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IN SILICO APPLICATIONS IN MICROBIOLOGICAL FOOD SAFETY AND RISK ASSESSMENT: THE ANALYSIS OF WHOLE GENOME SEQUENCE

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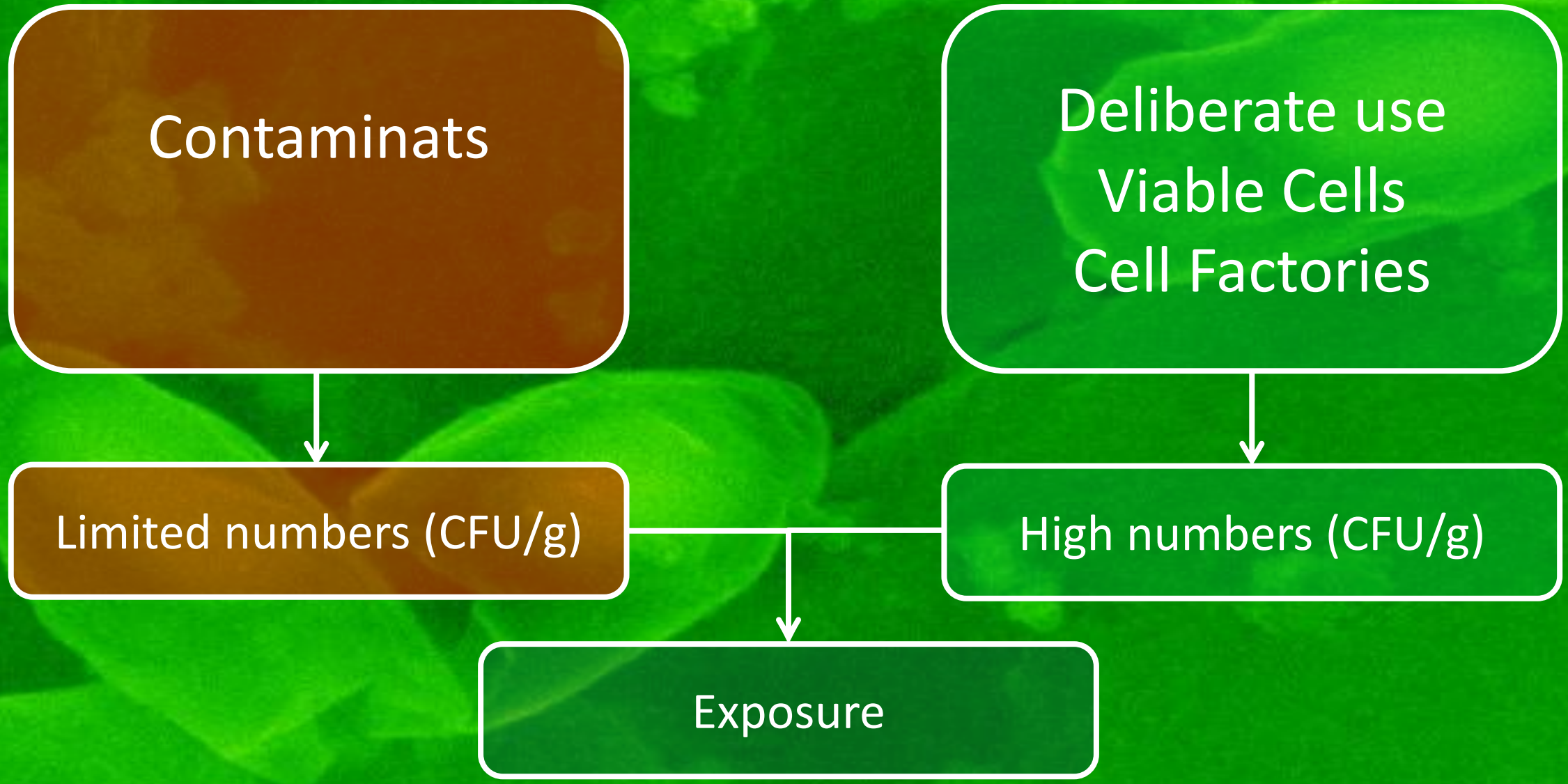
Microorganisms in the Food Chain



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THE INTENTIONAL USE OF MICROORGANISMS IN THE FOOD CHAIN

USE	Authorisation
Food starter cultures	Not required
Human probiotics	Health Claims
Animal probiotics	Feed Additives
Feed fermentation-silage	Feed Additives
Enzyme production	Feed Additives - Food Additives
Amino acid production	Feed Additives - Food Additives
Vitamin production	Feed Additives - Food Additives
Biopesticide	Plant Protection Products
Genetically Modified Microorg.	Feed Additives - Food Additives- GMO

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The theoretic risk assessment

KNOWLEDGE AND DATA

HAZARD IDENTIFICATION

HAZARD CHARACTERIZATION

EXPOSURE ASSESSMENT

RISK CHARACTERIZATION

OPINION FOR RISK MANAGERS

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Microbiological Risk Assessment in Practice:

CONTAMINANTS, BIOLOGICAL HAZARDS…….

