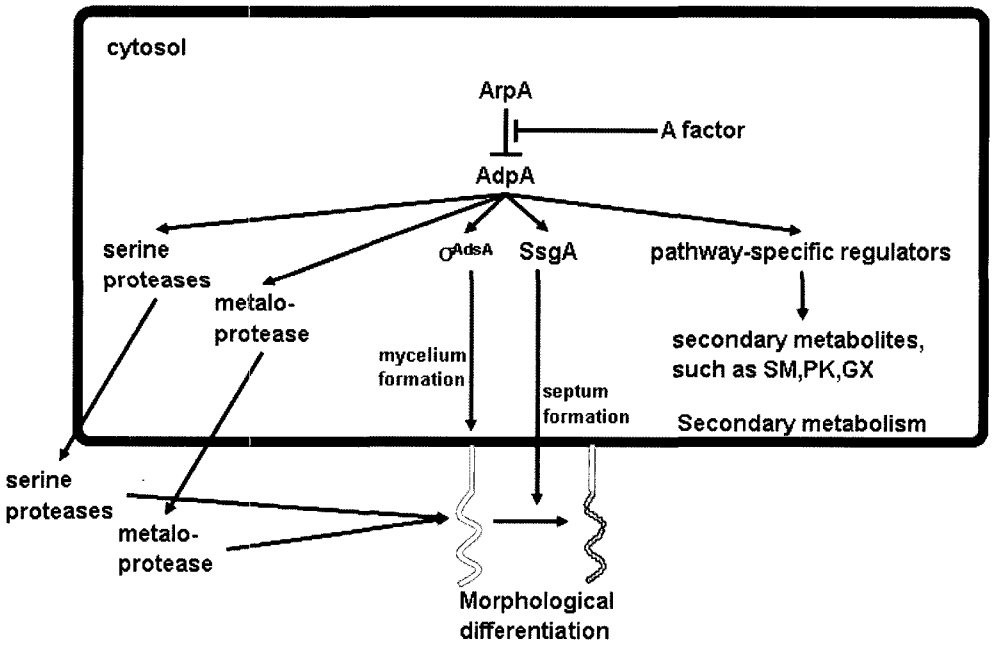

Fig. 3. AdpA regulons. AdpA, which is regulated by A factor, affects secondary metabolism and morphological differentiaion. SM, streptomycin; PK, polyketide; and GX, grixazone (modified from ref.88).

induction at low cell density does not help to enhance the final yield of antibiotics. As this quorum sensing mechanism is very tightly controlled by the cell [23], even significant changes in the AdpA expression owing to high fluctuations of γ-butyrolactone concentration will be rapidly dampened off during transition period. In addition, similar structures of γ-butyrolactones can give the same, but different levels of the effects to the other strains of *Streptomyces* on the antibiotics production and cell differentiation [49]. Recently, we have found that each ScbR-like receptor show different substrate specificity to various different ã-butyrolactones.

Unlike N-acyl homoserine lactones from Gram-negative bacteria [113], the butanolides from *Streptomyces* strains are produced with very small quantity, so that large volumes (i.e. from 300 l to 1,200 l) of the culture are required to accomplish NMR analysis as well as functional studies. To overcome such a difficulty in analysis, affinity capturing technology was introduced, and it showed the power to profile the structures of target molecules captured by the affinity tag [146]. Using receptor protein as a bait, *Streptomyces coelicolor* quorum sensing molecules were collected with small volume of cell supernant, and analyzed by ESI-MS/MS (Fig. 4).

The cell growth inhibition above threshold concentration of quorum molecule is recognized as a new approach to inhibit cell growth at certain condition to prevent abrupt cell growth and biofilm. This kind of affinity capture-mass spectrometric techniques can lead us to have enough structural information of target molecule and perform high-throughput screening (HTS) of meaningful activators for the antibiotic production as well as quorum inhibitors, and save time and efforts to find other possible novel metabolic products.

