Targeted screening for microbial bioactivity

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Abstract

High throughput screening technology has allowed significant advances to be made in the discovery of lead agents for use in the pharmaceutical and agrichemical industries. However, economic and practical constraints have limited the use of this technology in the identification of bioactive microbes targeted at crop pests and diseases. Smaller scale targeted screening programmes have generally provided greater success in identifying microbial bioactivity. This paper describes a strategy for targeted selection of bioactive *Trichoderma* spp. Isolates are selected for biological characteristics that best match the biocontrol blueprint developed for the target pathogen and are then put through a series of standardised bioassays. This strategy provides a rapid and cost-effective means of identifying biocontrol agents with commercial potential.

Keywords: bioactive microbes, biocontrol blueprint, high throughput screening, targeted screens, *Trichoderma*.

Introduction

When developing a drug, pesticide or biological control agent, a diversity of synthetic chemicals, natural products and microbes are filtered through screening programmes to identify a lead agent that will potentially control the disease of interest. Such screening programmes are usually initiated by selecting a target and then developing an assay to screen for activity against the target. Subsequently, screening takes place to identify lead agents and selected leads undergo further assessments, such as optimisation and toxicology trials (Campbell 1989; Carnero 2006; Mishra et al. 2008). Depending on the use of the product, preclinical and clinical trials or greenhouse and field trials take place with the ultimate goal of commercialisation. The basic framework of a screening programme may be applied to various research fields, but the scale and methods can vary depending on the screening source, target of interest and available resources (Ward & Bernasconi 1999).

High throughput screening

Screening of synthetic chemicals to identify active compounds for pharmaceutical and agrichemical development has been conducted by high throughput screening (HTS) (Janzen 1996; Ridley et al. 1998; Pereira & Williams 2007). High throughput screening is the random screening of large numbers of compounds to identify leads that express activity against a selected target (Janzen 1996; Harvey 2002; Mishra et al. 2008). Prior to the invention of HTS, traditional drug development

programmes screened 20 to 50 compounds a week, but the introduction of HTS allowed screening as many as 100,000 samples a week (Pereira & Williams 2007). Advances were made by using robotics to decrease labour, replacing test tubes with 96-well microtitre plates to miniaturise assays, and computerising data analysis to increase speed (Carnero 2006; Pereira & Williams 2007). High throughput screening capability has also improved by identifying molecular targets through genomics research and enriching screening collections through the adoption of combinatorial chemistry (Hertzberg & Pope 2000; Koehn & Carter 2005; Carnero 2006). For example, in the pharmaceutical industry, the availability of the human genome sequence and information on biochemical pathways of human diseases allow screening to be focussed by targeting molecular structures (Ridley et al. 1998; Steinrucken & Hermann 2000). In contrast, in the agrichemical industry, which has a global market of one tenth of pharmaceuticals (Ward & Bernasconi 1999), the diversity of hosts and limited molecular information on pests and diseases often necessitates the targeting of whole organisms thereby diminishing the effective use of HTS technologies (Ridley et al. 1998; Steinrucken & Hermann 2000; Tietjen et al. 2005). Despite the advances in technologies that allow screening of many compounds over a short period of time, HTS has a high attrition rate (Hertzberg & Pope 2000; Carnero 2006). It is estimated that one drug is produced for every 1,000,000 compounds whereas one agrichemical is produced for every 140,000 that are screened (McDougall 2003; Carnero 2006). An advantage in agrichemical development is the feasibility to test leads directly on plants at an early stage in comparison to clinical tests with pharmaceuticals (Ward & Bernasconi 1999; Tietjen et al. 2005).

Natural products

Natural products are another major source of active ingredient used in the pharmaceutical and agrichemical industries that can be found by HTS (Harvey 2002). The discovery of penicillin from the fungus *Penicillium notatum* is a well-known example (Demain 2006). Natural products are considered a valuable screening source as they provide greater structural diversity than synthetic chemicals and contain compounds that have been selected by evolutionary pressures (Knight et al. 1997; Harvey 2002; Koehn & Carter 2005; Harvey 2007). However, in contrast to synthetic chemicals that can be produced in the laboratory, sources for natural product screening require sampling from the environment (Harvey 2002; Verkman 2004). A diversity of environments can be sampled, including the air, water sources, soil, plants and microbial culture collections (R. Hill, personal communication). In particular, the unexplored biodiversity in marine environments is increasingly searched for novel products (Harvey 2007; Lam 2007). From the collected samples, crude extracts that contain mixtures of compounds are derived for HTS (Koehn & Carter 2005; Harvey 2007). To follow up on a positive result, purification of the active compound from the crude extract is necessary (Koehn & Carter 2005; Harvey 2007).

