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CEP Panel Guidance

Characterisation of microorganisms used for the production of food enzymes

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STATEMENT

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Characterisation of microorganisms used for the production of food enzymes

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP)*,
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Background

- 2018: FEEDAP Guidance on the characterisation of microorganisms used as feed additives or as production organisms
  - Up-to-date information requests for production strains
  - FIP participation with staff and experts

- Inconvenient to use document from another Panel
- Different purposes
- Applicants' requests for clarity

Self-task: CEP Statement on the characterisation of production strains

Assessent of microbial producurs from FEED additive to FOOD enzyme

	Section	Feed additives containing viable microorganisms		Fermentation products	
		Bacteria	Fungi – yeasts	Bacteria	Fungi – yeasts
Identification	2.1	✓	✓	✓	✓
Antimicrobial susceptibility	2.2	✓		✓	
Antimicrobial production	2.3	✓	✓	✓	✓
Toxigenicity and pathogenicity	2.4	✓	✓	✓	✓
Genetic modification	2.5			For GMMs only	For GMMs only
Absence of the production strain	3.1			✓	✓
Presence of DNA from the production strain	3.2			Where relevant	Where relevant
Compatibility with other authorised additives	4.2	Where relevant	Where relevant		

SCOPE

- **to assist in the preparation and presentation of applications** to market food enzymes produced with microorganisms by fermentation
- the term **microorganism** includes archaea, bacteria, yeasts and filamentous fungi.
- only **aspects linked to the production organism**, including the safety aspects of any genetic modifications, are considered.
- for other elements of the assessment refer to the other relevant CEF Panel documents
- the characterisation of microorganisms used in the production of food enzymes should be made at the **production strain level**.

The Structure

1. Characterisation of the microorganism

- 1.1 Identification
- 1.2 Use of whole genome sequence for characterisation
- 1.3 Antimicrobial susceptibility
- 1.4 Toxigenicity and pathogenicity
  - 1.4.1 QPS
  - 1.4.2 Non-QPS
- 1.5 Genetic modifications
  - 1.5.1 Purpose of the genetic modification
  - 1.5.2 Characteristics of the modified sequences
  - 1.5.3 Structure of the genetic modification

2. Viable cells and DNA of the production strain

- 2.1 Viable cells of the production strain
- 2.2 DNA from the production strain

1.1 IDENTIFICATION
Unambiguous identification at the species level

Bacteria

- Computational approach using WGS

  (e.g. ANI or dDNA hybridisation).

- Target sequence comparison (16S rRNA or housekeeping genes) may be acceptable

- If the species cannot be identified—› phylogenetic position

- New names —› No consequences. EFSA opinion will mention: "New name (formerly known as Old name)"

1.1 IDENTIFICATION
Unambiguous identification at the species level

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Yeast

- Computational approach using WGS
- This should be done by phylogenomic analysis (e.g. using a concatenation of several conserved genes to produce a phylogeny against available related genomes).
- New names —› No consequences. EFSA opinion will mention: “New name (formerly known as Old name)”

1.1 IDENTIFICATION
Unambiguous identification at the species level

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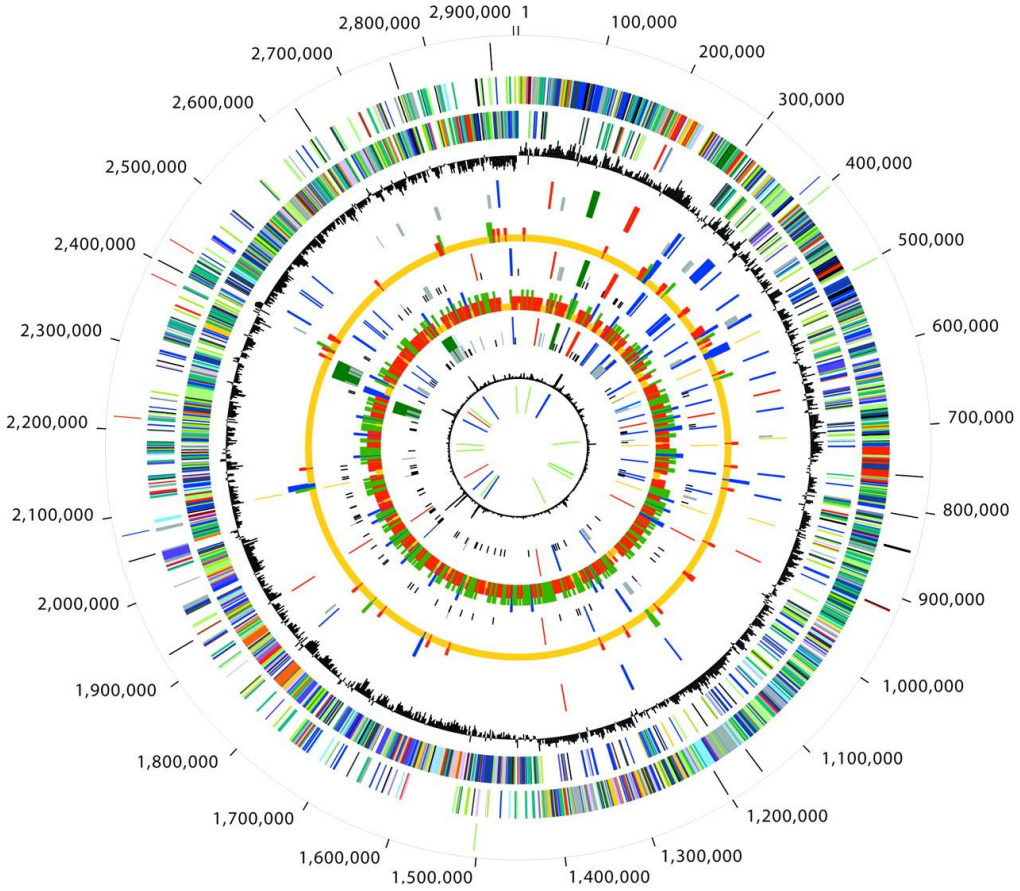
Filamentous Fungi

- When WGS is available, identification should be made by a phylogenomic analysis comparing the genome against available related genomes.

