Genomics update

Natural products genomics

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Secondary metabolites (or natural products) are often synthesized by multi-modular, multi-domain proteins called non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS). Various well-known metabolites produced by microorganisms are listed in Table 1, and examples of structures are shown in Fig. 1. In particular, Streptomyces species are known for their ability to produce a wide variety of secondary metabolites such as antibiotics, herbicides, parasitocides, siderophores and pharmacologically active substances including antitumour agents and immunosuppressants. Genome sequencing of Streptomyces coelicolor (Bentley et al., 2002) and S. avermitilis (Omura et al., 2001) revealed over 20 gene clusters for biosynthesis of secondary metabolites, while only a few of their natural products were known prior to sequencing. High-throughput genome sequencing of hundreds of other bacterial species and strains is now rapidly increasing the repertoire of identified gene clusters for biosynthesis of natural products (Donadio et al., 2007).

Here we give a brief update of the current status of genome mining and bioinformatic tools to identify novel NRPS and PKS systems.

Polyketide and non-ribosomal peptide biosynthesis

Both NRPS and PKS systems are molecular assembly lines for successive linking of multiple-amino/hydroxy acids or acyl-CoA precursors, respectively, into complex polymers which are often further modified into unique structures (Table 1, Fig. 1). The basic steps of both systems are initiation, elongation and termination performed by separate modules of the synthases (Fig. 2). These modules and others are usually encoded in large gene clusters (Khosla et al., 1999; Crosa and Walsh, 2002; Donadio et al., 2007; Rokem et al., 2007).

Non-ribosomal peptide synthetase modules can contain four principal domains (Fig. 2A): an adenylation domain (A) that selects, activates and loads the building blocks (proteinogenic and non-proteinogenic amino acids or carboxylic acids), a thiolation domain (T), also known as peptidyl carrier protein (PCP) that covalently fixes the amino acid on the synthetase, a condensation domain (C) that catalyses the peptide bond formation, and a thioesterase domain (Te) that releases the assembled peptide from the synthetase (Sieber and Marahiel, 2005; Wenzel and Muller, 2005). The diversity in structure and composition of the products is achieved due to different specificities of the A domains and further modifications by gene cluster-embedded or stand-alone additional domains such as methyltransferase (MT), epimerization (E), cyclization (Cy) and others (Walsh et al., 2001). The assembled final peptide structures range from linear [such as the pentadecapeptide gramicidin (Kessler et al., 2004)], to branched [such as vibriobactin (Keating et al., 2000)], partially cyclic [such as daptomycin (McHenney et al., 1998)], cyclic [such as gramicidin S (Erlanger and Goode, 1960)] or bicyclic [such as actinomycin (Pfennig et al., 1999)].

Polyketide synthase modules can contain four core domains (Fig. 2B): an acyltransferase (AT) domain that selects and activates the acyl-CoA building blocks (such as acetyl-CoA, malonyl-CoA, methylmalonyl-CoA and ethylmalonyl-CoA), an acyl carrier (ACP) domain, a ketoacylsynthase (KS) condensation domain and a releasing thio-esterase (Te) domain. The modules may contain other modification domains such as ketoreductase (KR), dehydratase (DH) and enoylreductase (ER). Polyketide synthases generate enzyme-bound ketoacyl intermediates in stepwise decarboxylylative condensations between the extender building blocks and the growing polyketide chain in a process similar to fatty acid synthesis. An example of such an assembly process is shown in Fig. 3.

Prediction of structure of non-ribosomally synthesized peptides

In most of the NRPS systems known so far, the order and structure of building blocks present in the secondary
Table 1. Examples of microbial natural products produced by NRPS/PKS systems.

