Progress needed to characterise the active agent in biologicals

by Dr Oni Oluwatobi

In recent decades, biopesticides have attracted significant attention in pest management, evidenced by valuations of the biopesticide market close to $3 billion in 2016 and for some $6.6 billion by 2022. The increasing global popularity of biopesticides in crop protection compared with their synthetic counterparts can partly be put down to their lower development cost, suitability for use in sustainable agriculture and as an additional tool to manage resistance in integrated pest management schemes.

The US and Canada has made the registration of biopesticide products more efficient through the development of modified test methodologies and clear guidance documents. However, in the EU, biopesticides are still registered under the same regulatory framework as chemical pesticides. The EU's registration process is complex with limited guidance on key areas of biopesticide risk assessment. The EU is encouraging the registration of more plant protection products of biological origin through "low-risk" categories. However, challenges still lie ahead in the registration processes that hinder safe and successful commercialisation of novel biopesticides.

There are three generic categories of biopesticides in the EU: (i) microbial pesticides – pesticides with micro-organisms (bacteria, virus or fungi) as the active ingredient; (ii) biochemicals – such as secondary metabolites produced by plants to deter insects from feeding on them; (iii) semiochemicals - chemical signals produced by one organism to cause a change in the behaviour of another organism of the same or different species. Most biopesticides on the market are microbial pesticides. Bacteria-based pesticides (mainly *Bacillus thuringiensis*-based products) are the most common form of microbial pesticides. Bacteria (especially *Bacillus* strains) are relatively easier and cheaper to produce through fermentation in comparison to fungal biological control agents, thus attracting commercial development of bacterial strains for use in biological control[1].

To ensure the safe use of bacterial strains, it is necessary to conduct a thorough assessment of hazard and risk to humans and the environment. Similar to synthetic chemical pesticides, the first and perhaps most vital step in the risk assessment of a bacterium are its taxonomic identification and characterisation. The EU, according to Regulation 283/2013 (part B) requires that each microbial active substance be

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identified, characterised and named at the strain level. Identification and characterisation of bacterial species with potential for biological control at strain level is necessary to distinguish them from closely-related pathogenic variants. Unlike synthetic chemical pesticides, there is limited guidance in the EU on the characterisation of micro-organisms. The only guidance document recently released, caters to the characterisation of micro-organisms used as feed additives[2]. However, none so far have been adapted for the same purpose with micro-organisms used as active ingredients in plant protection products.

Citing literature reports on bacterial strains similar to the biocontrol agent is a common approach but may be insufficient and misleading in drawing physiological and pathogenic comparisons. This is because a few base pair differences in the genetic sequences of diagnostic marker genes from micro-organisms of the same species or strains may be enough to result in pronounced differences in physiological (for example, metabolite profiles) or pathogenicity traits. Besides, reports describing and characterising the reference strain may be inaccurate as the strains may have been falsely assigned to certain taxonomic groups. This is particularly common in bacterial groups with a tight assemblage of closely-related species or strains, for example, the *B subtilis* complex, *B cereus* group or *Burkholderia cepacia* complex that harbours strains with significant potential for use as biological control agents. Clearly, better methods of characterisation or ways of interpreting data from existing methods are required. Currently, significant progress is being made through advanced gene technologies and a substantial foundation of prior knowledge to help designate novel members of these bacterial strains to the correct taxonomic groups and clearly distinguish them from very closely-related pathogenic strains. However, unequivocal strain level identification and characterisation of bacteria is still a challenging process. This is particularly problematic for novel strains from taxonomic groups about

which not much is known about cultured reference strains.

A handful of genetic methods for strain typing currently exist and have been widely applied by industries and contract research laboratories to identify and characterise novel bacterial strains for use in biological control. Examples of available methods include:

- *Pulsed-field gel electrophoresis (PFGE)*: a fingerprinting technique that involves electrophoretic separation of DNA fragments produced from the use of restriction enzymes to digest DNA. The resultant genetic information in highly definitive patterns can be compared with those of reference strains.

- *Multi-locus sequence typing (MLST)*: one of the most commonly used techniques for strain typing. It characterises microbial species by identifying small variations on fragments (400-500 base pairs) of multiple housekeeping genes.

- *Multi-locus variable number tandem repeats analysis*: a DNA fingerprinting technique which involves amplification and sequencing of multiple regions of the genome where nucleotides are arranged in tandem repeats. The number or length of repeats is variable in different microbial strains, thus forming a pattern characteristic of the analysed strain.

- *Repetitive sequence-based polymerase chain reaction*: (rep-PCR): exploits the variation in the arrangement of repetitive consensus sequences between different bacterial strains. Primers complimentary to the repetitive sequences enable their amplification via PCR. Amplicons of varying sizes are generated and separated by electrophoresis. The resulting fingerprint, specific for each bacterial strain can be compared.

- *PCR-ribotyping*: relies on the polymorphism of the 16S and 23S rRNA genes. It involves amplification and partial sequencing of the 16S and 23S rRNA genes as well as the intergenic space between them. The amplicons generated are digested by restriction enzymes. The resulting DNA fragments are fractionated by electrophoresis and visualised using fluorescent dyes.

The techniques described above are generally based on PCR and restriction digests. They can be prone to bias arising from the choice of primers or restriction enzymes. The methods can also produce varying results for the same strain or bacterial isolate analysed. For example, a limitation of MLST was recently highlighted in a study in the journal, Scientific Reports[3]. MLST failed to fully represent phylogeny from whole genome sequencing for many tested bacterial strains. Such discrepancies in results from different strain typing techniques may complicate taxonomic assignments of bacterial strains. This makes the delineation between pathogenic and biocontrol bacterial strains challenging, requiring expert assessment.

The ideal way to definitively characterise a microbial strain is to sequence the whole genome. The cost of sequencing an entire microbial genome is much cheaper nowadays and has become a more practical option for microbial strain characterisation due to the advent of next-generation sequencing technologies. A bottleneck is still the analysis and interpretation of the huge amount of data generated from such highly sophisticated

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sequencing techniques. With rapid developments in the field of bioinformatics, powerful software is now being developed to accurately annotate genomes within a very short time. Alternatively, using a combination of different strain typing techniques can also serve as a valid way to identify and characterise novel bacterial biological control agents and distinguish them from pathogenic variants with a high degree of certainty. Nevertheless, expert opinion on the choice of methods for unambiguous strain-level characterisation of novel biological control strains will be needed. The biopesticide industry should also expect relevant expertise from competent authorities to agree and interpret the outcomes from suitable technologies for proper strain identification to facilitate the regulatory process and hence promote biopesticides to the European market.

In summary, regulatory guidance has not kept pace with scientific progress in the characterisation of bacterial strains used as biocontrol agents. It is arguable that guidance is voluminously produced in other areas of pesticide regulation in the absence of adequate scientific validation and the limited progress for biocontrol characterisation is hampering the regulatory process and consequently the rate of appearance of new products on to the market.

Sources:

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