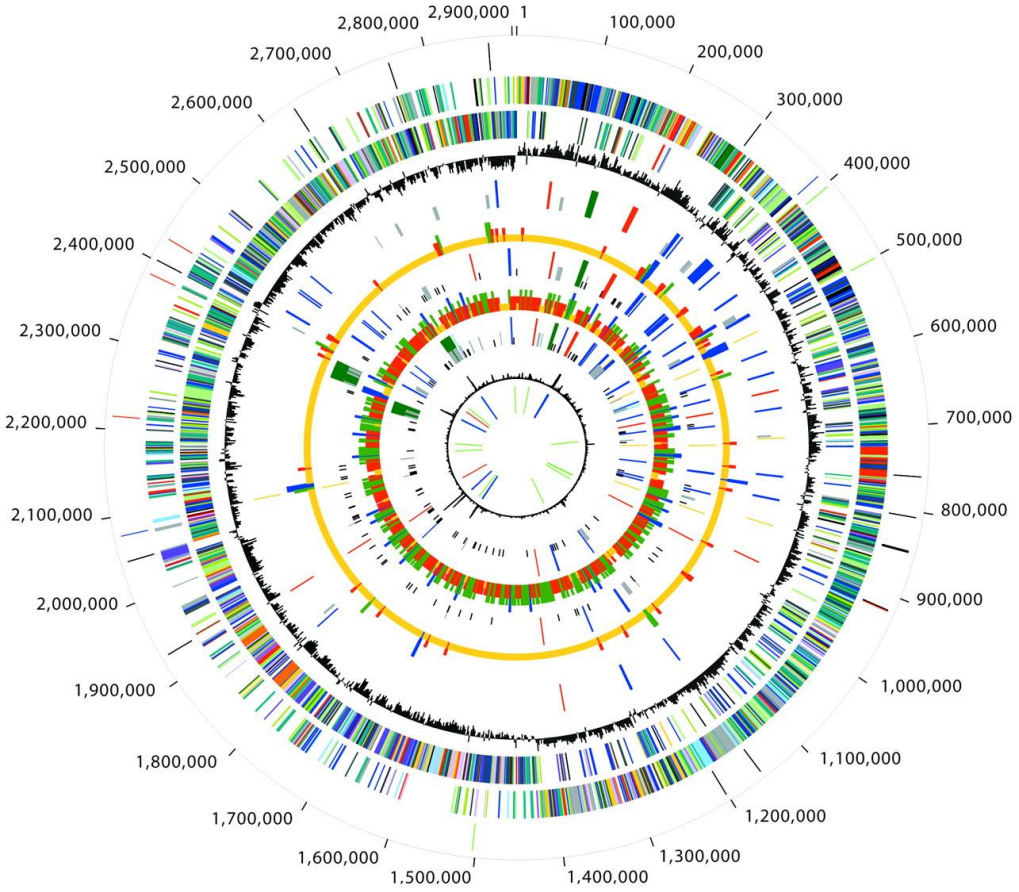
- Alternatively, identification may be made by comparing the 18S rRNA gene and/or internal transcribed spacer (ITS) regions and other characteristic genes (e.g. tubulin) with sequences deposited in databases.

1.2 WGS analysis



- Species identification
- Search for antimicrobial resistance genes
- Search for genes involved in toxins/virulence factors
- Characterisation of genetic modifications

1.2 WGS data



- DNA extraction method
- sequencing strategy and instrumentation used
- assembly method applied
- statistical measure of sequence quality
- FASTA file(s) of the WGS
- total length of contigs relative to the expected genome size
- annotation protocol used
- for fungi: information on the quality of the annotations obtained from relevant databases

1.3 ANTIMICROBIAL SUSCEPTIBILITY

- Applicable to all bacteria
- Relevant antimicrobials: CIAs or HIAs (WHO)
- Mainly based on WGS – Search for genes encoding resistance
- Phenotypic analysis (MIC determination) in case of uncertainty
  - incomplete coding sequences
  - low percentage of identity

