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Ministry of Health, Welfare and Sport

Assessing **antibiotic resistance** of microbial plant protection products using whole genome sequencing

RIVM letter report 2023-0150
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Synopsis

Recommendation for evaluating the antimicrobial resistance of microbiological plant protection products

Plant protection products safeguard plants against pathogenic organisms, such as fungi and insects. In recent years, plant protection products that contain micro-organisms have become increasingly common. These products contain viruses and bacteria that kill pathogens in plants, but are not harmful to humans.

Plant protection products undergo extensive testing to ensure that they are safe for humans, animals and the environment. RIVM has now drafted a recommendation for the agencies that evaluate the safety of microbiological plant protection products.

One aspect for consideration when evaluating products that contain bacteria is whether those bacteria are resistant to antibiotics. 'Resistant' means that a bacterium does not respond to antibiotics, making them less effective. In some cases, this resistance can be transferred to other types of bacteria. When it is transferred to bacteria that cause illness in humans, it becomes harder to treat that illness using antibiotics. For this reason, there is a ban on the use in plant protection products of bacteria that are capable of transferring antimicrobial resistance to other kinds of bacteria.

The recommendation describes how extensive DNA analyses (whole genome sequencing) can be used to determine whether bacteria intended for use in plant protection products are resistant to antibiotics. It also sets out how to determine whether this resistance can be transferred to other types of bacteria.

Keywords: microbiological plant protection products, antimicrobial resistance, whole genome sequencing

Publiekssamenvatting

Aanbevelingen voor de beoordeling van antibiotica resistentie van microbiologische gewasbeschermingsmiddelen

Gewasbeschermingsmiddelen beschermen planten tegen organismen waar ze ziek van kunnen worden, zoals schimmels en insecten. De laatste jaren worden steeds meer gewasbeschermingsmiddelen gebruikt waar micro-organismen in zitten. Dit kunnen zowel virussen als bacteriën zijn die ziekmakers van planten doden maar niet schadelijk zijn voor mensen.

Om te zorgen dat gewasbeschermingsmiddelen veilig zijn voor mens, dier en milieu worden ze uitgebreid getest. Het RIVM heeft nu een aanbeveling geschreven voor instanties die de veiligheid van microbiologische gewasbeschermingsmiddelen beoordelen.

In de beoordeling van middelen met bacteriën wordt onder andere onderzocht of deze resistent zijn tegen antibiotica. Resistentie betekent dat een bacterie ongevoelig is voor antibiotica waardoor die minder goed werken. Deze resistentie kan soms worden overgedragen op andere bacteriesoorten. Als dat gebeurt naar bacteriën waar mensen ziek van kunnen worden, kan die ziekte minder goed worden behandeld met antibiotica. Daarom mogen bacteriën die de resistente eigenschap tegen antibiotica kunnen overdragen op andere soorten bacteriën, niet als gewasbeschermingsmiddel worden gebruikt.

De aanbeveling beschrijft hoe met uitgebreide DNA-analyses (Whole Genome Sequencing) kan worden onderzocht of bacteriën die bedoeld zijn om als gewasbeschermingsmiddelen te worden gebruikt, resistent zijn tegen antibiotica. Daarnaast wordt beschreven hoe kan worden onderzocht of die resistentie kan worden overgedragen op andere bacteriesoorten.

Kernwoorden: microbiologische gewasbeschermingsmiddelen, antibiotica resistentie, whole genome sequencing

Contents

Summary

The number of applications for the approval of microbial active substances for plant protection use has been increasing in recent years. Microbial active substances are active substances based on microorganisms, including bacteria, fungi and viruses that are capable of replication or of transferring genetic material. Within the EU these microorganisms are assessed for their safety with regard to humans, animals and the environment in accordance to the legal framework laid down in Regulation (EC) No 1107/2009. One of the elements assessed within this legal framework is the antimicrobial resistance (AMR) potential of microorganisms. For bacteria there is the possibility that AMR genes could be transferred to bacteria which are pathogenic to humans. These human pathogens would then become more difficult to treat with antibiotics. This concern does not relate to other microorganisms such viruses or fungi.

For bacteria, applicants must demonstrate that relevant AMR genes are not present in their genome. Bacteria that contain transferable AMR genes can therefore not be approved for plant protection use under Regulation (EC) 1272/2009. The current report provides guidance to risk assessors on how whole genome sequencing (WGS) data can be used to evaluate the antibiotic resistance of bacteria.

This report provides an indication which AMR genes can be expected in the genera to which the bacteria approved as active substance in the EU belong to. It also provides evaluators with a set of criteria for assessing the quality of the WGS data submitted by applicants. In addition, it provides examples of public databases that can be used to evaluate the WGS data when screening for AMR genes. These databases are selected as they meet minimal quality standards and similar results are expected independent of the database used. When an AMR gene is found in the genome of the bacteria, applicants should investigate whether the gene is located on a mobile genetic element, since these mobile genetic elements could be transferred to human pathogens. This report provides guidance how this evaluation can be done.

It should be noted that there is not just one acceptable approach to evaluate WGS data for antibiotic resistance. This report describes the generally used procedure and tools to evaluate WGS data. The information provided by applicants may include minor deviations from these procedures or use a different tool that may still lead to acceptable results. Evaluators should assess on a case-by-case basis whether these deviations are acceptable.

1 Introduction

The number of applications for the approval of microbial active substances for plant protection use has been increasing in recent years. Microbial active substances are active substances based on microorganisms, including bacteria, fungi and viruses that are capable of replication or of transferring genetic material. These microorganisms are assessed for their safety with regard to humans, animals and the environment in accordance with the legal framework laid down in Regulation (EC) No 1107/2009. One of the elements to be considered in the risk assessment is potential antimicrobial resistance (AMR) of the microorganism. AMR means the intrinsic or acquired ability of a microorganism to multiply in the presence of an antimicrobial agent at concentrations which are relevant for therapeutic measures. The Uniform Principles for plant protection products (Regulation (EU) 546/2011) outline that in case the microorganism is resistant to antimicrobials it should be demonstrated that this resistance or the possible transferability of the AMR genes does not interfere with the effectiveness of antimicrobials used in human and animal health care. When the antimicrobial resistance can be transferred to other microorganisms, including human and animal pathogens, the microorganism cannot not be approved.

The concern for possible transfer of resistance genes relates to bacteria and not to fungi or viruses (EC, 2020):

- Viruses (excluding bacteriophages) have not been reported in the scientific literature as contributors to the AMR concern.
- The acquisition of antimicrobial resistance in fungi is multifactorial. Therefore, transfer of AMR genes between fungi appears to be very rare and this is not associated with specific mechanisms, as described for bacteria (for instance through plasmid exchange).