Sigma Factors. Sigma factor is an essential component of RNA holopolymerase, and reversibly associates with the core RNA polymerase complex. It can be replaced by alternative factors that co-ordinately express genes involved in diverse functions, such as stress responses and morphological development. *Streptomyces* have extremely many sigma factors (ca. 70–80, 87 sigma factors including anti sigma factor and anti-anti sigma factor) are found in Sanger SCOCYC database. Especially, 66 sigma factors are found in *S. coelicolor*, since they undergo cell differentiation such as mycelial growth and form exospores. Sometimes each sigma factor has its own regulon or a couple of sigma factors share the same regulons. In the case endospore formation of *B. subtilis* [117], mother cell and endospore have different sigma factors (sigE, F, G, and K) in each compartment during the process of cell differentiation. Major sigma factor during vegetative growth is sig70 family (SCO5820, which is equivalent to rpoD in *E. coli*, sigA in *Bacillus*), SigB is active under oxidative stress and

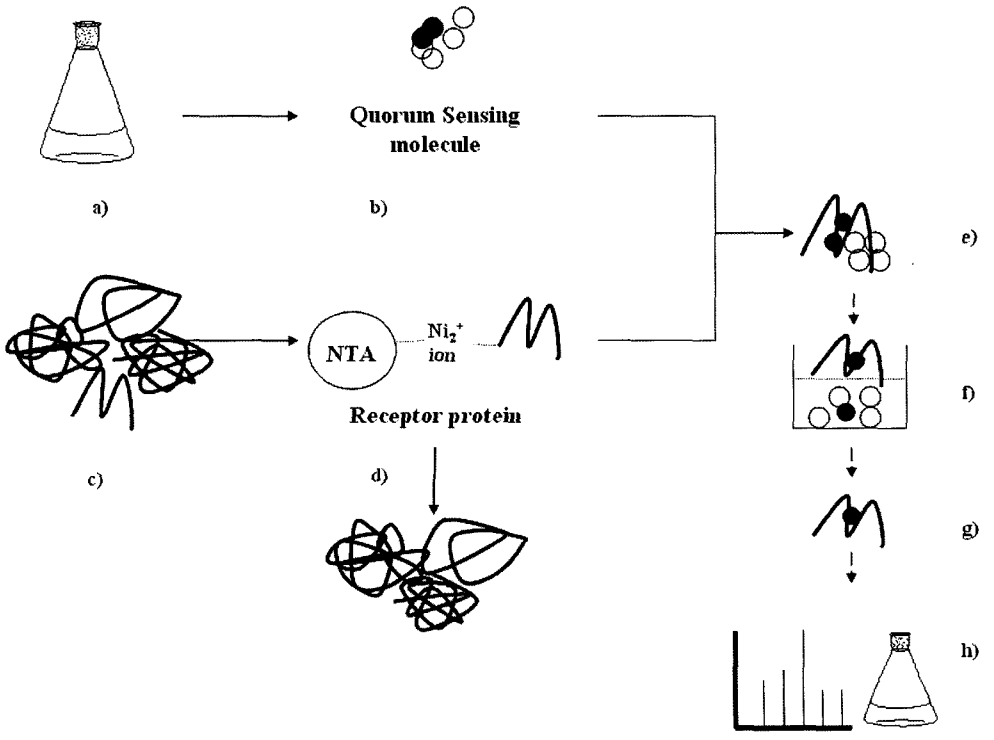

Fig. 4. Scheme for our current method.
a) *S. coelicolor* culture in 250-ml flask. b) Extraction with ethyl acetate. c) Overexpression of ScbR in *E. coli*. d) His-tag purification and washing out of unbound proteins. e) Binding reaction between ScbR and SCB1. f) Ultrafiltration. g) Elution by pH change and boiling. h) ESI-MS/MS and bioassay.