OUTCOME

Strain with acquired resistance genes —› absence of DNA

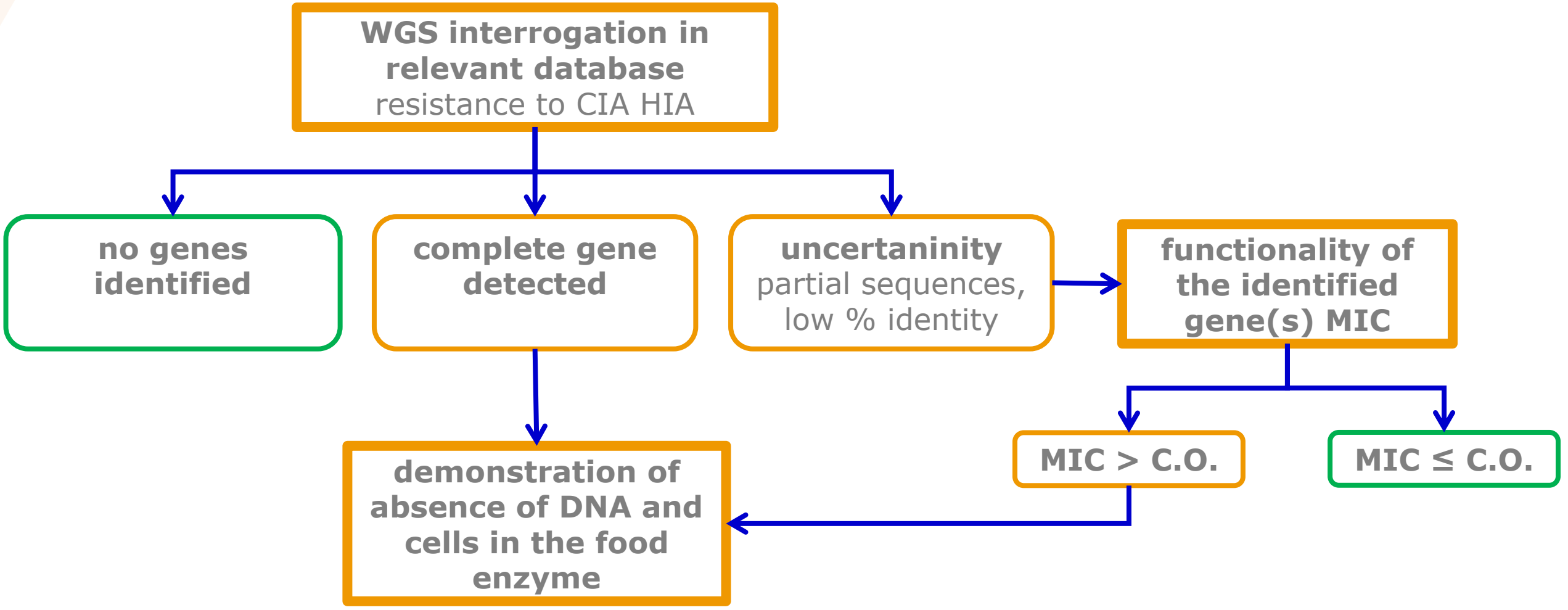
Cut-Off VALUES

Table 1: Microbiological cut-off values (mg/L)[7]

	Bacillus	*Corynebacterium* and other Gram-positive	*Pseudomonas*	Enterobacteriaceae
Ampicillin	n.r.	1	n.r.	8
Piperacillin	n.r.	n.r.	16	n.r.
Vancomycin	4	4	n.r.	n.r.
Gentamicin	4	4	8	2
Kanamycin	8	16	n.r.	8
Streptomycin	8	8	n.r.	16
Erythromycin	4	1	n.r.	n.r.
Clindamycin	4	4	n.r.	n.r.
Tetracycline	8	2	n.r.	8
Chloramphenicol	8	4	n.r.	n.r.
Tylosine	n.r.	n.r.	n.r.	n.r.
Ciprofloxacin	n.r.	n.r.	0.5	0.06
Colistine	n.r.	n.r.	4	2
Fosfomycin	n.r.	n.r.	n.r.	8

n.r. not required.

1.3 ANTIMICROBIAL SUSCEPTIBILITY

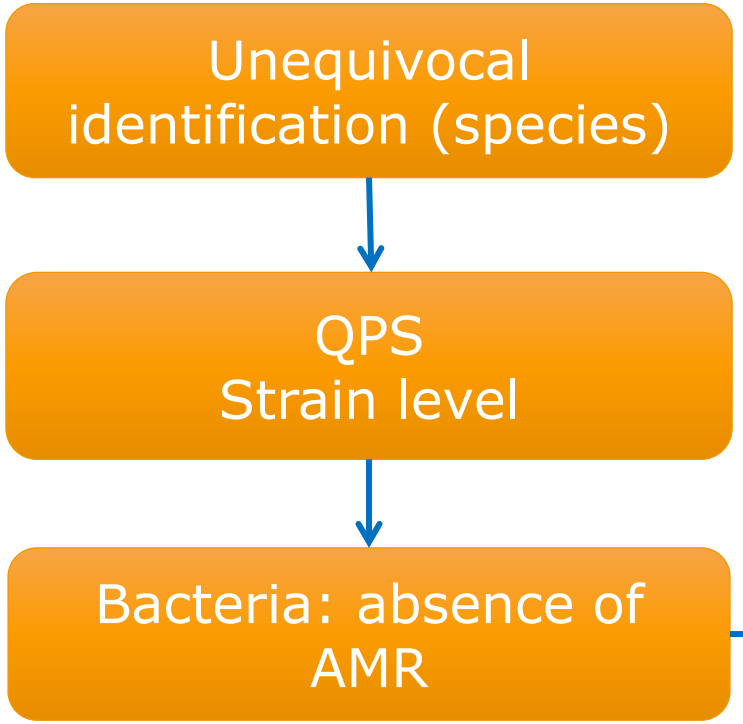


outcome:

Strain with AMR genes —› absence of DNA

1.4 TOXIGENICITY AND PATHOGENICITY

QPS strains: considered toxicologically safe



Bacillus:
cytotoxicity test on the production strain —› **parental not acceptable**