| Natural product | Microorganism                      | NRP/PK |
|-----------------|------------------------------------|--------|
| Antibiotics     |                                    |        |
| Penicillin      | Penicillium chrysogenum (fungi)    | NRP    |
| Bacitracin      | Bacillus licheniformis             | NRP    |
| Tyrocidin       | Bacillus brevis                    | NRP    |
| Cephalosporin   | Streptomyces clavuligerus          | NRP    |
| Erythromycin    | Saccharopolyspora erythrea         | PK     |
| Tetracycline    | Streptomyces aureofaciens          | PK     |
| Actinomycin     | Streptomyces chrysomallus          | NRP    |
| Antitumour agents |                                |        |
| Dolastatin 10   | Symploca species (cyanobacteria)   | NRP    |
| Bleomycin       | Streptomyces verticillus           | Hybrid NRP/PK |
| Chondramide     | Chondromyces crocatus              | Hybrid NRP/PK |
| Epothilone      | Sorangium cellulosum              | Hybrid NRP/PK |
| Immunosuppressants |                                |        |
| Cyclosporin     | Tolypocladium inflatum (fungi)     | NRP    |
| Rapamycin       | Streptomyces hygroscopicus         | PK     |
| FK506           | Streptomyces sp.                   | PK     |
| FK520           | Streptomyces hygroscopicus         | PK     |
| Protease inhibitors |                                |        |
| Anabenaopeptin  | Anabaena flos-aquae (cyanobacteria)| NRP    |
| Oscillamide     | Oscillatoria agardii (cyanobacteria)| NRP    |
| Siderophores    |                                    |        |
| Mycobactin      | Mycobacterium tuberculosis         | NRP    |
| Bacillibactin   | Bacillus subtilis                  | NRP    |
| Enterobactin    | Escherichia coli                   | NRP    |
| Yersiniabactin  | Yersinia pestis                    | Hybrid NRP/PK |
| Toxins          |                                    |        |
| Mycolactone     | Mycobacterium ulcerans             | PK     |
| Naphthazarins   | Fusarium oxysporum (fungi)         | PK     |
| HC-toxin        | Cochliobolus carbonum (fungi)      | NRP    |

Bacteria unless otherwise indicated.

Fig. 1. Examples of some chemical structures of (A) polyketides, (B) non-ribosomal peptides and (C) mixed NRP-PK compounds. Reprinted with permission from Watanabe and Oikawa (2007). Copyright Royal Society of Chemistry.
Polypeptide product are reflected by the modular architecture of the NRPS. This relation between the template and the product is referred to as co-linearity rule. The specificity of A domains as well as the role of the other modifying domains will specify the composition of the produced polypeptide. General rules for predicting substrate specificity of A domains were initially developed based on the crystal structure of an adenylation domain of gramicidin synthetase (Stachelhaus et al., 1999; Challis et al., 2000). The NRPSpredictor (http://www.ab.informatik.uni-tuebingen.de/toolbox) uses transductive support vector machines (TSVMs) as a predictive tool for detecting substrate specificities of A domains (Rausch et al., 2005) based on the physicochemical properties of substrate-binding pocket residues.

**In silico genome screening for NRPS/PKS gene clusters**

There are several bioinformatic tools available for searching NRPS/PKS systems in genome sequences. The NRPS-PKS tool is web-based software (http://www.nii.res.in/nrps-pks.html) for analysing the large multi-enzymatic, multi-domain megasynthases (Ansari et al., 2004). The results of these analyses have been organized as four searchable databases for elucidating domain organization and substrate specificity of NRPS and PKS. These databases provide an interface to correlate chemical structures of these natural products with the domains and modules in the corresponding PKS or NRPS. ASMPKS is a web-based tool (http://gate.smallsoft.co.kr:8008/~hstae/asmpks/index.html) for computational analysis of PKS systems against genome sequences (Tae et al., 2007). The ASMPKS can predict functional modules for each protein sequence, estimate the chemical composition of a polyketide synthesized from the modules, and display the carbon chain structure on the web interface. Another recent method to accurately predict PK/NRP structures from genome sequences is described by Minowa and colleagues (2007). Norine (http://bioinfo.lifl.fr/norine) is a platform that includes a database of non-ribosomal peptides (currently more than...
700) together with tools for their analysis. The Norine database stores peptide structures as well as various annotations such as the biological activity, producing organisms, bibliographical references and others (Caboche et al., 2008).

