Award Lecture

Genome Analysis System for Actinomycetes:
Development and Application

Jun Ishikawa

Department of Bioactive Molecules, National Institute of Infectious Diseases,
1-23-1, Toyama, Shinjuku, Tokyo 162-8640, Japan

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In 1986, I moved from Prof. Y. Koyama's laboratory (Toho University) to Dr. K. Hotta's laboratory (National Institute of Health), and began a study on the cryptic kanamycin resistance gene of a streptomycin-producing *Streptomyces griseus*. It was not long before I cloned a 1.8-kb DNA fragment containing the *kan* gene encoding an aminoglycoside 3-*N*-acetyltransferase (AAC(3); Hotta *et al.*, 1988; Ishikawa *et al.*, 1988). Subsequently, I determined its nucleotide sequence to specify the coding region (Ishikawa & Hotta, 1991). Initially, it was believed that obtaining the nucleotide sequence would allow identification of the gene interest. Unexpectedly, however, the coding region of the *kan* gene could not be readily specified because the 1.8-kb sequence contained many candidate start and stop codons as well as open reading frames. However, the coding region of the *kan* gene was predicted quite easily by FRAME analysis (Bibb *et al.*, 1984), a gene prediction method based on the distribution of third-letter G+C content of each codon in high G+C content bacteria. This experience led me to the computational analysis of sequence information, which has recently been called "bioinformatics".

When FRAME analysis was developed by Bibb *et al.*, the software was implemented on only a few platforms such as the VAX systems. Therefore, I developed the FramePlot program from scratch in a BASIC language and distributed it to my colleagues. After several years, I re-implemented the FramePlot program as a web-based program with the invention of the World Wide Web in the mid-1990s. The graphical user interface and integration of the NCBI BLAST search made the FramePlot program an essential tool to analyze actinomycete genes because of its platform independence and ease of use (Fig. 1).

In 2000, Prof. Haruo Ikeda (Kitasato University) began the genome sequencing of *S. avermitilis*, an industrial microorganism producing avermectin. I agreed to help in sequence assembly and annotation of the genome sequence, because it was exciting work to unveil the longest bacterial genome sequence at that time. First, I modified the FramePlot program so that the program could handle genome-scale sequence data. In addition, I had to develop a great many shell- and Perl-scripts to handle hundreds of thousands of sequence traces, database search results, *etc.* This was very arduous work but a good opportunity to relearn computer programming. After one year, we over-

came many difficulties and obtained a genome sequence as five supercontigs. Since this sequence was estimated to cover more than 99% of the genome, we could deduce the ability of this organism to produce secondary metabolites (Omura *et al.*, 2001). Surprisingly, the *S. avermitilis* genome was found to have 25 secondary metabolite gene clusters, only a few of which were known before the determination of the genome sequence. On December 27, 2001, we finally completed the genome sequence. The final gap contained an 18 G-run (Fig. 2). The completion of the genome sequence yielded the discovery of five additional gene clusters (Ikeda *et al.*, 2003), which could not have been identified before completion of the genome sequence due to the loss of gene context. Currently, 32 secondary metabolite gene clusters have been recognized by the continuous efforts of Prof. Ikeda's group. Furthermore, the relationships between genes and metabolites have been demonstrated experimentally for some of these clusters.

Thus, I established a genome analysis system, especially for high-G+C bacterial genomes. After sequencing of the *S. avermitilis* genome, I worked on genome sequencing projects of *Mycoplasma penetrans* (Sasaki *et al.*, 2002), *Symbiobacterium thermophilum* (Ueda *et al.*, 2004), *Nocardia farcinica* (Ishikawa *et al.*, 2004), and *S. griseus* (Ohnishi *et al.*, 2008). At present, six genome sequencing projects, including *Kitasatospora setae* and *Actinoplanes*

Fig. 1. Usage statistics of FramePlot.

Fig. 2. Nucleotide sequence contained in the final gap of the *S. avermitilis* genome sequence assembly. The sequence was determined by transcriptional sequencing (Sasaki *et al.*, 1998).

missouriensis, are using my system.

Completion of the genome sequence is not the final goal of my work, and of course the genome sequence must be used for post-genome sequence studies. Recently, I collaborated with Prof. Tohru Dairi's group (Toyama Prefectural University) in the discovery of a novel biosynthetic pathway for menaquinone (Hiratsuka *et al.*, 2008). He performed detailed comparisons of published genome sequences and found that some bacteria, such as *Streptomyces coelicolor* A3(2), *Helicobacter pylori* and *Campylobacter jejuni*, lack genes for the known menaquinone biosynthetic pathway, although they synthesize this essential compound for their life. Prof. Dairi requested me a fantastic demand: "Can you specify which genes are responsible for menaquinone biosynthesis in the *S. coelicolor* A3(2) genome?". This request perplexed me but I soon had an idea. I considered that genes for a novel pathway could be specified by comparing orthologous genes between bacterial genomes having and lacking the

known pathway, because menaquinone biosynthetic genes are essential for the bacteria. I confirmed my strategy by repeated simulations and finally narrowed down the likely candidates to 50 of 7,500 genes. Subsequently, Prof. Dairi's group demonstrated successfully that at least four hypothetical genes of *S. coelicolor* A3(2) were involved in the novel pathway. The work was commented to be "a tour de force" (Drahl, 2008).