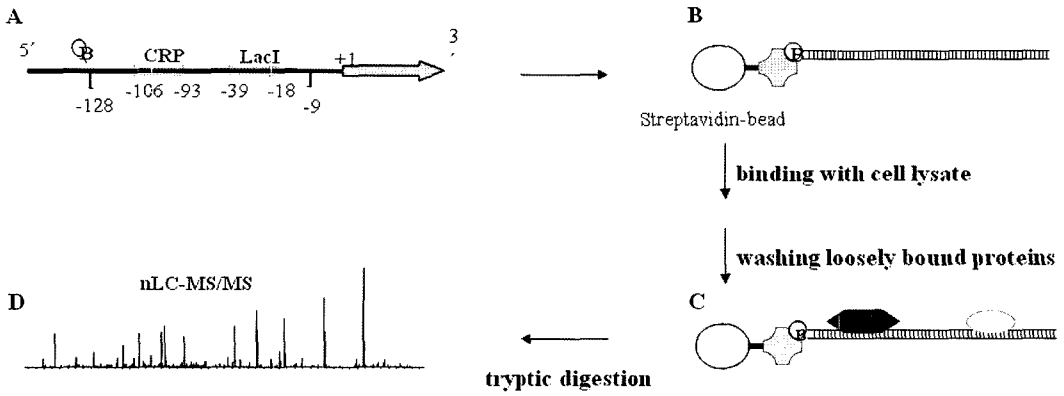

Fig. 5. Schematic view of the procedure of DNA-affnity capture assay.
(A) The promoter region of *lac* operon, which biotinylated at the 5_ terminus of the forward. (B) After incubation of annealed oligonucleotides with beads, biotin was added to isolate specific DNA-binding proteins. (C) The loosely bound proteins are washed out. (D) Analysis of tryptic peptides using nLC-MS/MS.

SigR is active for GroEL/ES expression and redox stress. WhiG is active for exospore formation and belongs to extracytoplasmic function (ECF) family. According to the very limited information of exosporulation, about 10 heat shock protein related sigma factors and 49 ECF family sigma factors are found in *S. coelicolor* [8]. Among the 49 ECF sigma factors, the functions of only three sigma factors are understood. Most of sigB and sigR family have sigma factor antagonist called "anti-sigma factor" in the same system, so that when cognate antagonist lose affinity for sigma factor, then corresponding sigma factor is released and activate the transcription of target operon [98].

In some cases, anti-anti-sigma factor is found as well. The mass spectrometry analysis can be used to identify the interactions among sigma factor/anti sigma factor/anti-anti sigma factors.

Pathway Specific Regulators

Pathway specific regulators are lower level transcriptional regulators functioning to operons of specific biosynthetic pathways, such as DnrI for doxorubicin synthesis in *S. peucetius*, RedD for undecylprodigiosin, ActII-ORF4 for actinorhodin synthesis in *S. coelicolor*, StrR for streptomycin biosynthesis in *S. griseus*. The pathway specific regulators are sometimes linked to multi-level control network, so that another regulator controls the lower level pathway specific regulator. For example, newly found transcriptional regulator, AtrA [130] activates the transcription of ActII-ORF4. In addition, certain pathway-specific activators are regulated at post-transcriptional level by the availability of the *bldA* leucyl-tRNA, which recognizes a rare codon [78]. Recently, adpA is also known to contain TTA (Leu) codon, so that it is governed by translational level.

For the search of pathway specific regulatory proteins, conventional procedures involve chromosome library construction, screening gene clusters of biosynthetic pathway, and subsequent sequence analysis of the gene cluster and analysis of the function of putative transcriptional regulators nearby the biosynthetic gene cluster. Gel retardation method using isotoped DNA and target regulator protein is quite commonly used *in vitro* to confirm specific DNA-protein interaction using cell extract. Although the effect of transcriptional regulators on target genes can be identified using constructing a deletion mutant, whole procedure is very tedious, time consuming and painstaking. Recent progress in mass spectrometric techniques provides a smart affinity capture and sequencing tool using DNA-protein interaction reaction, where immobilized upstream promoter regions DNA (250–400 base pairs)of all the ORFs of biosynthetic genes are used as baits, and bound proteins are applied to tryptic digestion, peptide sequencing and identification using mass spectrometry and bioinformatics (Fig. 5) [38]. Here, biotinylated DNA and magnetic streptavidine were used as bait, and DNA-binding proteins were successfully screened. As a result of these techniques, many putative and important regulators were found: AtrA, that is a pathway-specific activator of the actinorhodin biosynthetic gene cluster in *S. coelicolor*, Aur1P, that is essential for auricin production in *Streptomyces aureofaciens* CCM 3239, CcaR that controls clavulanic acid biosynthesis, and Brp encoding a butyrolactone receptor protein in *S. clavuligerus*, and so on have been reported. These newly found regulators can possibly improve the yield and productivity of the target products in the specified strains.