Therefore, the focus of this report was on bacteria.

A guidance was recently adopted, stating that for bacteria applicants must show that relevant AMR genes are not present in the genome, e.g. by submitting Whole Genome Sequencing (WGS) data, screening for the presence of AMR genes (EC 2020). This requirement is in addition to phenotypic susceptibility testing for antibiotics. How the submitted WGS data must be assessed by authorities has not yet been sufficiently laid down in this guidance and requires further elaboration. The current report provides guidance to risk assessors on the assessment of WGS data on antimicrobial resistance. It should be noted that when it comes to analysing WGS data there is not just one acceptable approach. This report describes the generally used procedure and tools to evaluate WGS data. In the information provided by applicants there might be slight deviations from the procedures described in this report or use a different tool. Evaluators should assess on a case-by-case basis if these deviations are acceptable.

To develop the guidance, first an inventory was made of current protocols that are used in other regulatory domains to assess antimicrobial resistance using WGS data (chapter 2). Second an inventory was made of the AMR genes that can be expected in the genera to which the bacteria approved as active substances in the European Union (EU) belong (chapter 3). In addition, a set of quality criteria was established, which risk assessors can use to evaluate if the submitted WGS data meets minimal requirements (chapter 4.1). Furthermore, the report provides examples of public databases that can be used to evaluate the WGS data when screening for AMR genes and how these results should be evaluated (chapter 4.2 and 4.3). Chapter 4.4 provides an example of these analysis in public databases using the publicly available complete genome of *B. thuringiensis* strain ABTS-351.

2 Inventory of current risk assessment practices in other regulatory domains

An inventory was made of current protocols that are used in other regulatory domains to assess AMR using WGS. For this purpose the following documents and websites were assessed:

- Documents from the Inter-European Union Reference Laboratories (EURLs) Working Group on Next Generation Sequencing (NGS) via the EURL-*Salmonella* website[1]:
  - Bioinformatics tools for basic analysis of Next Generation Sequencing data
  - Guidance document for WGS-laboratory procedures
- European Food Safety Authority (EFSA), 2021. EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain.
- EU Reference Laboratory – Antimicrobial Resistance website on WGS[2]
- GLASS whole-genome sequencing for surveillance of antimicrobial resistance. Geneva: World Health Organization; 2020ISO 23418:2022. Microbiology of the food chain — Whole genome sequencing for typing and genomic characterization of bacteria — General requirements and guidance.

The information provided in these protocols was used as input to prepare the guidance on how to evaluate the WGS data for antimicrobial resistance described in chapter 4 of this report.

[1] https://www.eurlsalmonella.eu/
[2] https://www.eurl-ar.eu/wgs.aspx

3 Inventory of approved bacteria approved in the EU for plant protection use and AMR genes that can be expected in these species

3.1 Approved bacteria

As a first stap to gather information on AMR genes that can be expected an overview was made of the bacteria currently approved or pending approval as active substance in the EU. To this end the full list of active substances reported in the EU pesticide database[3] was screened (accessed on January 11th 2022, approval status updated on February 24 2023).

The screening revealed 31 bacteria, with 24 of them approved and 7 bacteria pending for approval. The most common bacterial genus among them was *Bacillus* (n=26), but the list also includes *Pseudomonas* (n=2), *Streptomyces* (n=2) and *Pasteuria* (n=1). Table 1 shows detailed information of the 31 bacteria. The genera to which these bacteria belong were later screened to determine the potential AMR genes.

Table 1 Overview of bacteria approved in the EU or pending approval .

Substance	**Status[1]**
Bacillus amyloliquefaciens (formerly *subtilis*) str. QST 713	Approved
Bacillus amyloliquefaciens AH2	Approved
Bacillus amyloliquefaciens MBI 600	Approved
Bacillus amyloliquefaciens strain FZB24	Approved
Bacillus amyloliquefaciens subsp. *plantarum* D747	Approved
Bacillus amyloliquefaciens IT-45	Approved
Bacillus firmus I-1582	Approved
Bacillus pumilus QST 2808	Approved
Bacillus subtilis strain IAB/BS03	Approved
Bacillus thuringiensis subsp. *aizawai* strain ABTS-1857	Approved
Bacillus thuringiensis subsp. *aizawai* strain GC-91	Approved
Bacillus thuringiensis subsp. *aizawai* strains ABTS-1857, GC-91	Approved
Bacillus thuringiensis subsp. *israeliensis* strain AM65-52	Approved
Bacillus thuringiensis subsp. *kurstaki* strain ABTS 351	Approved
Bacillus thuringiensis subsp. *kurstaki* strain EG 2348	Approved
Bacillus thuringiensis subsp. *kurstaki* strain PB 54	Approved
Bacillus thuringiensis subsp. *kurstaki* strain SA 11	Approved
Bacillus thuringiensis subsp. *kurstaki* strain SA 12	Approved
Bacillus thuringiensis subsp. *kurstaki* strains ABTS 351, PB 54, SA 11, SA12 and EG 2348	Approved
Pasteuria nishizawae Pn1	Approved
Pseudomonas chlororaphis strain MA342	Approved
Pseudomonas sp. strain DSMZ 13134	Approved
Streptomyces strain K61 (formerly *S. griseoviridis*)	Approved
Streptomyces lydicus WYEC 108	Approved
Bacillus amyloliquefaciens AT-332	Pending

[3] https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances

Substance	Status[1]
Bacillus amyloliquefaciens FZB42	Pending
Bacillus licheniformis strain FMCH001	Pending
Bacillus nakamurai F727	Pending
Bacillus subtilis strain FMCH002	Pending
Bacillus subtilis strain RTI477	Pending
Bacillus velezensis strain RTI301	Pending

Note: [1] under Regulation (EC) No 1107/2009.

3.2 Antibiotic classes and number of AMR gene types

Antimicrobial agents, for which AMR genes encode resistance, were grouped in various different antibiotics classes (see Table 2). Each antibiotic class contains multiple types of AMR genes that encode resistance against the respective antibiotic class. Moreover, one type of AMR gene can comprise of one to hundreds of allelic variants. Table 2 shows the approximate number of known acquired AMR gene families among the various antibiotics classes. Acquired AMR genes are commonly located on mobile genetic elements, like for example plasmids and transposons. These genes consequently pose the highest risk for transfer to other microorganisms and thus pose a risk for public health in general.

Table 2 Acquired antimicrobial resistance (AMR) gene types and allelic variant numbers among the different antibiotics classes.