Any other qualification

For GMMs: QPS parental = QPS concept applicable to the GM

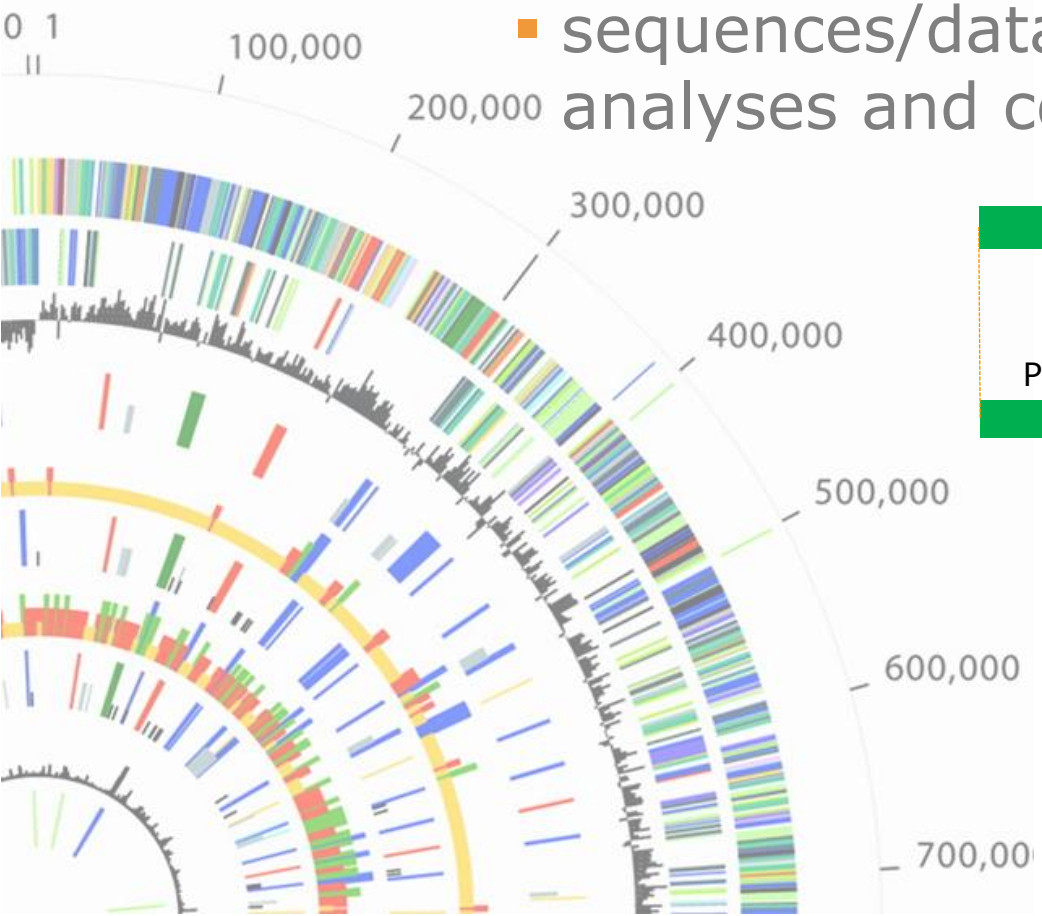
1.4 TOXIGENICITY AND PATHOGENICITY
Non-QPS strains

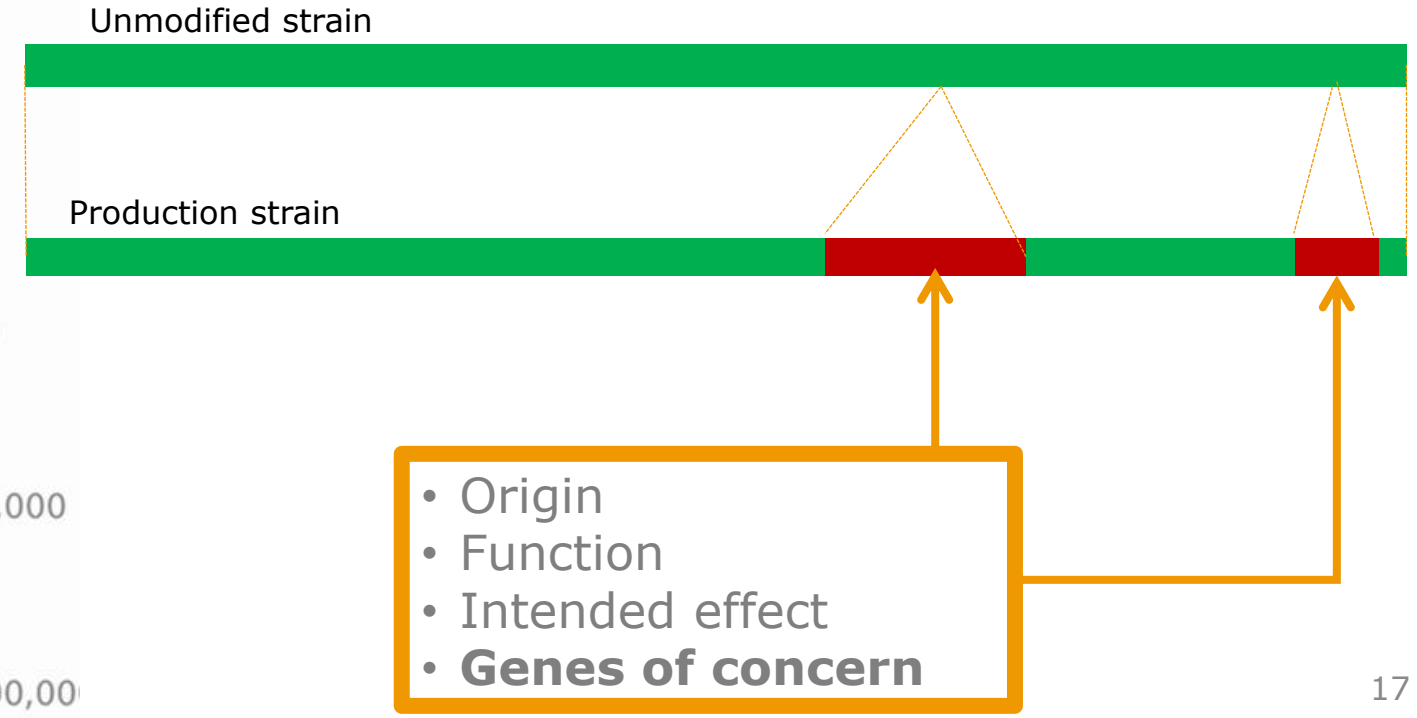
- **Bacteria**: WGS analysis —› Search for sequences coding for known virulence factors:
  - matches to be assessed on a case-by-case basis
  - may trigger further phenotypic testing
- **Eukaryotes**: Data from literature searches
  - If WGS available: targeted searches against known sequences encoding toxin production pathways