Metabolic Engineering and Proteomics

In order to see the effect of specific nutrient or metabolite effects on cell metabolism and growth, DNA microarray is the best methods since it can provide high density data on transient changes in mRNA level of all the genes in the cell. However, eventually we need to confirm the effects by examining protein expression level. In addition, quantitative

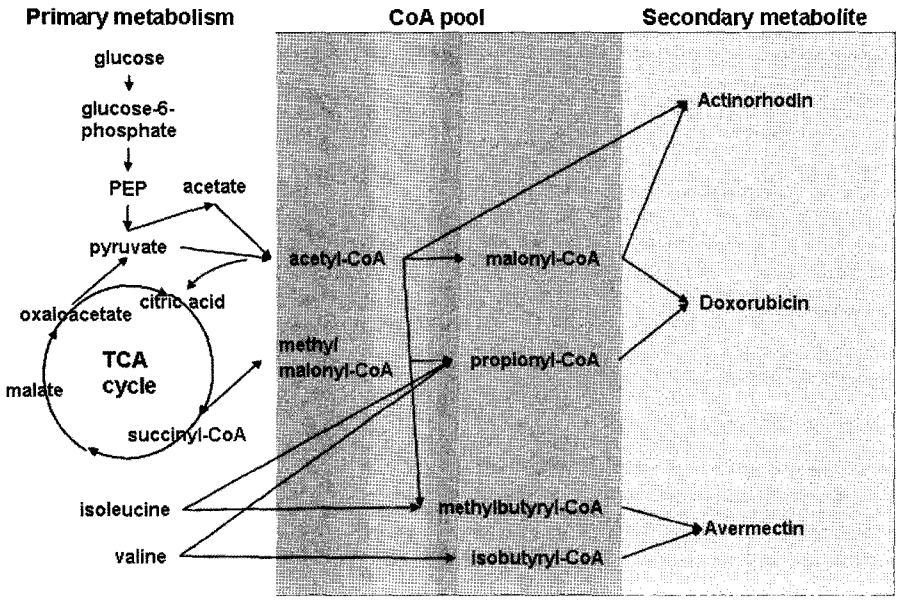

Fig. 6. Acyl-CoA flux for secondary metabolites.

analysis of metabolite concentration using triple quadrapole is essential to identify rate-determining steps (RDS) in the pathway, which then guide us to construct some useful mutants to solve the bottleneck [100]. Especially, in the case of the branch point connected with circular pathway (i.e. TCA), isotopomer flux distribution profile using isotope-labeled amino acid is necessary to measure accurate metabolic flux distribution through the branch point, and to accurately underpin the RDS [71, 85]. To compare the differences in specific protein expression level, 2D-gel-MS analysis is still powerful method to grasp a whole pathway proteome picture [47]. But it requires too much time to generate a reference gel. Instead, since antibody (Ab) generation is quite easy or commercially available Abs are plentiful nowadays, comparative pathway specific proteome analysis can be carried out using fluorescence dye-tagged Ab assay in 2D-gel [129].

Acyl-CoA and Polyketide Synthase

As most valuable products from *Streptomyces* are secondary metabolite antibiotics, proteomics study is very closely related to quantitative analysis of the intermediates of antibiotics and final product itself. Major concerns in terms of production yield point of view can be summarized as follows: 1) achieving high cell density 2) sufficient supply of precursor metabolites for synthesis for antibiotics, 3) removing all the unnecessary side products or pathways, 4) removal of product inhibition or developing product resistance. Here, we would like to focus more on the issues of the precursor supply, since this issue is related with the types of antibiotics and production of each type of antibiotics will be somewhat different depending upon the types of precursor as shown in Fig. 6 and Table 3 [114]. Polyketide synthase (PKS) type antibiotics use acyl-CoA

Table 3. Different types of PKS and the name of antibiotics each strain produces.