Antibiotic class	Number of AMR gene types[1]	Number of allelic variants
Aminoglycoside	>200	>650
β-Lactam	>200	>3850
Colistin	10	>75
Fosfomycin	>25	>55
Fusidic Acid	5	7
Glycopeptide	>20	>200
MLSKO	>100	>250
Nitroimidazole	9	9
Phenicol	>15	>125
Quinolone	12	>175
Rifampicin	11	19
Sulphonamide	4	>35
Tetracycline	>70	>225
Trimethoprim	10	>125

Note: [1] The number of AMR gene types in some antibiotic classes increases constantly, that is why a 'larger than (>)' symbol is used.

3.3 AMR genes among bacterial genera approved at EU level

Online DNA databases (e.g. https://www.patricbrc.org/, https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/) as well as some public literature (e.g. https://faculty.washington.edu/marilynr/) were specifically investigated for possible AMR genes among the genera to which the above mentioned approved bacteria below to. The search focussed on acquired AMR. The screening was not intended to obtain a complete overview, but to provide some examples of the possible AMR genes within the antibiotic classes present among the bacteria approved for plant protection products (Table 3).

Table 3 Possible acquired AMR genes among the bacterial genera approved for plant protection products

Antibiotic class	Genera/species[1]	Gene
Aminoglycoside	*Bacillus* spp.	*aadK*, *ant*(4')-Ib
	Pseudomonas spp.	*aac*(3)-Ia, *aac(3)-Id*, *aac(3)-IIIc*, *aac(6')-Ib*, *aac(6')-IIc*, *aac(6')-29*, *aac(6')-31*, *aacA4*, *aacC1*, *aadA6*, *aadA11*, *aadA13*, *aadA15*, *aadB*, *ant(2'')-Ia*, *ant(4')-IIb*, *aph(3'')-Ib*, *aph(3')-II*, *aph(3')-IIb*, *aph(3')-VI*, *aph(6)-Id*, *neo*
	Streptomyces spp.	*aac(3)-VIIa*, *aac(3)-VIII, aac(3)-X*, *aac(6')-Ib*, *aacA4*, *aph(3')-Ia*, *aph(3')-V*, *aph(3')-VIII*, *aph(6)-Ia*, *aph(6)-Ib*, *aph(7'')-Ia*, *aphD*, *aphE* *hygR*, *neo*
β-Lactam	*Bacillus* spp.	*bcI*, *bcII*, *bla1*, *bla2*, *blaI*, *blm*, *penP*
	Pseudomonas spp.	bla_{IMP-12}, bla_{VIM-6}
Fosfomycin	*Bacillus* spp.	*fosB*
	Pseudomonas spp.	*fosB*, *fosE*
	Streptomyces spp.	*fosB*
Fusidic Acid	*Streptomyces* spp.	*fusH*
Glycopeptide	*Bacillus* spp.	*vanZ*
	Pseudomonas spp.	*ble*
	Streptomyces spp.	*vanJ*
MLSKO	*Bacillus* spp.	*cfr*, *clbA*, *clbC*, *erm*(A), *erm*(C), *erm*(D), *erm*(G), *erm*(34), *lnu*(A), *lnu*(B), *lnu*(D), *lsa*(B), *mph*(B), *mph*(K), *mph*(L), *mph*(M), *vat*, *vgaA*, *vgb*(A), *vgb*(B), *vlmR*
	Pasteuria spp.	*erm*(42)
	Pseudomonas spp.	*ere*(A), *ere*(B), *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(V), *erm*(X), *lnu*(A), *msr*(A), *msr*(D), *msr*(E), *mef*(A), *mph*(A), *mph*(B), *mph*(D), *mph*(E), *mph*(F)
	Streptomyces spp.	*car*(A), *erm*(E), *erm*(H), *erm*(I), *erm*(N), *erm*(O), *erm*(S), *erm*(U), *erm*(V), *erm*(Z), *erm*(30), *erm*(31), *erm*(32), *lmr*(A), *lmr*(C), *lnu*(A), *msr*(A), *ole*(B), *ole*(C), *srm*(B), *tlr*(C), *varM*
Nitroimidazole	*Pseudomonas* spp.	*nimB*, *nimT*
Phenicol	*Bacillus* spp.	*catA1*, *catA4*, *catA6*, *catA7*, *catA9*, *catA15*, *catB*, *catQ*
	Pseudomonas spp.	*catA1*, *catA4*, *catB*
	Streptomyces spp.	*catA1*, *catA4*, *catA5*, *catB*, *cmlA*, *cmlR*, *cmlV*
Quinolone	*Bacillus* spp.	*qnrB10*
	Pseudomonas spp.	*qnrB1*, *qnrB10*
	Streptomyces spp.	*qnrB10*
Tetracycline	*Bacillus* spp.	*otr*(A), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(O), *tet*(Q), *tet*(T), *tet*(W), *tet*(39), *tet*(42), *tet*(45)
	Pasteuria spp.	*tet*(H),
	Pseudomonas spp.	*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tet*(M), *tet*(O),

Antibiotic class	Genera/species[1]	Gene
		tet(T), *tet*(W), *tet*(X), *tet*(Y), *tet*(34), *tet*(35), *tet*(38), *tet*(39), *tet*(42)
	Streptomyces spp.	*otr*(A), *otr*(B), *otr*(C), *tet*(K), *tet*(L), *tet*(M), *tet*(W), *tet*(X), *tet*(33), *tcr3*
Trimethoprim	*Bacillus* spp.	*dfrA1*, *dfrA2*, *dfrA3*, *dfrC*, *dfrD*, *dfrE*, *dfrG*, *dfrK*, *folA*
	Pseudomonas spp.	*dfrA1*, *dfrA14*, *dfrA15*, *dfrA21*, *dfrA22*, *dfrA27*, *dfrA47*, *dfrA5*, *dfrA7*, *dfrB1*, *dfrB3*, *dfrB6*, *folA*
	Streptomyces spp.	*folA*

Note: [1] *P. aeruginosa* was excluded from the screening. As a human pathogen it is often linked to AMR, which might cloud the list of (acquired) AMR genes for the other *Pseudomonas* species.

4 Guidance on how to evaluate submitted WGS data on antimicrobial resistance

The following chapter provides guidance on how to evaluate submitted WGS data on antimicrobial resistance. Please note that chapter 7 provides some additional explanation on the terms and definitions used in this chapter.

4.1 Criteria for assessing the quality of the submitted WGS data

Wet lab procedures
The first step in the evaluation is to assess the quality of the WGS data submitted by applicants. The following topics regarding the wet lab procedures of the WGS data should be reported by the applicant and checked by the evaluator.