Possible production of toxic compounds ➡ **OUTCOME** evidence of no concern { By analysis / By tox studies

1.5 GENETIC MODIFICATIONS - WGS

- **bacteria and yeasts** (optional for fungi) —› **WGS**
  - Map/graphic of all genomic regions harboring genetic modifications (ORFs and non-coding sequence/s)
  - sequences/databases and the methodology used for analyses and comparison





1.5 GENETIC MODIFICATIONS

- **Inserted sequences from defined organisms**
  - nucleotide sequence of all inserted elements including a functional annotation and the physical map of all the functional elements
  - name, derived amino acid sequence(s) and function(s) of the encoded protein(s).

- **Inserted sequences - designed.**
  - rationale and strategy for the design
  - DNA sequence and a physical map of the functional elements
  - identify the functional domains of the recombinant protein;

- **Deletion**
- **Base pair substitutions and frameshift mutations**

1.5 GENETIC MODIFICATIONS – Structure of the GM **without WGS data**

- **all the steps** should be described.
- identification of **all genetic material** potentially introduced into the recipient/parental microorganism.
  - Characteristics of the **vector(s)**
  - Information relating to the **genetic modification process**
  - Structure of any **vector and/or donor** nucleic acid remaining in the GMM
  - **Genes of concern**

2.1 Absence of viable production strain

- **Required for all cases except QPS**
- Culture-based method
  - Molecular methods less sensitive
- Production strain ≠ contaminating microbiota
- Recovery of possible stressed cells
- **≥ 1 g or ml of product**
- **9 samples from at least 3 batches**
- **Positive control**

2.2 Absence of DNA from the production strain

- **Requested for:**
  - GM production strains
  - non-GM production strains with acquired AMR genes

- **PCR-based methodology. Indications on:**
  - Target sequence (<1kb) or the smallest gene of concern (e.g AMR)
  - Amount of sample (**≥ 1 g or 1 ml**)
  - Number of batches – 9 replicates (3 x 3)
  - Controls

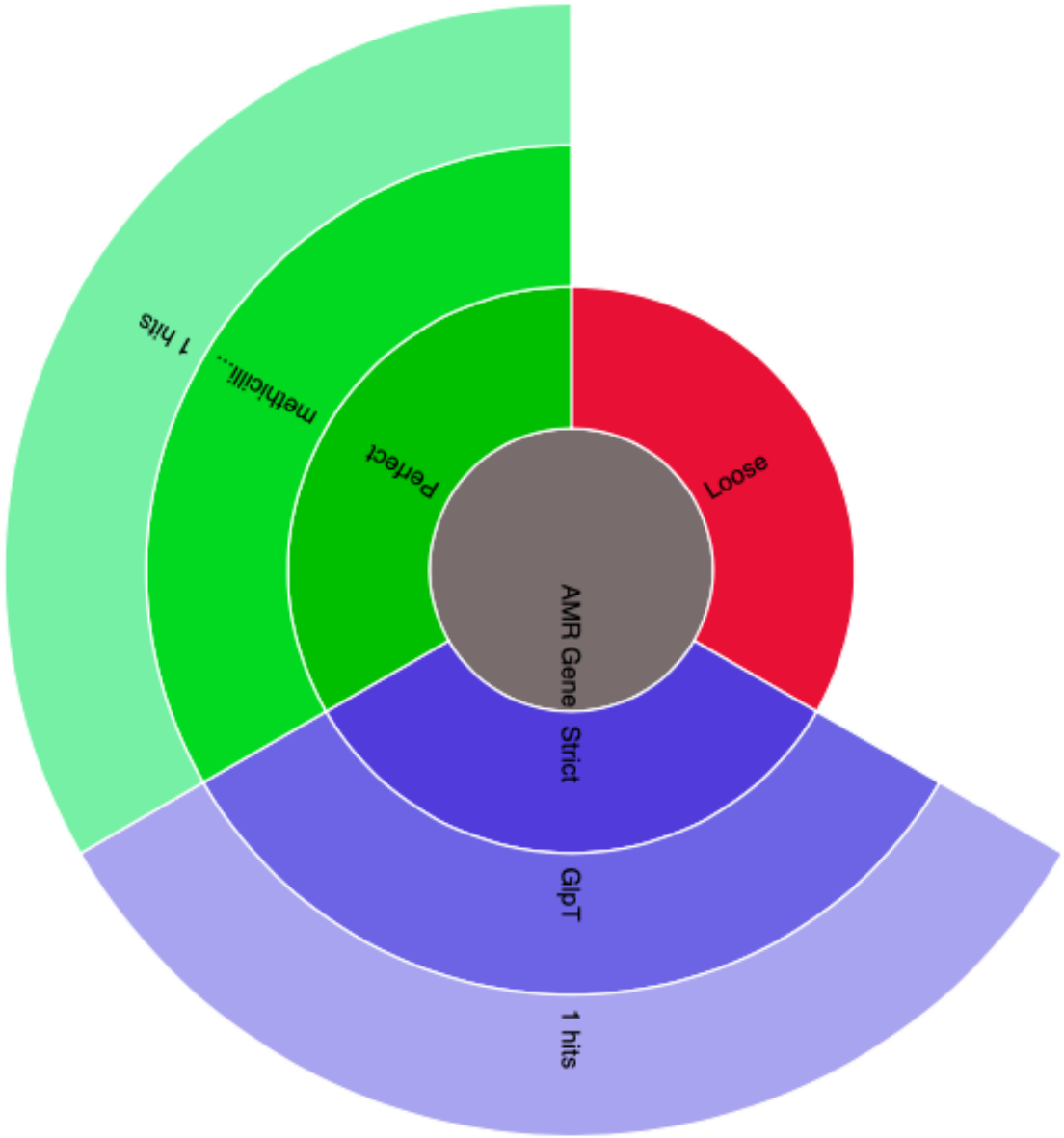
- **Threshold: 10 ng control DNA per g or mL of product**