The post-genome sequence analysis of *N. farcinica* accounts for the majority of my present work, which are aimed at understanding its characteristics such as pathogenicity, drug resistance, secondary metabolism and conversion of chemicals. At present, the most remarkable result from this work is probably the discovery of the duplication of RNA polymerase β subunit gene (Ishikawa *et al.*, 2006). We demonstrated the presence of two different RNA polymerase β subunit genes, *rpoB* and *rpoB2*, in the *N. farcinica* IFM 10152 genome. These two genes show 88% identity and 94% similarity at the deduced amino acid level. Moreover, comparison of their amino acid sequences with those of other bacterial RpoB proteins suggested that the nocardial RpoB protein is likely to be sensitive to rifampin, whereas RpoB2 protein contains substitutions at the rifampin-binding region likely to confer rifampin resistance. The introduction of *rpoB2* conferred rifampin resistance to *N. asteroides* IFM 0319, which has neither rifampin resistance nor *rpoB* duplication. Furthermore, unmarked *rpoB2* deletion mutants of *N. farcinica* IFM 10152 showed no significant resistance to RIF (Table 1). These results indicated that *rpoB2* was responsible for rifampin resistance in *N. farcinica* IFM 10152. We also demonstrated that *rpoB* duplication was widespread in *Nocardia* species and correlated with the rifampin-resistant phenotype (Table 1). In addition to *rpoB2*, the *N. farcinica* genome contains many candidate genes for drug resistance, such as aminoglycoside phosphotransferase, β -lactamase,

Table 1. *rpoB* duplication and rifampin resistance in *Nocardia*.

Species, strain/plasmid	<i>rpoB</i> duplication*	Resistance (μ g/ml)**
<i>N. farcinica</i> IFM 10152	+	>100
<i>N. farcinica</i> IFM 10152 Δ <i>rpoB2</i>	—	<5
<i>N. farcinica</i> IFM 10152 Δ <i>rpoB2</i> /pNV <i>rpoB2</i>	—	>100
<i>N. farcinica</i> IFM 0284 ^T	+	>100
<i>N. farcinica</i> IFM 10125	+	>100
<i>N. asteroides</i> IFM 0319 ^T	—	<5
<i>N. asteroides</i> IFM 0319 ^T /pNV <i>rpoB2</i>	—	>100
<i>N. asteroides</i> IFM 10159	+	25
<i>N. asteroides</i> IFM 10162	—	<5
<i>N. brasiliensis</i> IFM 0236 ^T	+	50
<i>N. brasiliensis</i> IFM 1032	+	>100
<i>N. brasiliensis</i> IFM 10160	+	50
<i>N. terpenica</i> IFM 0406	+	<5

*Estimated by Southern hybridization experiments.

**Determined after incubation at 37°C for 48 h on BHI agar plates containing various concentrations of rifampin.

drug efflux pump, and ribosomal RNA methyltransferase genes. These candidate genes could explain almost all drug resistance profiles of *N. farcinica*. We will experimentally demonstrate not only their roles but also all the resistance genes of the genome (resistome) in future studies.

As part of the post-genome sequence study, we have developed genetic tools for *Nocardia* (Chiba *et al.*, 2007) and have first succeeded in gene knockout (Ishikawa *et al.*, 2006) in this microorganism. To facilitate studies on *Nocardia*, we have distributed our tools and techniques as well as the genome resources of *N. farcinica*. To date, we have provided pNV vectors to 18 researchers or research groups and 35 genomic DNA clones from the ordered plasmid library of the *N. farcinica* genome. Two reports have been published using our resources. Agematu *et al.* (2006) reported that Nfa33880 and Nfa53110 are able to hydroxylate the 16 α - or 17-position of testosterone, respectively. More recently, Martins *et al.* reported that *nfa18420* gene encodes an arylamine *N*-acetyltransferase and discussed its putative role in isoniazid resistance (Martins *et al.*, 2008). I hope that our resources will continue to facilitate further valuable works.

Nocardia species have many important, interesting and valuable features. However, since the post-genome sequence analysis of *N. farcinica* has just begun, we do not have sufficient results to present here yet. However, the result of several on-going studies will be published elsewhere in the near future.

One of the most important subjects of actinomycetes genome analysis is the understanding of secondary metabolism. Although there have been a number of efforts to discover new secondary metabolites, it seems that most secondary metabolites remain to be discovered. Because, as mentioned above, over 90% of genes or gene clusters for secondary metabolite biosynthesis have gone unnoticed. If we could activate these phenotypically silent genes or gene clusters for secondary metabolite biosynthesis, it may be possible to discover large numbers of new antibiotics more efficiently. Based on this notion, I began a study, which I call ASAP (Activation of Silent Antibiotics Productivity). The ASAP approach does not refer to a particular technique but to an idea for discovering new antibiotics as soon as possible. There will be various ways to activate silent antibiotics productivity, for example artificial control of the SARP-family transcriptional regulator genes. New approaches may free us from time-consuming and laborious classical approaches, such as improvement of culture conditions and obtaining mutant strains.

Ten years ago, genome sequencing was possible for only a few limited researchers due to the very high costs and requirement for large-scale facilities. However, both the lowering of the cost and the appearance of next-generation sequencers have enabled many researchers to perform genome sequencing. As a result, there has been an explosion in the number of genome sequences in literatures and databases. Although this was our awaited state, we are

far from capable of obtaining out information efficiently or easily from genome sequences. We are still struggling in a sea of genome sequence data rather than swimming. The development of new technology to analyze genome sequences is a major priority so as to prevent drowning.

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