- Description that the bacterial strain was cultivated before DNA extraction as a pure culture to minimize the risk of contamination, which might hamper downstream analysis.
- Description that the bacterial strain as well as the extracted genomic DNA (chromosome and possible plasmid(s)) was handled in a way that minimizes the risk of sample degradation, misidentification, and cross contamination.
- Description of the sequencing strategy; i.e. short-read and/or long-read sequencing.
- Description of the library construction method, including DNA fragmentation method and selection of fragments. The manufacturer's protocol of these methods is recommended. Procedures may be adapted for specific requirements, but modifications have to be documented.
- Description of the type of sequencing instrument; e.g. Illumina platforms (Illumina), Ion Torrent system (ThermoFischer Scientific), MinION based (Oxford Nanopore Technologies), PacBio system (PacBio).
- Description of the sequence depth chosen (in DNA sequencing this is the number of unique reads that includes a given nucleotide in the reconstructed sequence).

Dry lab procedures
It should be checked whether the following topics regarding the dry lab procedures of the WGS data are reported by the applicant.

- Description of a quality check method used (e.g. fastQC) and the result of it (e.g. total number of reads, coverage, average read length, average GC%, average read Phred score).
    - The coverage depends on the application and sequenced bacterial species but should be ≥20X.
    - Average read lengths (which can be deduced for example from fastQC output files) should be in the range of what is expected from the selected library. E.g. if a 2x150 bp paired-end library was used, an average read length of 150 is expected.
    - Average GC% should be in the range of what is expected for the bacterial species. For example *B. thuringiensis* subsp.

aizawai strain ABTS-351: 34.9%, *B. thuringiensis* subsp. *kurstaki* strain SA-11: 35.0%, *Streptomyces* strain K61: 72.4%, *S. lydicus* WYEC 108: 70.8%.
    - Average read Phred score values ≥30 are considered acceptable.
- Description of the trimming protocol (e.g. Trimmomatic) to remove adaptors and low quality sequences.
- Description of the procedure to determine the potential contamination level.
    - The contamination level should be less than 5%.

It is possible to map sequence reads directly to the gene targets using a short reads aligner like KMA (k-mer alignment) (Clausen et al., 2018) or in software such as Ridom SeqSphere+ (Ridom GmbH, Germany). However, the most suitable method to screen for AMR genes would be via *de novo* genome assembly, because this reconstruction of the bacterial genome also allows to check whether the AMR determinant is located on the chromosome or on a mobile genetic element like a plasmid. Looking at the flanking regions of the AMR determinant will help determining its location.

Several published assembly programs suitable for bacterial genomes are freely available. Some popular assemblers for Illumina data of bacterial organisms are SKESA (Souvorov et al., 2018[4]), SPAdes (Bankevich et al., 2012[5]) and Velvet (Zerbino and Birney, 2008[6]). The SPAdes genome assembler is the most frequently used nowadays.

Prior to starting downstream analyses, the quality of the generated assembly should be assessed. The following data should be reported by the applicant as general indicators of assembly quality:

- The read depth. (this needs to be sufficient to ensure variants are reliably detected in the assembly)
  Preferably a value between 30-100 is achieved.
- The number of contigs (low coverage and/or small contigs can be removed prior to reporting).
  Preferably a value lower than 300 is achieved when only contigs > 500 nucleotides are included.
- N50 (after sorting the contigs from large to small, determine the cumulative sum of the lengths of the contigs until >50% of the assembly genome size is reached, the length of that contig is N50)
  Preferably a N50 of >2% of the assembled genome length is obtained.
- The length of the longest contig.
- The total length of all contigs included should approximate the known genome size of the target organism.

4.2 Tools and public databases to be used to evaluate the data

Table 4 provides an overview of a number of available tools to screen for the presence of acquired AMR genes. All described tools are acceptable

[4] https://github.com/ncbi/SKESA
[5] https://github.com/ablab/spades
[6] https://github.com/dzerbino/velvet

and similar results are expected independent of the tool used. The described tools make use of public AMR genes databases (Table 5). Instead of using one or several of these tools, one of the public AMR databases can also be downloaded by the applicant and used in a local BLAST pipeline. The use of a curated database is recommended when the latter procedure was chosen.

When an AMR gene is found, the applicant should investigate whether this gene is located on a mobile genetic element.
Possible tools to screen for mobile genetic elements are presented in Table 6.

Table 4 Overview of tools to screen for acquired antimicrobial resistance genes.

Name tool	Explanation	Source
ABRicate	Uses mass screening of contigs for antimicrobial resistance or virulence genes.	Installation via: https://github.com/tseemann/abricate
AMRFinderPlus (Feldgarden et al., 2021)	A tool that identifies AMR genes, resistance-associated point mutations, and selects other classes of genes using protein annotations and/or assembled nucleotide sequence.	Installation via: https://github.com/ncbi/amr
ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation (Gupta et al., 2014))	This was a bioinformatic tool to detect existing and putative new AMR genes in bacterial genomes. It used a local BLAST program to analyse sequences without a Web interface and only supported contigs, not FASTQ reads.	No longer maintained
ARIBA (Antimicrobial Resistance Identification By Assembly (Hunt et al., 2017))	A tool that identifies AMR genes and single nucleotide polymorphisms directly from paired short sequencing reads. Various reference AMR gene lists can be selected.	Installation via: https://github.com/sanger-pathogens/ariba
KmerResistance (Clausen et al., 2016, Clausen et al., 2018)	A tool for the identification of acquired antibiotic resistance genes using kmers. Both fastq (raw NGS data files) and fasta (assembly files) formats are supported, but fastq is recommended.	Online access: http://www.genomicepidemiology.org/services/ Installation via https://bitbucket.org/genomicepidemiology/kmerresistance/src/master/
ResFinder (Zankari et al., 2013, Bortolaia et al., 2020)	A tool which identifies acquired AMR genes and/or finds chromosomal mutations mediating antimicrobial resistance using assembled genomes/contigs, but single or paired end read files (fastq) are also possible. From ResFinder versions 4.0 onwards also *in silico* antibiograms are predicted.	Online access: http://www.genomicepidemiology.org/services/ Installation via: https://bitbucket.org/genomicepidemiology/resfinder/src/master/
RGI (Resistance Gene Identifier)	Predicts the resistome from protein or nucleotide data based on homology and SNP models.	Web portal: https://card.mcmaster.ca/analyze/rgi Installation via: https://github.com/arpcard/rgi

Name tool	Explanation	Source
SRST2 (Inouye et al., 2014)	A read mapping-based tool for detection of genes (e.g. resistance genes, virulence genes, etc), alleles and multi-locus sequence types (MLST) from Illumina WGS data.	Installation via: https://github.com/katholt/srst2

Table 5 Overview of public antimicrobial resistance genes databases.