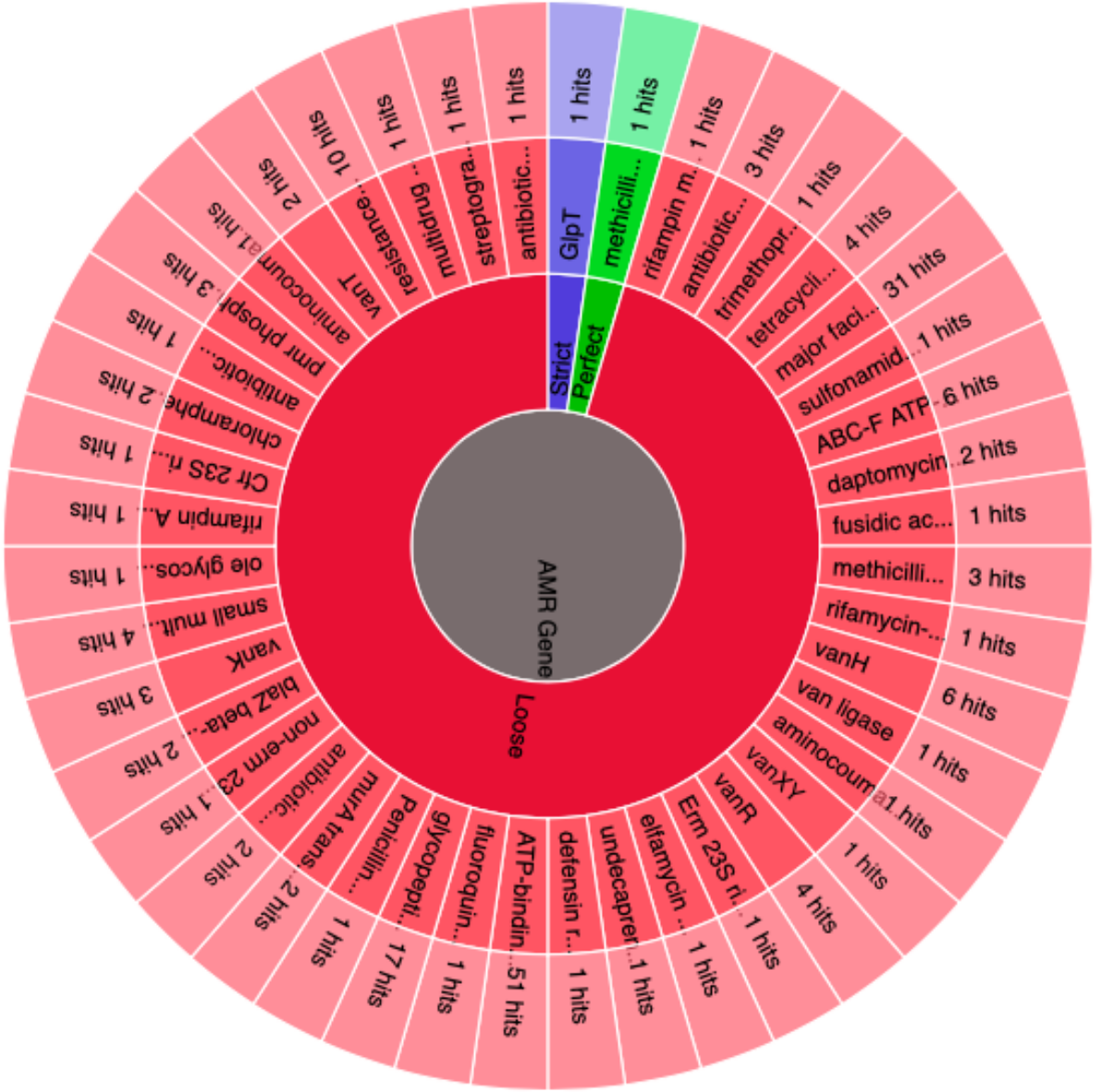
PCR controls and sensitivity tests
DNA extraction

- DNA from the **production strain**
- DNA from the **production strain**, added to the product sample before the **DNA extraction**, known quantity and **dilutions until DNA extinction**,
- DNA from the production strain, added to the DNA extracted from each of the three batches of the product tested, to check for any **factors causing PCR failure**
- **negative** control without sample
- **extraction** using a methodology suitable for all cellular forms in (e.g., vegetative cells, spores)
- **PCR failure** is encountered, the causes should be investigated