The success rate of natural products over synthetic chemicals is unclear, but some reports suggest a higher success rate with natural products (Harvey 2002; Berdy 2005). At Sphinx Pharmaceuticals Corp., both natural products and combinatorial

chemistry compounds are screened, and an average screen tests 125,000 compounds over 4 months (Janzen 1996). At Biodiscovery New Zealand Ltd., more than 10,000 samples are screened every year with an average of one sample out of every 10,000 leading to commercialisation (R. Hill, personal communication). Natural products can also be useful as templates to synthesise chemicals with similar structures (Harvey 2007). An example is the development of azoxystrobin fungicides from strobilurin, a fungicidal compound found in a few Basidiomycete fungi (Knight et al. 1997). Despite the fact that half of successful pharmaceuticals are derived from or inspired by natural products (Demain 2006), screening of natural products has decreased in the last 15 years (Harvey 2002; Koehn & Carter 2005; Harvey 2007; Mishra et al. 2008). Reasons include the incompatibilities with highly automated technologies developed for screening synthetic chemicals (Rollinger et al. 2008), difficulties in accessing biological sources and complications with handling mixtures of compounds (Harvey 2002; Koehn & Carter 2005; Mishra et al. 2008). However, more recently, the importance of natural products is being revisited as they prove to be a valuable source in pharmaceutical and agrichemical development (Knight et al. 1997; Harvey 2002; Koehn & Carter 2005; Harvey 2007; Mishra et al. 2008).

Microbial bioactivity

In contrast to HTS of synthetic chemicals and natural products, smaller-scale targeted screening programmes are used to identify microbial bioactivity. Examples of such microbes include probiotics that improve human and animal health as well as biological control agents (BCAs) that suppress agricultural diseases (Campbell 1989; Fuller 1989). Prior to screening, isolation of source microbes is necessary, similar to that required for sampling of natural products (Campbell 1989; Montesinos 2003). However, unlike the diverse sampling of natural products, isolation of source microbes is usually more focused on ecological niches that demonstrate evidence of beneficial bioactivity associated with the host of interest (Campbell 1989; Montesinos 2003). For instance, common sources to screen for human probiotics come from human intestines and likewise, common sources to screen for BCAs for postharvest fruit diseases come from fruit (O'Sullivan 2001; Janisiewicz & Korsten 2002; Fravel 2005; Taqarort et al. 2008; Vinderola et al. 2008). As microbial bioactivity can be employed through multiple modes of action (Campbell 1989; Howell 2003; Roberts & Lohrke 2003; Kesarcodi-Watson et al. 2008), the target for microbial screening is usually not molecular but the whole disease-causing organism itself. Initial screening commonly consists of *in vitro* laboratory assays examining growth inhibition on agar plates (Campbell 1989, 1994; Fravel 2005; Kesarcodi-Watson et al. 2008). Such tests are easy to conduct and clear to read, but do not always reflect *in vivo* performance (Campbell 1989, 1994; Fravel 2005; Kesarcodi-Watson et al. 2008). Plate tests select for microbes with particular modes of actions, such as the production of inhibitory substances or lytic activity on a specific medium; therefore, microbes with modes of action that are not expressed on plate tests, such as the ability to induce host resistance or production of enzymes, may be overlooked (Campbell 1989; Kesarcodi-Watson et al. 2008). Thus, although time-consuming and costly, *in vivo* screening with the host plant or animal is essential (Campbell 1989, 1994; Kesarcodi-Watson et al.

2008). The scale of microbial screening is smaller than synthetic chemical or natural product screening (Campbell 1994), ranging from approximately 50 isolates to a few hundred isolates (Clarkson et al. 2002; Idris et al. 2007; Taqarort et al. 2008; Vinderola et al. 2008; Missotten et al. 2009). For BCA development, it is estimated that approximately 1% of the original microbe source is taken to field trials (Campbell 1989; Montesinos 2003). In comparison to the high attrition rate of HTS and high costs in pharmaceutical industries where developing a drug can cost US $802 million (DiMasi et al. 2003), attrition rates and costs for BCA development are lower (Andrews 1992; Campbell 1994). This allows smaller research groups to pursue BCA development as a niche area of research without having to make large investments into HTS technologies.

Trichoderma case study

As mentioned previously, one of the main strategies for selection of microbial BCAs has been to isolate strains from the same ecological niche as the pest/pathogen that is being targeted for control (Cook & Baker 1983). This was the strategy used by the Plant Disease Biocontrol Research Group at Lincoln University during the early stages of development of its biocontrol research programmes. For example, survival structures were used to bait microbes from soil that could parasitise selected pathogens such as *Sclerotinia* (Jones & Stewart 2000), and microbes were isolated from the phylloplane of grape leaves and flowers to test for their activity against *Botrytis cinerea* grey mould (Walter et al. 2006). Surprisingly, what was observed from numerous isolations of this nature was that *Trichoderma* spp could be routinely isolated from all of the different ecological niches (roots, soil, pathogen survival structures, leaves, fruit, internal plant tissues). This was expected for soil-based niches but not for above-ground niches since *Trichoderma* is a recognised soil-borne saprophyte. *Trichoderma* spp. have been isolated from a diverse range of plant species (monocots and dicots), from wide geographical sources and across a broad environmental range (Klein & Eveleigh 1998). From the literature there are 104 known species, many of which include reported biocontrol agents, of which several have been developed as commercial products (Stewart 2001). An important attribute that favours the use of *Trichoderma* species for biocontrol is their multiple modes of action with isolates reported to act via mycoparasitism, antibiosis, competition, induced resistance and plant growth promotion (Harman 2006; Vinale et al. 2008). This has led to our view that the *Trichoderma* genus possesses isolates with such broad ranging biological attributes that there is a high likelihood of finding one or more isolates active against all of the key target plant pathogens of interest. This is further supported by repeated observations showing that isolates of *Trichoderma* obtained from the soil can perform equally well as biocontrol agents on foliage/flowers and other above-ground environments.