Name database	Explanation
ARDB (Antibiotic Resistance Genes Database (Liu and Pop, 2009))	This database is no longer being maintained, but all data can be found in CARD (see below). All data underlying ARDB are available for download at: ftp://ftp.cbcb.umd.edu/pub/data/ARDB/ARDBflatFiles.tar.gz
AMRFinderPlus (Feldgarden et al., 2021)	This database focuses on acquired or intrinsic AMR gene products including point mutations in a limited set of taxa. The most recent database release can be found in https://ftp.ncbi.nlm.nih.gov/pathogen/Antimicrobial_resistance/AMRFinderPlus/database/latest
CARD (Comprehensive Antibiotic Resistance Database (MacArthur et al., 2013)	Bioinformatic database of resistance genes, their products and associated phenotypes. The database can be accessed via https://card.mcmaster.ca/
MEGARes (Lakin et al., 2016, Doster et al., 2020)	This database contains sequence data for approximately 8,000 hand-curated AMR genes. In version 2.0 of the database also metal and biocide resistance determinants were incorporated. The database can be accessed via https://megares.meglab.org
NCBI's Pathogen Detection AMR Reference Gene Catalog (Feldgarden et al., 2021)	A curated database of an ever-growing reference set of AMR genes and proteins, point mutations, and is now starting to incorporate stress response (biocide, metal, heat resistance) and virulence determinants as well. The database can be accessed at https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/
PATRIC (Pathosystems Resource Integration Center (Wattam et al., 2014)	This database is an online resource of more than 10,000 annotated bacterial genomes including AMR and virulence genes. The database can be searched at http://www.patricbrc.org
ResFinder_db (Zankari et al., 2012)	Curated database of acquired resistance genes, which can be found at https://bitbucket.org/genomicepidemiology/resfinder_db/src/master/

Table 6 Overview of tools to screen for mobile genetic elements.

Name tool	**Explanation**
BLAST (Basic Local Alignment Search Tool (Altschul et al., 1990))	Sequence similarity search program that can be used to quickly search a sequence database for matches to a query sequence. Blast can be performed online at https://blast.ncbi.nlm.nih.gov/
BLAST+ (Camacho et al., 2009)	Improved blast user interface of the command-line applications. Installation via: ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST
MobileElementFinder (Johansson et al., 2021)	Tool developed to enable rapid detection of MGEs and their genetic context in assembled sequence data. The online version can be assessed at https://cge.cbs.dtu.dk/services/MobileElementFinder/
oriTfinder (Li et al., 2018)	Web server that facilitates the rapid identification of the origin of transfer site (oriT) of a conjugative plasmid or chromosome-borne integrative and conjugative element.
PlasmidFinder (Carattoli et al., 2014, Clausen et al., 2018, Camacho et al., 2009)	Identifies plasmids in total or partial sequenced bacterial isolates. Can be performed online at https://cge.food.dtu.dk/services/PlasmidFinder/

4.3 Assessing the results of the tools and public databases

To assess the results from the tools and databases described in section 4.2 it is recommended that the following elements are checked:

- Determine whether one of the tools described in Table 4 in section 4.2 was used by the applicant. If this was not the case, determine whether the procedure followed is equally applicable as the ones mentioned in the previous section.
- Determine whether one of the public databases described in Table 5 in section 4.2 was used by the applicant. If this was not the case, determine whether the database used is equally complete as the ones mentioned in the previous section.
- If an AMR gene was found, determine whether its location was established by the applicant following one of the tools described in Table 6 in section 4.2.

4.4 Examples to analyse the WGS data provided

If raw sequence data (*fastq.gz file(s)) or assembly files (*.fa or *.fasta) are provided in the application for a bacteria, the analyses of the data could be performed by an evaluator using an online tool such as; ResFinder (https://cge.food.dtu.dk/services/ResFinder/).

For example, running ResFinder with a fasta file that contains the generated contigs after assembly of the filtered and trimmed raw sequence data would look like Figure 1. At the ResFinder website select the "Acquired antimicrobial resistance genes" option, followed by the selection of the "Antimicrobial configuration" (which antibiotic classes have to be investigated). Then select 90% or 95% at "threshold for %ID" (range 30-100%, with 30% producing a lot of noise/unrelated fragments/genes, and 100% is regarded as very strict, not allowing any mismatches) and either 60% or 80% at "minimum length" (range 20-100%, 20% producing a lot of noise/unrelated fragments/genes, and 100% is regarded as very strict, not allowing any gene length differences). Select "Other" at the "select species" drop down menu, since most microbial plant protection substances are not available. Choose the type of file(s) to analyse ("type of your reads"); in case of a fasta file this should be "Assembled Genome/Contigs". Finally select the fasta file from a folder on the computer and press "Upload". After this is successfully carried out, the window will be refreshed, stating "Your job is being processed.
Wait here to watch the progress of your job, or fill in the form below to get an email message upon completion. This page will update itself automatically."

The result of the example ResFinder analysis in Figure 1 is shown in Annex A. The example fasta file contained the publicly available complete genome of *B. thuringiensis* strain ABTS-351; i.e. a chromosome and twelve plasmids.
A fosfomycin resistance gene *fosB1* was displayed. The extended output of the results (Annex B) show that in strain ABTS-351 this gene has three mismatches compared to the reference sequence of *fosB1* (accession number CP001903), resulting in a 99.28% identity match, and a 100% length match.