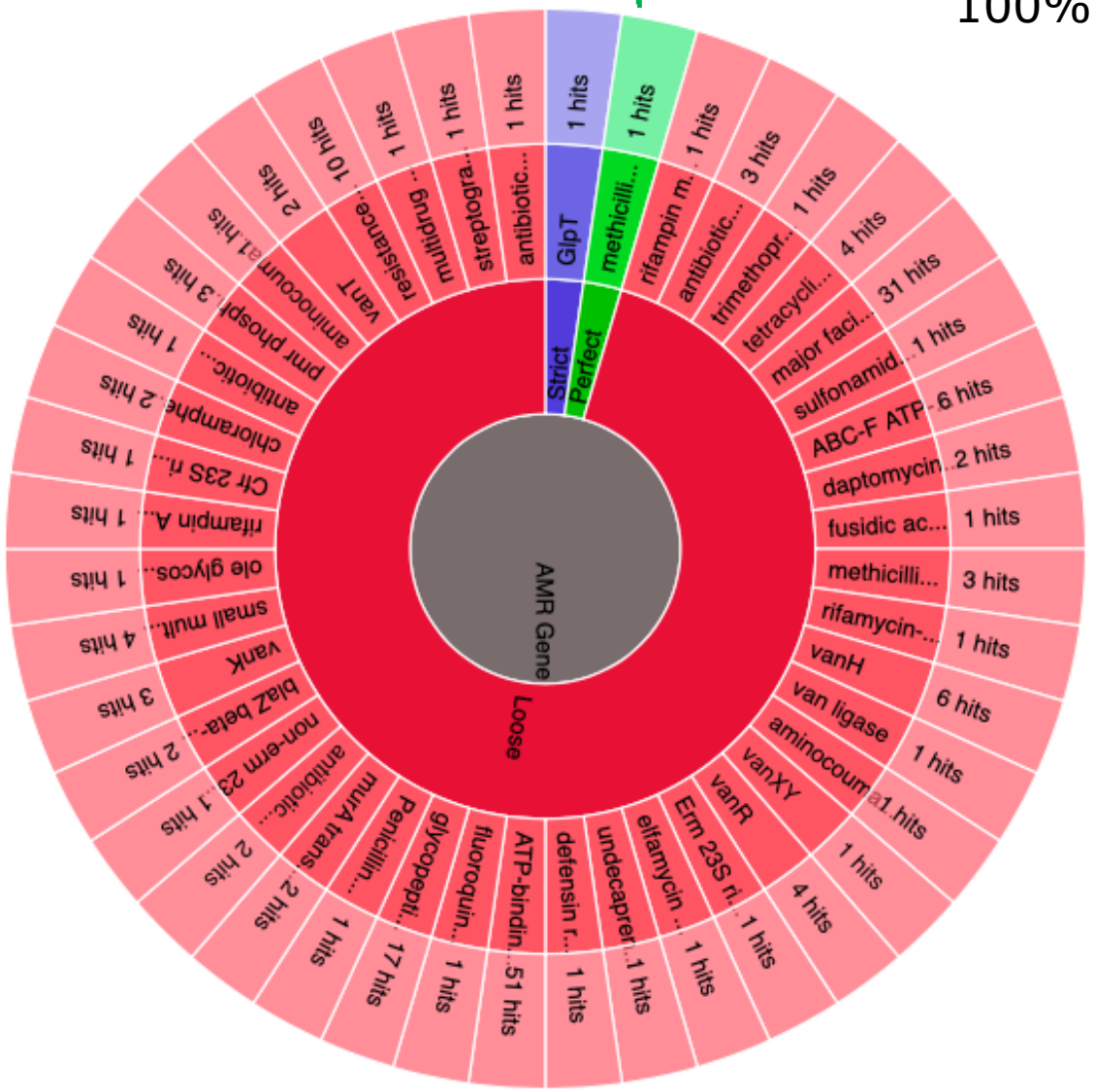
CONCLUSIONS

AMR LOW vs HIGH identity





AMR LOW vs HIGH identity



100% identity

BLAST Alignment *mecC*

```
Query  MKKIYISVLVLLLIMIIITWLFKDDDIEKTISSIEKGNYNEVYKNSSEKSKLAYGEEEIV
       MKKIYISVLVLLLIMIIITWLFKDDDIEKTISSIEKGNYNEVYKNSSEKSKLAYGEEEIV
CARD   MKKIYISVLVLLLIMIIITWLFKDDDIEKTISSIEKGNYNEVYKNSSEKSKLAYGEEEIV

Query  DRNKKIYKDLSVNNLKITNHEIKKTGKDKKQVDVKYNIYTKYGTIRRNTQLNFIYEDKHW
       DRNKKIYKDLSVNNLKITNHEIKKTGKDKKQVDVKYNIYTKYGTIRRNTQLNFIYEDKHW
CARD   DRNKKIYKDLSVNNLKITNHEIKKTGKDKKQVDVKYNIYTKYGTIRRNTQLNFIYEDKHW

Query  KLDWRPDVIVPGLKNGQKINIETLKSERGKIKDRNGIELAKTGNTYEIGIVPNKTPKEKY
       KLDWRPDVIVPGLKNGQKINIETLKSERGKIKDRNGIELAKTGNTYEIGIVPNKTPKEKY
CARD   KLDWRPDVIVPGLKNGQKINIETLKSERGKIKDRNGIELAKTGNTYEIGIVPNKTPKEKY

Query  DDIARDLQIDTKAITNKVNQKWVQPDSFVPIKKINKQDEYIDKLIKSYNLQINTIKSRVY
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CARD   DDIARDLQIDTKAITNKVNQKWVQPDSFVPIKKINKQDEYIDKLIKSYNLQINTIKSRVY