Class	Antibiotics	Strain	Target	Application
PKS(I)	Avermectin	*S. avermitilis*	Chloride ion channels	Antiparastic
	Erythromycin	*S. erythraea*	Ribosome binding	Antibacterial
	Olendomycin	*S. antibioticus*		Growth promoter
	Tylosin	*S. fradiae*		
PKS(II)	Daunorubicin	*S. peucetius*	DNA intercalation	Antitumor
	Doxorubicin	*S. peucetius*	Ribosome binding	Antibacterial
	Tetracycline	*S. aureofaciens*		
Aminoglycoside	Gentamycin	*Micromonospora*	Ribosome binding	Antibacterial
	Kanamycin	*S. kanamyceticus*		
	Ribostamycin	*S. ribosifidicus*		
Glycopeptide	Teicoplanin	*Actinoplanes teichomyceticus*	Peptidoglycan	Antibacterial
	Vancomycin	*Amycolatopsis orientalis*		

compounds as starting unit (or loading unit) and extending unit for PKS substrates [87], whereas aminoglycoside and glycopeptide require glucose-6-PO4/myo-inositol-1-phosphate and amino acids, respectively. To provide sufficient amount of acyl-CoA for the antibiotics synthesis, selection of good carbon sources in the medium is the most important, because most acyl-CoAs are provided either by glycolysis or β-oxidation of fatty acids (or from oil). It suggests that antibiotics production is very closely related with primary metabolism as well. As shown in the figure, malonyl-CoA is synthesized from 2 acetyl-CoA by removing CO_2, and methylmalonyl-CoA, methylbutyryl-CoA, isobutyryl-CoA are from succinyl-CoA, isoleucine, valine, respectively. Propionyl-CoA is from acetyl-CoA or isoleucine via methylacetoacetyl-CoA or valine via isobutyryl-CoA. Therefore, branched amino acid concentrations are very significant to supply enough branched acyl-CoA precursors. Other strategies to supply sufficient acetyl-CoA draw our attention, since there are various ways for *Streptomyces* to provide it through amino acid degradation, β-oxidation of fatty acid or pyruvate dehydrogenation. Amino acid degradation and β-oxidation of fatty acid can be controlled by media optimization. In terms of pyruvate to acetyl-CoA conversion, it is known that three pathways exist in bacteria: i.e. i) direct conversion of pyruvate to acetyl-CoA using pyruvate dehydrogenase complex, ii) from pyruvate to acetyl-CoA via acetyl-phosphate using pyruvate oxidase and phosphate acetyltransferase, iii) from pyruvate to acetyl-CoA via acetate using pyruvate oxidase and acetyl-CoA synthetase. Among them, Streptomyces appears to have i) and iii) pathways. Amplification of these pathways tends to increase the yield of antibiotics which use acetyl-CoA as the precursor of the structure (unpublished data).

Another approach to increase acyl-CoA concentration is to provide sufficient CoA pool, which is synthesized from pantothenic acid via five enzyme steps, among them the first step pantothenate kinase (panK) is known to be a rate-limiting enzyme [104]. The amplification of panK increased various PKS type antibiotics, suggesting that excess supply of CoA somewhat accompany acyl-CoA production, leading to yield improvement of PKS type antibiotics (unpublished result). For the hybrid antibiotics synthesis, feeding of appropriate starting unit precursors is sometimes essential, so that substrate specificities of PKS for the precursors is very critical to increase the yield of antibiotics [17, 59]. Various starting unit precursors for the PKS are well documented in the literature [87].

Many PKS type and glycopeptide antibiotics are consisted of glycon and aglycon moieties. In the case of aminoglycoside, pseudosugar, i.e. 2-deoxystreptamine synthesized from myo-inositol, becomes a component of the antibiotics [60]. The glycon is often involved in binding to target molecules [73]. As the synthesis rate of aglycon unit is not always coupled with glycon unit, the yield of complete antibiotics become low, and such cases are more common in the production of hybrid antibiotics [54]. Therefore, the amplification of the biosynthetic pathway for glycon as well as improvement of glycosyltransferase activity becomes an issue in the production of antibiotics with high yield. The same problem was observed with panK mutant of *S. peucetius* producing doxorubicin. The major product was ε-rhodomycinone without glycon, while doxorubicin production was even reduced by panK amplication (unpublished data). When this issue goes to combinatorial biosynthesis, substrate specificity of glycosyltransferase is very critical, so that evolution or screening of glycosyltransferase having very broad substrate specificity is required [80, 142]. In addition, it would be very interesting to elucidate the control mechanism of glycan synthesis and the relationship between glycan synthesis and primary metabolite production, such as acetyl CoA.