Analysis of over 220 completed bacterial genomes up to 2005 revealed that PKS and NRPS systems are mainly found in actinobacteria, $\beta$-proteobacteria, $\gamma$-proteobacteria, firmicutes and cyanobacteria (Donadio et al., 2007). We have now analysed the 140 most recently sequenced microbial genomes (July 2007–April 2008; GOLD database http://www.genomesonline.org/) using Hidden Markov Model profiles of all core domains of both NRPS and PKS. Many of these genomes are publicly accessible in the NCBI database but have not been described in the scientific literature yet (Siezen and Wilson, 2008). Numerous NRPS/PKS systems were found, and Table 2 lists the genomes with three or more systems; several are described in more detail below. They are mainly found in microorganisms with genomes larger than 4 Mb isolated from soil or aquatic environments. In addition, at least two NRPS or PKS systems are predicted in Yersinia pseudotuberculosis IP 31758, Azorhizobium caulinodans ORS 571, Marinomonas sp. MWY1 and Bacillus cereus cytotoxins NVH 391-98, while at least one system is predicted in Escherichia coli HS, Coxiella burnetii Dugway 7E9-12, Enterobacter sakazakii ATCC BAA-894, Staphylococcus aureus ssp. aureus Mu3, Vibrio harveyi BB120, Serratia proteamaculans 568, Delftia acidovorans SPH-1, Salmonella enterica arizonae sv. 62:z4,z23 RSK2980, Klebsiella pneumonia MGH78578 and Kineococcus radiotolerans SRS30216. Quite a number of the latter bacteria are human pathogens.

**Recently sequenced microbial genomes with large potential for production of NRPS/PKS natural products**

*Sorangium cellulosum* is a soil-dwelling $\delta$-proteobacterium of the group myxobacteria. The genus Sorangium synthesizes approximately half of the secondary metabolites isolated from myxobacteria, including the anticancer metabolite epothilone. Seventeen secondary metabolite loci are encoded in the genome of strain So ce56 (Schneiker et al., 2007), mostly PKS and NRPS systems (Table 2). Known products are chivosazol, etnangien and myxochelin, while others are still unknown. Metabolites secreted by *S. cellulosum* known as epothilones have been noted to have antineoplastic activity. This has led to the development of analogues that mimic its activity. One such analogue, known as ixabepilone, is a US Food and Drug Administration (FDA)-
approved chemotherapy agent for the treatment of metastatic breast cancer.

The soil actinomycete *Streptomyces griseus* produces the well-known antituberculosis agent streptomycin. Recent sequencing of the genome of *S. griseus* IFO 13350 shows that it has 34 gene clusters or genes for biosynthesis of secondary metabolites, of which 14 PKS or NRPS gene clusters seem to be specific for this species (Ohnishi et al., 2008). These clusters presumably direct the synthesis of various as yet unknown secondary metabolites.

Actinomycetes of the marine-dwelling genus *Salinispora* are a rich source of drug-like molecules. *Salinispora* strains are commonly isolated from tropical marine sediment, and many isolates produce compounds that inhibit cancer cells, such as salinosporamide A (Feling et al., 2003). The *Salinispora tropica* CNB-440 genome dedicates nearly 10% of its genome to natural product assembly (Udwary et al., 2007), which is greater than *S. coelicolor* and *S. avermitilis* as well as other secondary metabolite-producing actinomycetes. The *S. tropica* genome features PKS systems of every known formally classified family, NRPS systems and several hybrid clusters. The majority of the 17 biosynthetic loci are novel. Genomic sequencing is ongoing of *Salinispora arenicola* CNS-205, a producer of the bioactive compounds staurospong and rifamycin which may be useful in the treatment of cancer. Other marine actinobacteria are also potential sources of bioactive natural products (Bull and Stach, 2007).

*Frankia* species form a separate lineage among the high % G+C Gram-positive *Actinobacteria*. They are filamentous ‘euactinomycetes’ that grow by hyphal branching and tip extension and thus resemble the antibiotic-producing *Streptomyces* species. *Frankia* species form a symbiotic nitrogen-fixing association with a number of plants. These symbioses add a large proportion of new nitrogen to several ecosystems. The genome of *Frankia* sp. strain EAN1pec has all housekeeping genes necessary for saprophytic existence plus genes for sporulation, vesicle development, symbiosis, N2 fixation and secondary metabolite production. Ten putative NRPS/PKS

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**Table 2.** Recently sequenced bacterial genomes (1 July 2007 to April 2008) with at least three predicted NRPS/PKS gene clusters.