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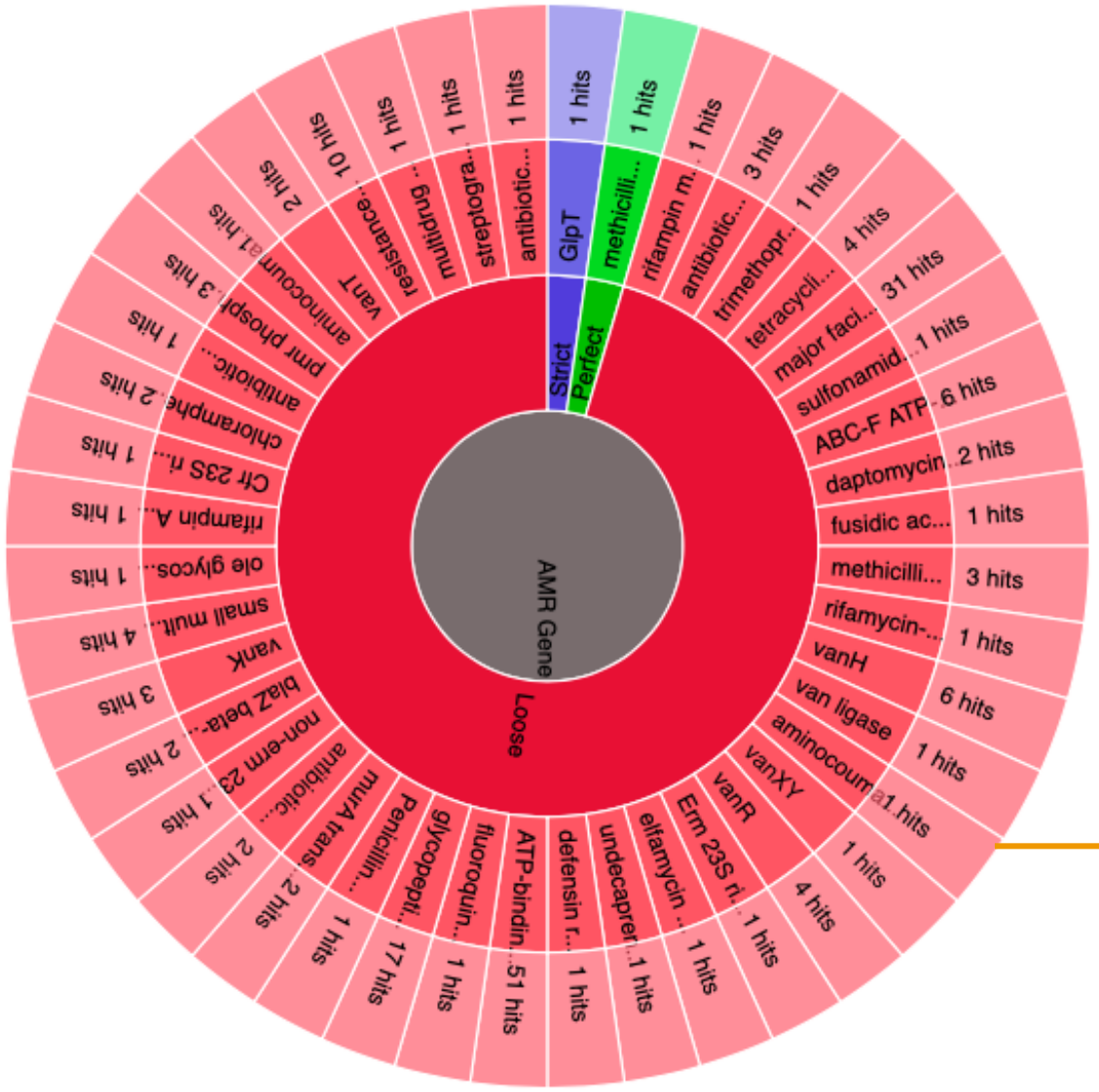
```

AMR LOW vs HIGH identity

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CARD  AYGLIKTFGDNRAVDGVDLNVRTGTIYGVLGPNGAGKTTTIRMLATLLRPDAGSARIFGH

Query --KPDNRQYQKQIGVVFQKSVLDNQLTVWQNL---KSRADMYQGVTLTPESELITAFGLT
        + +++  ++ IGV  Q + +D  L+  +NL        + +         EL+  FGL+
CARD  DVQAESQIVRQLIGVTGQYASVDESLSATENLIIFSRLLGLGRKEARRKAEELLEEFGLS

Query SILKQTYGTLSGGQRRRVDIARALIHQPKLLFLDEPSTGLDIQTRTVIWQTLNQLRQQQG
         K+     SGG RRR+D+A +LI QP L+FLDEP+TGLD +TR+ +W T+ +L    G
CARD  EAAKRPLKNFSGGMRRRLDLAASLIAQPPLIFLDEPTTGLDPRTRSQMWDTIRRL-VNTG

Query LTIILTTPYLEEAAE-ADFVYVIDHGQIIAADTVEQLQATYAQSQLMIETD
       T++LTT YLEEA + AD + VID+G+++A  T ++L+ +   S L +  +
CARD  STVLLTTQYLEEADQLADRIAVIDYGRVVAEGTADELKMSVGTSSLHLTVE
```

34% identity