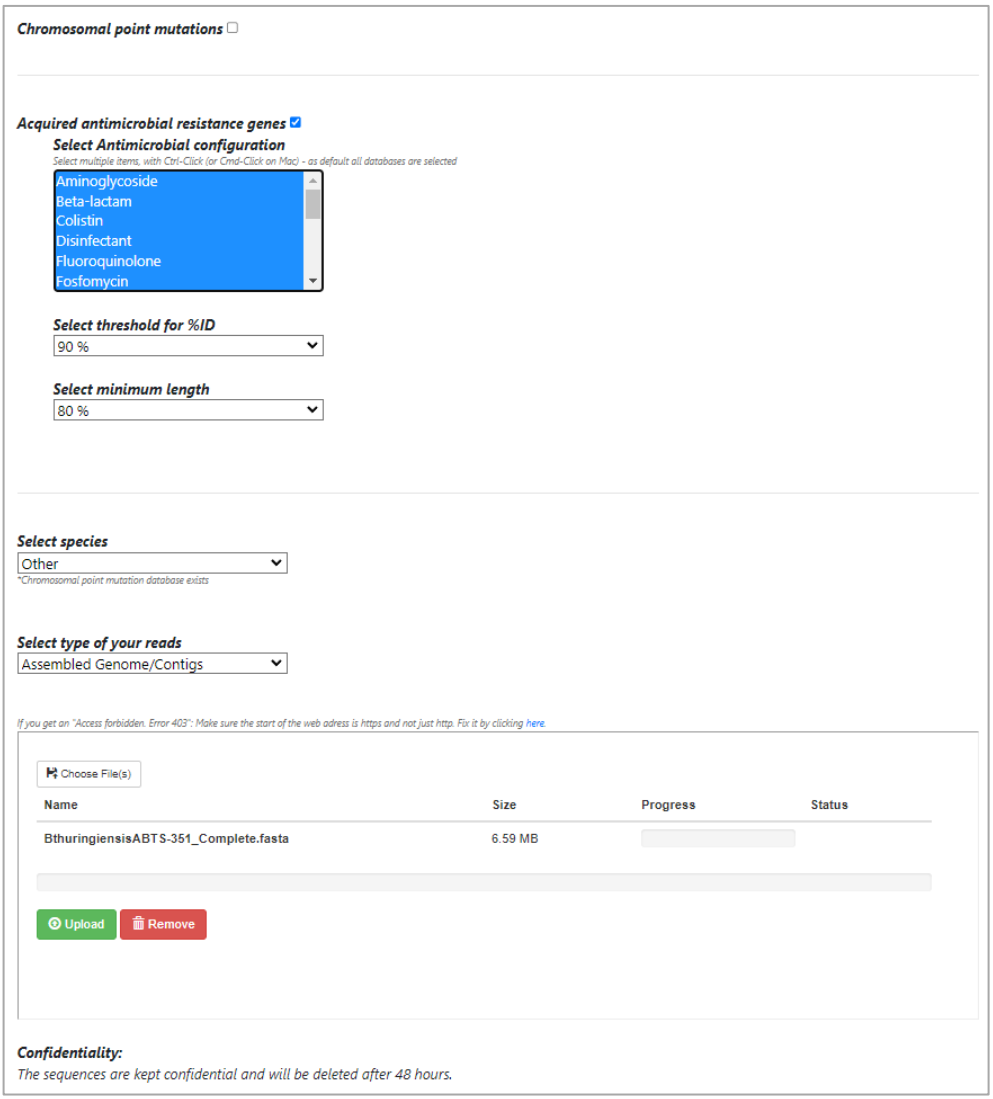

Figure 1 Screenshot of the settings of a ResFinder analysis with an example fasta file.

If an AMR gene is identified and a fasta file is provided, the search for its location could be repeated by an evaluator using an online tool such as MobileElementFinder (https://cge.food.dtu.dk/services/MobileElementFinder/).

For example, running MobileElementFinder would look like Figure 2. At the MobileElementFinder website only select the "Acquired Antimicrobial Resistance genes (ResFinder)" option. Add the fasta file via the "Isolate File" button from a folder on the computer and press "Upload". After this is successfully carried out, the window will be refreshed, stating "Your job is being processed. Wait here to watch the progress of your job, or fill in the form below to get an email message upon completion. This page will update itself automatically."

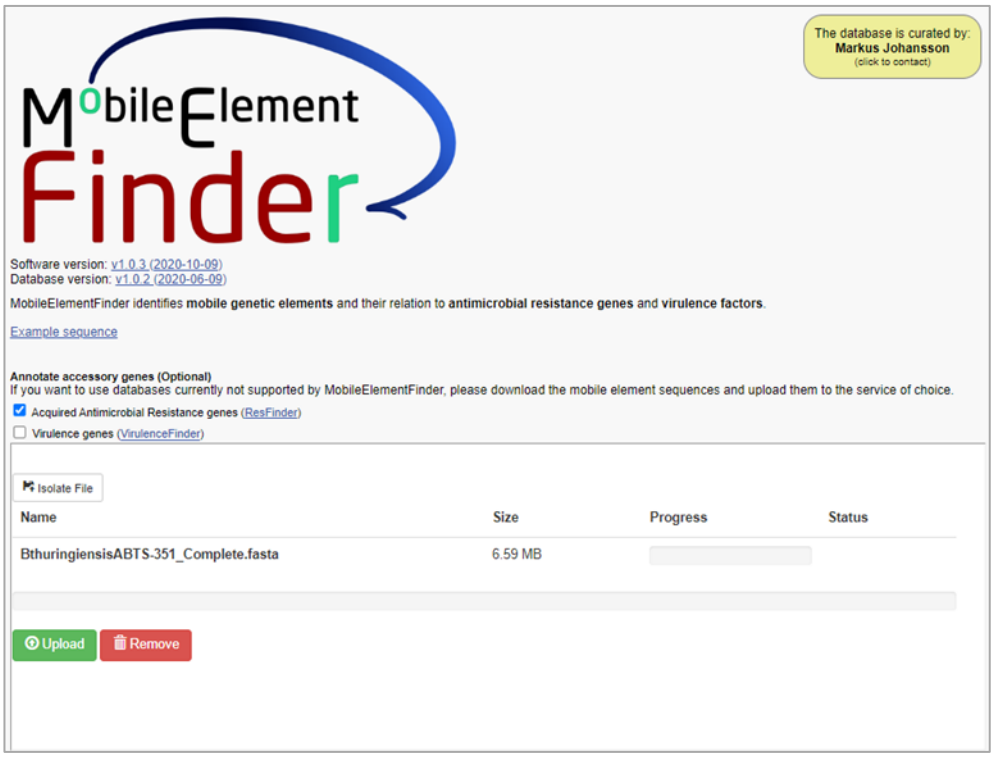

Figure 2 Screenshot of the settings of a MobileElementFinder analysis with an example fasta file.

The result of the MobileElementFinder analysis in Figure 2 is shown in Annex C. Again the example fasta file was the publicly available complete genome of *B. thuringiensis* strain ABTS-351.
The analysis showed that the *fosB1* fosfomycin resistance gene is present on the chromosome of strain ABTS-351 with no insertion sequences (only three resistance genes are shown in Annex C) flanking it. So this AMR gene is not located on a mobile genetic element.

5 Conclusions

This report provides guidance to risk assessors on how WGS data can be evaluated to screen for the presence of AMR genes. It shows an overview of which AMR genes can be expected for the bacteria currently approved or pending approval as active substance on the EU market. It also provides evaluators a set of criteria to assess the quality of the WGS data submitted by applicants, and it gives examples of public databases that can be used to evaluate the data to screen for AMR genes. These databases are all acceptable and similar results are expected. When an AMR gene is found, applicants should investigate whether it is located on a mobile genetic element. This report describes guidance on how this can be done.