S-Adenosyl Methionine and other Amino Acids

S-adenosyl methionine (SAM) is an essential cell metabolite involved in biological methylation [18, 25] and homocysteine transfer, such polyamine synthesis [13] and tRNA modification [10, 58], so that cell would die without it [24].Recently, it was known that additional SAM feeding to the cell broth or *metK* (SAM synthetase, SCO1476) amplification in *Streptomyces* dramatically increases antibiotics production and inhibit sporulation [66, 93], and that the enhancing effect is more pronounced in methylated antibiotics, such as oleandomycin and avermectin [56]. The newly found result from the subsequent experiments is that SAM is playing a role as a signal triggering molecule in *E. coli* chemotactics via O-methylation [147] and plant hormone synthesis [139], which is a very intriguing in that some essential metabolites like amino acid (e.g. glutamate) are involved in cell metabolism as well as gene regulation. SAM is also known as the substrate for autoinducer synthesis in quorum sensing system of *Vibrio Harveyi* [141], suggesting that it is involved in control mechanism of cell density. Our recent result is that SAM can also play a role as a precursor to increase the pool of Acyl-CoA via several reaction steps (unpublished data). Therefore, supply of sufficient SAM by amplifying MetK (SAM synthetase) would be a good strategy to enhance various antibiotics productions. Interestingly, *S. coelicolor* and *S. avermetilis* has one *metK* gene, whereas *S. peucetius* has two *metK* genes. The function of each *metK* gene is remained to be seen. As the SAM effect is also closely linked with the supply of sufficient methionine, cysteine, aspartate, serine, *etc.* in order, regulations of TCA cycle and glycolysis are closely link as well. Therefore, it appears to me that one small chemical compound itself acts like a global regulatory molecule.

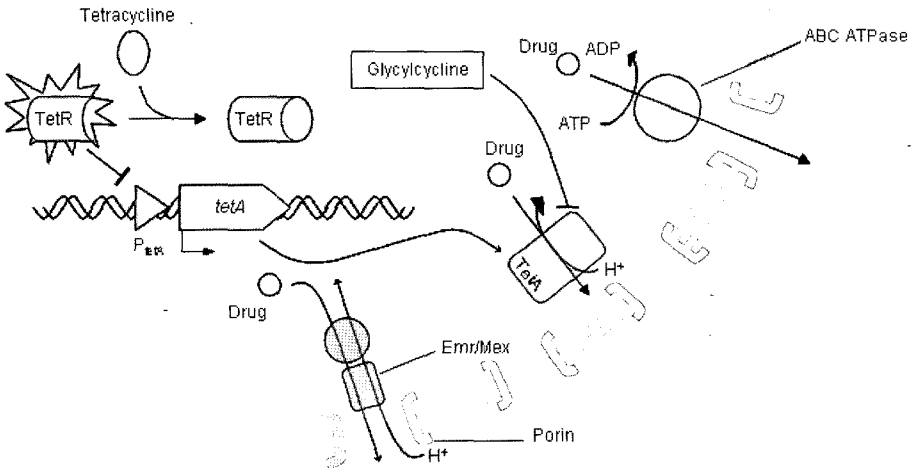

Fig. 7. Resistance by action of H^+ and ATP-coupled efflux pumps in bacterial membranes (adapted from ref.) (Christopher Walsh ANTIBIOTICS Chap8 107–124).