On the basis of these observations, a decision was made to focus the group's biocontrol research primarily on the selection of bioactive *Trichoderma* spp. A large-scale isolation programme was established in 2003 to collect *Trichoderma* isolates from different regions, soil types and cropping systems from throughout NZ. The Trichobank™ culture collection currently houses >1000 isolates representing

21 species and provides the underlying microbial resource for the research group's screening programmes. Over the past decade, the group has progressively characterised the isolates held in the culture collection based upon growth characteristics (temp, pH range) and biological characteristics (competition, antibiosis, parasitism, rhizosphere competence, induced resistance capability). This is an on-going process with the ultimate goal of creating a database of fully characterised isolates that can be searched for targeted bioactivity.

Biocontrol blueprint

When a biocontrol programme commences at the Bio-Protection Research Centre, a set of draft technical specifications for the potential BCA, a blueprint, is developed based upon knowledge of the pathogen's lifecycle, environmental niche, optimum mode/s of action and likely formulation/application strategy. The blueprint is then compared with information known on the isolates held in the Trichobank™ collection and the best 20-30 matches identified. These 'best bet' test microbes are then put through a series of standardised bioassays developed by the research group to further identify bioactivity. Tests include a necrotic leaf disc assay to identify competitive saprophytic behaviour, a dual culture assay to identify antibiotic production by the test microbe, a plant growth promotion assay, a mycoparasitism assay and a spore production assay. Threshold levels of activity are set for each of these assays and the comparative performance of test microbes assessed. The 10 best-performing isolates are then selected for further testing in glasshouse/growth room pathogen challenge trials. These trials are repeated several times under variable pathogen challenge and/or BCA inoculum density, different environmental conditions and with different basic BCA formulations.

At this stage in the biocontrol programme, a commercial business partner is identified and technical specifications for a prototype product are developed that set thresholds for field performance, compatibility with current crop management practices, spore production capability and quality assurance parameters, e.g. viability, shelf life. The best performing isolates (3-5) from the glasshouse trials undergo intensive field testing for 2 years and then the top candidate is selected for commercial development.

Biocontrol successes

This targeted screening strategy was used successfully by the research group to develop a biocontrol agent for Sclerotinia disease of lettuce. Fifteen isolates were selected from the Trichobank™ collection based upon relevant biological attributes and tested in laboratory and glasshouse trials over a 3-year period (Rabeendran et al. 2006). Three isolates were selected for field testing in collaboration with our business partners, Agrimm Technologies Ltd, and one isolate *Trichoderma hamatum* 6SR4 was selected for commercial development. This isolate was shown to be highly competitive in the soil environment and was an effective mycelial and sclerotial parasite of *Sclerotinia minor*. When applied as a solid substrate (flake formulation) into the nursery potting mix at the rate of 2 kg/m^3 followed by a seedling drench prior to transplanting at the rate of 100 g/50 litres, the biocontrol

agent gave 50-75% control of Sclerotinia disease, which was equal to or better than the standard fungicide treatment (Rabeendran et al. 2006). Two products Lettucemate™ Flake and Lettucemate™ WP were commercialised in 2005 for use in lettuce nurseries in NZ and Australia (www.vinevax.com).

A similar strategy was used to develop *Trichoderma atroviride* LU132 as a botryticide on grapes. This isolate had been initially developed as a biocontrol agent for use against onion white rot disease. The biological attributes of the biocontrol agent identified in this system (nutrient competition, localised antibiosis (Stewart & McLean 2007)) were recognised as having value for application in other systems, in particular grapevines where the environmental conditions (pH, temperature) on the leaf surface were a good match for this isolate. Intensive field testing of the isolate validated this proposition and the product Sentinel® was commercialised in 2006 for use against *Botrytis* on grapevines in NZ and an extension of the application to include tomatoes was approved in 2007 (www.vinevax.com). Current programmes that are utilising this targeted selection and screening strategy include Rhizoctonia diseases of potato and Sclerotinia disease of brassicas.

Conclusion

It is impractical and uneconomic for small biocontrol research groups to be able to implement intensive high throughput screening systems to identify microbes with specified bioactivity. The use of a targeted approach to selection and screening allows researchers to effectively rationalise the use of limited resources and make rapid progress in the identification of microbes with commercial potential. This has reduced the average length of time taken from initial screening to identification of the prototype product from 8-10 years down to 5-6 years, thereby allowing greater investment of time and money in the development of improved formulations and extensive field testing.

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