It should be noted that when it comes to analysing WGS data there is not just one acceptable approach. This report describes the generally used procedure and tools to evaluate WGS data. In the information provided by applicants there might be slight deviations from the procedures and tools described in this report. Evaluators should assess on a case-by-case basis if these deviations are acceptable.

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7 Terms and definitions

Assembly

Output from process of aligning and merging sequencing reads into larger contiguous sequences.

BLAST

Basic Local Alignment Search Tool (BLAST) finds regions of similarity between biological sequences. The program compares nucleotide or protein sequences with sequence databases and calculates the statistical significance.

Contigs

Contiguous stretch of DNA sequences that results from the assembly of smaller, overlapping DNA sequence reads.

Coverage

Number of times that a given base position is read in a sequencing run. An option to calculate the coverage is based on the number of reads divided by the target organism genome size.

Fastq

A human-readable file format that stores the untrimmed, unfiltered nucleotide base sequences (reads), the calculated confidence for each base in a sequence, and information describing the origin of the read down to its position on the sequencing platform used. Each read has four lines of data. The first line always begins with "@" and is often called the sequence identifier. The second line contains the raw nucleotide sequence. The third line is a spacer that will start with a "+". The fourth line contains the quality string.

Fasta

A text-based file format to store sequence data (DNA (nucleotide) or protein (amino acid codes)) commonly used for reference or consensus sequences. Each sequence has two lines. The first line starts with a ">", followed by a unique description of the sequence. The second line contains either the DNA or protein sequence.

Kmer

A nucleotide sequence of a certain length k in a string (e.g 8-mer).

MLST

Multi-locus sequence typing method is a genomic analysis procedure that identifies nucleotide variants within a predefined sets of housekeeping genes.

N50

The sequence length (N) of the shortest contig at 50% of the total genome length.

Phred score

A measure of the sequence quality which is defined by Q = −10 logP, where P is the probability that a base is incorrectly assigned at a given position in the sequence. For example, a score of Q30 indicates that there is a 1 in 1,000 chance that a base is incorrectly assigned (i.e. the base call is 99.9 % accurate).

Read

The nucleotide sequence inferred from a fragment of DNA or RNA.

SNP

Single nucleotide polymorphism.

8 Annexes

Annex A

Resfinder result from the complete genome of *B. thuringiensis* strain ABTS-351 (chromosome and 12 plasmids).

Center for Genomic Epidemiology

Home Services Instructions Output Overview of genes Article abstract

ResFinder-4.1 Server - Results

Input Files: *BthuringiensisABTS-351_Complete.fasta*

complete

Antimicrobial	Class	WGS-predicted phenotype	Genetic background
vancomycin	glycopeptide	No resistance	
mupirocin	pseudomonic acid	No resistance	
tobramycin	aminoglycoside	No resistance	
hygromycin	aminoglycoside	No resistance	
isepamicin	aminoglycoside	No resistance	
virginiamycin s	streptogramin b	No resistance	
hydrogen peroxide	peroxide	No resistance	
butirosin	aminoglycoside	No resistance	
ampicillin	beta-lactam	No resistance	
astromicin	aminoglycoside	No resistance	
lividomycin	aminoglycoside	No resistance	
sulfamethoxazole	folate pathway antagonist	No resistance	
temocillin	beta-lactam	No resistance	
trimethoprim	folate pathway antagonist	No resistance	
oleandomycin	macrolide	No resistance	
florfenicol	amphenicol	No resistance	
clindamycin	lincosamide	No resistance	
quinupristin	streptogramin b	No resistance	
fosfomycin	fosfomycin	Resistant	fosB1 (fosB1_CP001903)
cephalothin	beta-lactam	No resistance	
lincomycin	lincosamide	No resistance	
butiromycin	aminoglycoside	No resistance	
piperacillin+clavulanic acid	beta-lactam	No resistance	
paromomycin	aminoglycoside	No resistance	
fluoroquinolone	quinolone	No resistance	
amoxicillin+clavulanic acid	beta-lactam	No resistance	
telcoplanin	glycopeptide	No resistance	
tiamulin	pleuromutilin	No resistance	
ribostamycin	aminoglycoside	No resistance	
erythromycin	macrolide	No resistance	
kanamycin	aminoglycoside	No resistance	
gentamicin	aminoglycoside	No resistance	
amikacin	aminoglycoside	No resistance	
tigecycline	tetracycline	No resistance	
ticarcillin+clavulanic acid	beta-lactam	No resistance	
cephalotin	beta-lactam	No resistance	
cefoxitin	beta-lactam	No resistance	
virginiamycin m	streptogramin a	No resistance	
penicillin	beta-lactam	No resistance	
neomycin	aminoglycoside	No resistance	
ceftiofur	under_development	No resistance	
dalfopristin	streptogramin a	No resistance	
piperacillin	beta-lactam	No resistance	
telithromycin	macrolide	No resistance	
tetracycline	tetracycline	No resistance	