TRANSPORTER AND RESISTANCE GENE

There are four known bacterial antibiotic resistance mechanisms, which are drug inactivation, target alteration, prevention of target influx and active extrusion of drugs from the cell [102]. Among them, active extrusion of drugs from the cell is the only good strategy to construct a high antibiotics producing strain, since the other strategies will change the host genotype or phenotype or reduce the antibiotics yield *per se*. To improve the efficiency of efflux of transporter and, hence, to increase resistance, amplification of antibiotics transporter gene is the most common remedy to increase the resistance of a certain antibiotics. However, to study such transporters, analysis of membrane proteins is a major limitation of current 2D-PAGE, because membrane protein is insoluble in standard gel running buffer solution. Therefore, special techniques are needed to separate integral membrane proteins. However, amidosulfobetaine-14-solubilized membrane proteins by anion exchange chromatography (AIEC) even identified 50membrane-integral proteins among 170 protein shown in the 2D-gel, corresponding to 7.5% of predicted membrane proteins in *Corynebacterium* [110]. Another approach is using formic acid in the presence of cyanogen bromide [136] and organic solvents [11] increasing the solubility of membrane proteins. Until now, 2-D gel analysis of whole membrane proteins of *Streptomyces* has not been published yet. More advanced techniques are needed to treat membrane proteins properly in the near future.

In general, there are several kinds of transporters for mutidrug resistance by their functions and structures: i.e. ATP binding cassette transporters (ABC), major facilitator superfamily (MFS), resistance/nodulation/division proteins (RND), multidrug and toxic compound extrusion transporters (MATE), drug/metabolite transporters (DMT), multidrug endosomal transporters (MET), and small multidrug resistance family (SMR). Largely speaking, these transporters can be categorized into two groups: ABC type transporters and antiporters (Fig. 7). ABC transporters are using free energy generated by ATP hydrolysis to pump drugs out of the cells, and antiporters are using transmembrane electrochemical gradient of proton or sodium ions to drive extrusions of drugs from the cells like MFS, SMR and RND [89].

Until now, some transporters and resistance systems are revealed in *Streptomyces*. Nystatin transport genes in *Streptomyces noursei* [116], avilamycin transporter in the *Streptomyces viridochromogenes* Tu57 [137], oleandomycin ABC transporter in *Streptomyces antibioticus* [15], mithramycin transporter in *Streptomyces argillaceus* [33] etc. are studied. Especially, DrrABC system in *S. peucetius* is a representative and well-characterized example [37]. It is composed of transporters, which have ATP binding domain (DrrA) and channel for the export (DrrB), and DNA-binding protein (DrrC), which can inhibit or destabilize the binding of daunomycin and doxorubicin to genomic DNA, and is involved in excision repair of DNA. To study such specific antibiotic resistance and transporter system, incremental antibiotics dosage dependent mutation method using nitrosoguanidine (NTG) or ethylmethane sulfonate (EMS) can be widely used [69], which can screen more antibiotic-resistant strain without losing the ability of producing antibiotics. Then, such strains have high probability of having improved antibiotic resistance. Such strains can be compared with the wild type control strain using 2D-gel analysis and MudPIT shot gun analysis of whole proteome, which leads to identify meaningful proteins to examine and key factors involved.

As another approach to identify a certain antibiotic transporter, because of the difficulties in identifying interacting ligand for the transporter membrane proteins due to the

difficulties in harvesting membrane protein directly from cell extracts, many transporter and resistance related regulators can be studied and modified to improve antibiotic resistance and productivity as producing host. In terms of regulators to control transporter system, TetR from *E. coli* (Fig. 7) [103], BmrR from *Bacillus subtilis* [45], MexR from *P. aeruginosa* [2], etc are well documented (Fig. 7) [135].

SECRETOME

The extracellular proteome of S. coelicolor grown in a liquid medium was recently analyzed by using 2-D gel analysis and MALDI-TOF [47] and the protein secretion is revealed to be mainly a stationary phase phenomenon. As *Streptomyces* was well known for producer of small molecular weight secondary metabolites, i.e. antibiotics, secretion was not investigated thoroughly. On the other hand, secretion mechanism plays an important role in the production of extracellular enzymes such as proteases, lipases, cellulases, and chitinase, etc. However, very little information has been elucidated on the secretion systems of *Streptomyces*. Therefore, more studies are needed in this field of research.