| Species                  | Habitat                               | Genome size (Mb) | Gene clusters (predicted) | Reference and/or NCBI code |
|--------------------------|---------------------------------------|------------------|---------------------------|----------------------------|
| *Sorangium cellulosum* So ce56 | Soil                                  | 13.0             | 3 NRPS 6 PKS 4 NRPS/PKS   | Schneiker et al. (2007) NC_010162 |
| *Salinispora tropica* CNB-440   | Marine, sediment                      | 5.2              | 3 NRPS 6 PKS 4 NRPS/PKS   | Udwary et al. (2007) NC_009380 |
| *Streptomyces griseus* IFO13350 | Soil                                  | 8.5              | 9 NRPS 5 PKS 4 NRPS/PKS   | Ohnishi et al. (2008) NC_010572 |
| *Salinispora arenicola* CNS205   | Marine, sediment                      | 5.8              | 4 NRPS 2 PKS 2 PKS 2 ambiguous PKS | NC_009953 |
| *Frankia sp. EAN1pec*          | Plant symbiont, soil                  | 9.0              | 2 NRPS 3 PKS 5 ambiguous PKS | NC_009921 |
| *Bacillus amyloliquefaciens* FZB42 | Rhizosphere-colorizing, soil         | 3.9              | 4 NRPS 2 PKS 2 PKS 1 ambiguous NRPS | Chen et al. (2007) NC_009725 |
| *Herpetosiphon aurantiacu* ATCC 23779 | Aquatic                              | 6.4              | 5 NRPS 4 NRPS/PKS         | NC_009972 |
| *Pseudomonas aeruginosa* PA7    | Soil, aquatic, host (human)           | 6.6              | 5 NRPS                   | NC_009656 |
| *Xanthobacter autotrophicus* Py2 | Soil, aquatic, sediment              | 4.8              | 2 NRPS 2 ambiguous PKS 1 NRPS/PKS | NC_009720 |
| *Clostridium kluyveri* DSM 555  | Aquatic, mud                          | 4.0              | 1 NRPS 3 NRPS/PKS         | Seedorf et al. (2008) NC_009706 |
| *Bacillus pumilus* SAFR-032     | Soil                                  | 3.7              | 2 NRPS 1 NRPS/PKS         | Gioia et al. (2007) NC_009848 |
| *Citrobacter koseri* ATCC BAA-895 | Soil, aquatic, food, human intestine | 4.7              | 3 NRPS/PKS                | NC_009792 |

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clusters were identified in the genome sequence of strain EAN1pec.

*Bacillus amyloliquefaciens* is a Gram-positive bacterium belonging to the firmicutes. It is member of a group of free-living soil bacteria known to promote plant growth and suppress plant pathogenic bacteria and fungi. The *B. amyloliquefaciens* FZB42 genome reveals an unexpected potential to produce secondary metabolites, with more than 8.5% of the genome devoted to synthesizing antibiotics and siderophores by NRPS and PKS pathways (Chen *et al.*, 2007). Besides five gene clusters known from *Bacillus subtilis* to mediate biosynthesis of secondary metabolites (surfactin, fengycin, bacillibactin, bacilysin, bacillaene), an additional four giant gene clusters were identified for biosynthesis of bacillomycin D, macroysin, bacillaene), an additional four giant gene clusters predicted to encode nine NRPS/PKS systems of unknown function. A novel ‘genomisotopic’ approach uses a combination of genomic sequence analysis and isotope-guided fractionation (Zazopoulos *et al.*, 2003; McAlpine *et al.*, 2005). A novel ‘genomisotopic’ approach uses a combination of genomic sequence analysis and isotope-guided fractionation to identify unknown compounds synthesized by NRPS gene clusters (Gross *et al.*, 2007). A phage-display method was developed for high-throughput mining of gene clusters encoding PKS and NRPS systems, which can be applied to genomes of unknown sequence and metagenomes (Yin *et al.*, 2007), providing opportunities for exploiting the potentially rich source of natural products from unculturable microbes.

**High-throughput experimental screening for NRPS/PKS gene clusters**

The newly discovered gene clusters for NRP and PK synthesis represent a tremendous source of novel bioactive compounds, but in most cases the natural product is unknown. Classical methods to characterize the products include heterologous expression of gene clusters (Wenzel and Muller, 2005), metabolic profiling and assay-guided fractionation (Zazopoulos *et al.*, 2003; McAlpine *et al.*, 2005). The impact of systems biology to control and regulate secondary metabolite production has only recently been addressed (Rokem *et al.*, 2007). The ever-increasing pace of microbial genome sequencing is revealing a plethora of new NRPS/PKS gene clusters, mostly of unknown function. A major challenge for the next decade is to back this up with characterization of the chemical structures and biological activities of these secondary metabolites, so that we can chart Nature’s unique repertoire of natural products and exploit them for the directed synthesis of novel molecules of biotechnological, agricultural and pharmaceutical utility.

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