https://cge.food.dtu.dk//cgi-bin/webface.fcgi?jobid=62DA8F90000047B5CFFFA13F 1/3

Antimicrobial	Class	WGS-predicted phenotype	Genetic background
amoxicillin	beta-lactam	No resistance	
meropenem	beta-lactam	No resistance	
ethidium bromide	quaternary ammonium compound	No resistance	
sisomicin	aminoglycoside	No resistance	
unknown aminoglycoside	aminoglycoside	No resistance	
cefepime	beta-lactam	No resistance	
spectinomycin	aminocyclitol	No resistance	
doxycycline	tetracycline	No resistance	
piperacillin+tazobactam	beta-lactam	No resistance	
fusidic acid	steroid antibacterial	No resistance	
ciprofloxacin	quinolone	No resistance	
colistin	polymyxin	No resistance	
temperature	heat	No resistance	
imipenem	beta-lactam	No resistance	
arbekacin	aminoglycoside	No resistance	
nalidixic acid	quinolone	No resistance	
metronidazole	nitroimidazole	No resistance	
cefixime	beta-lactam	No resistance	
bleomycin	aminoglycoside	No resistance	
pristinamycin ia	streptogramin b	No resistance	
formaldehyde	aldehyde	No resistance	
tylosin	macrolide	No resistance	
benzylkonium chloride	quaternary ammonium compound	No resistance	
cefotaxime+clavulanic acid	beta-lactam	No resistance	
rifampicin	rifamycin	No resistance	
ceftriaxone	beta-lactam	No resistance	
ceftazidime	beta-lactam	No resistance	
fortimicin	aminoglycoside	No resistance	
carbomycin	macrolide	No resistance	
ticarcillin	beta-lactam	No resistance	
azithromycin	macrolide	No resistance	
chlorhexidine	quaternary ammonium compound	No resistance	
kasugamycin	aminoglycoside	No resistance	
chloramphenicol	amphenicol	No resistance	
cetylpyridinium chloride	quaternary ammonium compound	No resistance	
ampicillin+clavulanic acid	beta-lactam	No resistance	
cefotaxime	beta-lactam	No resistance	
quinupristin+dalfopristin	streptogramin a	No resistance	
ceftazidime+avibactam	beta-lactam	No resistance	
apramycin	aminoglycoside	No resistance	
spiramycin	macrolide	No resistance	
dibekacin	aminoglycoside	No resistance	
ertapenem	beta-lactam	No resistance	
unknown quinolone	quinolone	No resistance	
linezolid	oxazolidinone	No resistance	
netilmicin	aminoglycoside	No resistance	
minocycline	tetracycline	No resistance	
aztreonam	beta-lactam	No resistance	
unknown beta-lactam	beta-lactam	No resistance	
pristinamycin iia	streptogramin a	No resistance	
streptomycin	aminoglycoside	No resistance	

Download phenotype table (txt) | Download species specific phenotype table (txt)

Fosfomycin

Resistance gene	Identity	Alignment Length/Gene Length	Position in reference	Contig or Depth	Position in contig	Phenotype	PMID	Accession
fosB1	99.2805755396	417/417	1..417	NZ_CP063101.1 Bacillus thuringiensis strain ABTS-351 chromosome, complete genome	2947751..2948167	fosfomycin	20525827	CP001903

Download acquired AMR gene results:

Results as text | Hit in genome sequences | Resistance gene sequences | Results as tabseperated file

Selected %ID threshold for ResFinder: *90 %*

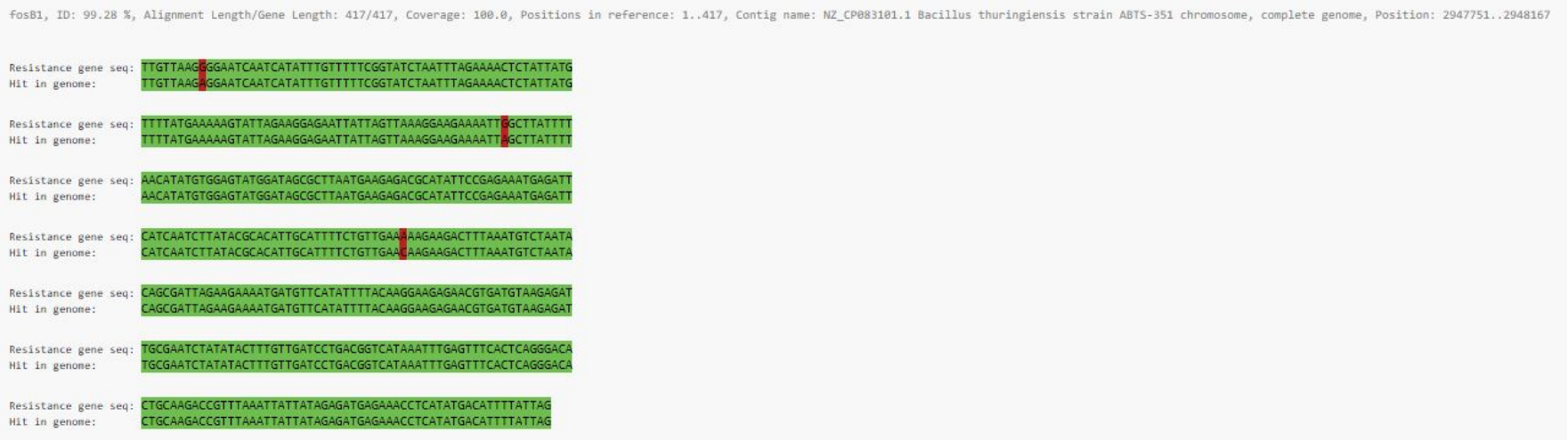
Selected minimum length for ResFinder: *60 %*

extended output

Support Scientific problems Technical problems

Annex B

Detailed *fosB1* result from the complete genome of *B. thuringiensis* strain ABTS-351 (chromosome and 12 plasmids)



Annex C

MobileElementFinder result from the complete genome of *B. thuringiensis* strain ABTS-351 (chromosome and 12 plasmids).

MobileElementFinder Results

Sample name: BthuringiensisABTS-351_Complete
Date:
MGEfinder version: 1.0.3
MGEdb version: 1.0.2

Displaying: 48 of 185 mobile elements

Contig	Plasmid	#MGEs	Resistance
NZ_CP083101.1 Bacillus thuring...		29	fosB1
NZ_CP083106.1 Bacillus thuring...	rep3	0	
NZ_CP083105.1 Bacillus thuring...	rep12	1	
NZ_CP083102.1 Bacillus thuring...		5	
NZ_CP083103.1 Bacillus thuring...		4	
NZ_CP083104.1 Bacillus thuring...		1	
NZ_CP083107.1 Bacillus thuring...		8	

Download result | Download MGE sequences

Contig: NZ_CP083101.1 Bacillus thuringiensis strain ABTS-351 chromosome, complete genome

Resistance results

Gene name	Phenotype	Accession	Position in contig	Coverage	Identity
fosB1	fosfomycin	CP001903	2947751-2948167	100%	99.28057553956835%

IS232

Synonyms	IS232A,IS232B,IS232C
Family	IS21
Type	Insertion sequence
Reference db	isfinder
Accession	M38370
Position in contig	1911856-1914039
Strand	reverse
Alignment coverage	100%; 2184 / 2184
Sequence identity	99.95%
Num Substitutions	1
E-value	0

Show MGE alignment

IS232

Synonyms	IS232A,IS232B,IS232C
Family	IS21
Type	Insertion sequence
Reference db	isfinder
Accession	M38370
Position in contig	4567176-4569359
Strand	reverse
Alignment coverage	100%; 2184 / 2184
Sequence identity	99.95%
Num Substitutions	1
E-value	0

Show MGE alignment

IS232

Synonyms	IS232A,IS232B,IS232C
Family	IS21
Type	Insertion sequence
Reference db	isfinder
Accession	M38370
Position in contig	1653380-1655562
Strand	reverse
Alignment coverage	99.95%; 2183 / 2184

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