Bacillus, the best characterized Gram-positive bacterium, secrets a lot of extracellular proteins (Fig. 8). There exist 5 distinct classes classified on the basis of signal peptides [125]. SRP-YEG (Sec) co-operation pathway, which is the major pathway in protein secretion, not only brings cytosolic proteins to the cell membrane and environment, but also localizes and assembles proteins subsequently for sporulation. Tat system (Twin-arginine translocation pathway), Sec-independent, might function in the secretion and membrane localization of some specific proteins [61]. In addition, both ABC transporters, related to the production of pheromones and peptide antibiotics bacteriocins [29], and COM-specific pathway which transports pseudopilin precursors [27, 28, 126] are known as SRP-Sec system independent. Finally, membrane protein liberation pathway is dependent on the SRP-Sec system [4, 127]. Now, it is quite possible and interesting to see what kinds of equivalent genes to bacillus are present in *Streptomyces* in terms of secretion systems. In addition, the secretion systems for protein exports should be confirmed using different growth conditions, mutant strains, and proteomic approaches. Furthermore, elucidating the characterization and modification of the secretion systems, and chimeric secretion systems might be the next step to increase extracellular production of proteins.

CONCLUSION

Streptomyces are known as a treasure box of natural organic compounds in itself as they produce over 75% of naturally-occurred bioactive compounds. Integration of genomics, proteomics, and metabolomics data which allow us to understand global and/or pathway-specific regulation, metabolism and signaling pathway, is essential for developing Streptomyces as an industrial host for antibiotics production. Owing to the burgeoning knowledge of the "-omics" data, many industrial *Streptomyces* strains undergone many rounds of random mutagenesis are on the verge of facing new destiny, such that several known defined mutations

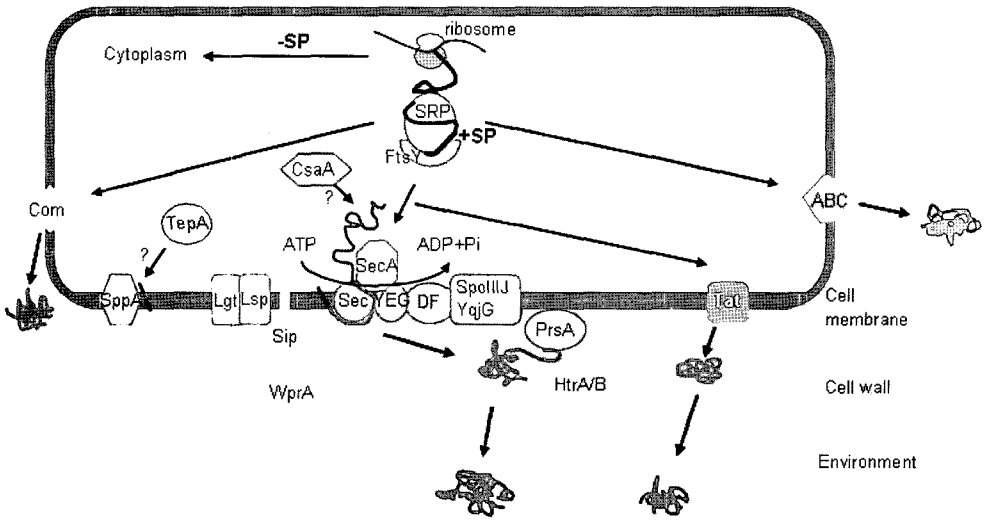

Fig. 8. Schematic overview of Bacillus secretion system.
Protein export pathways in *B. subtilis*. Ribosomally synthesized proteins can be sorted depending on the presence (+SP) or absence (-SP) of an N-terminal signal peptide and specific retention signals (modified from ref. 118).

can make the original wild type *Streptomyces* strain almost equivalent to the industrial strain. It is not surprising to see that happens in the near future like *E. coli* and *Corynebacterium* (personnel communication). New hybrid antibiotics made from combinatorial biosynthesis, which are known as a new age antibiotics to overcome current problems of antibiotics resistance, often show very low yield and productivity, which are immediate concerns to all the scientist working in the area of combinatorial biosynthesis. To improve their yields and productivities, understanding all the regulatory mechanisms involved in the biosynthetic pathways as well as global regulatory network is needed. To have such a picture, proteomics itself has its own limitation. Therefore, genomics and metabolomics informations are prerequisite to achieve such a goal. In addition, for some secreted peptides like peptide antibiotics or NRPS peptides which cannot be inferred from genome data due to post translational modification, peptidomics analyzing whole secreted oligopeptide populations can be a strong tool to elucidate their structural information, cell physiology under specified conditions, and secretion system.

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