SCIENTIFIC KNOWLEDGE AND DATA COLLECTION	available? reliable? harmonised?
HAZARD IDENTIFICATION	emerging risks?
HAZARD CHARACTERIZATION	uncertainities
EXPOSURE ASSESSMENT	quantitative data? consumption info?
RISK CHARACTERIZATION	✓
OPINION FOR RISK MANAGERS	Regulation

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Microbiological Risk Assessment in practice:

Regulated Products

KNOWLEDGE AND DATA	proprietary data: completeness, trustable?
HAZARD IDENTIFICATION	✓
HAZARD CHARACTERIZATION	uncertainities
EXPOSURE ASSESSMENT	✓
RISK CHARACTERIZATION	✓
OPINION FOR RISK MANAGERS	Regulation

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A wide variety of microorganisms are intentionally added at different stages into the food chain, either directly or as a source of additives or enzymes.

- More than 130 bacterial species

  Arthrobacter globiformis ..... Streptococcus thermophilus

- Filamentous fungi

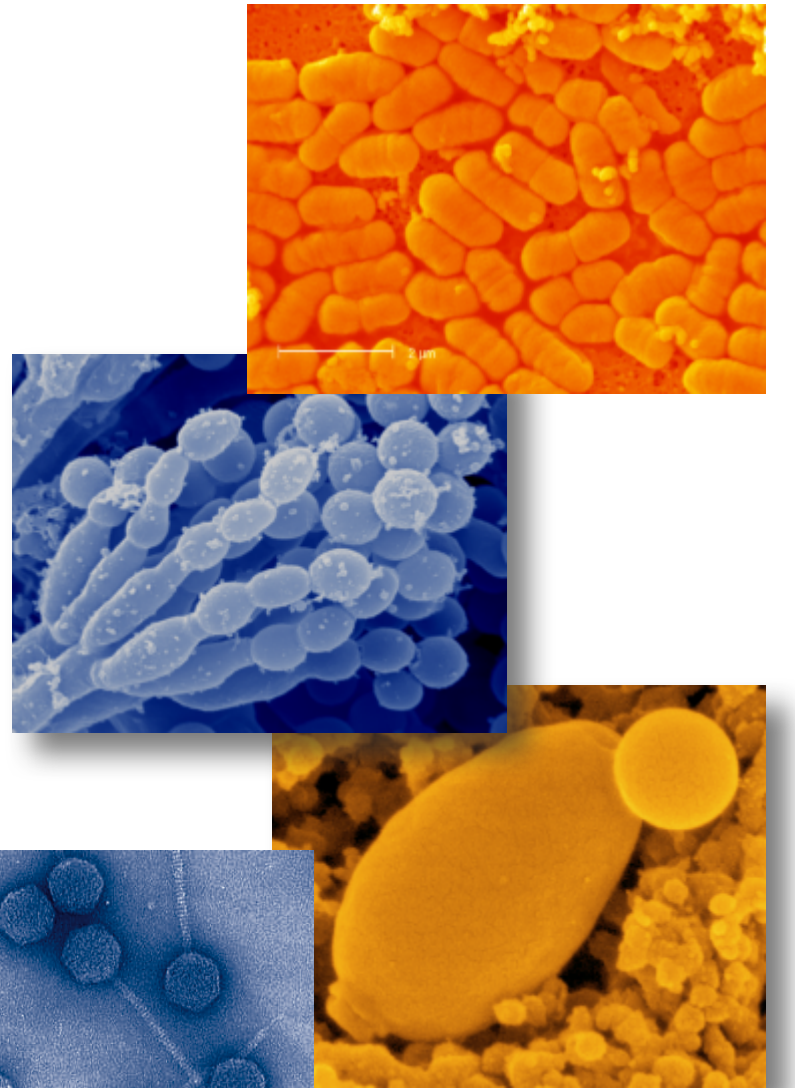
  Fusarium solani ..... Trichothecium domesticum

- Yeasts

  Candida famata .....Williopsis mrakii

- Virus

  Bacteriophages – Baculovirus -Potovirus



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Safety Assessment Guidelines and Guidance

Safety of microbial agents intended for deliberate introduction in the food chain and notified to EC-EFSA:

- for the consumer
- for the animals (when applicable)
- for the user/worker,
- for the environment

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Risk Assessment of Microbial products

- Microbial Agent
- Manufacturing process
- Product
  - Metabolism and residue studies
  - Toxicological studies
    - Acute toxicity
    - Genotoxicity studies (mutagenicity, clastogenicity)
    - Subchronic oral toxicity
    - Chronic oral toxicity/carcinogenicity
    - Reproduction toxicity including teratogenicity
  - Known toxins and virulence factors
  - Antibiotic susceptibility
  - Antibiotic production

How to assess the safety of a microbial product?

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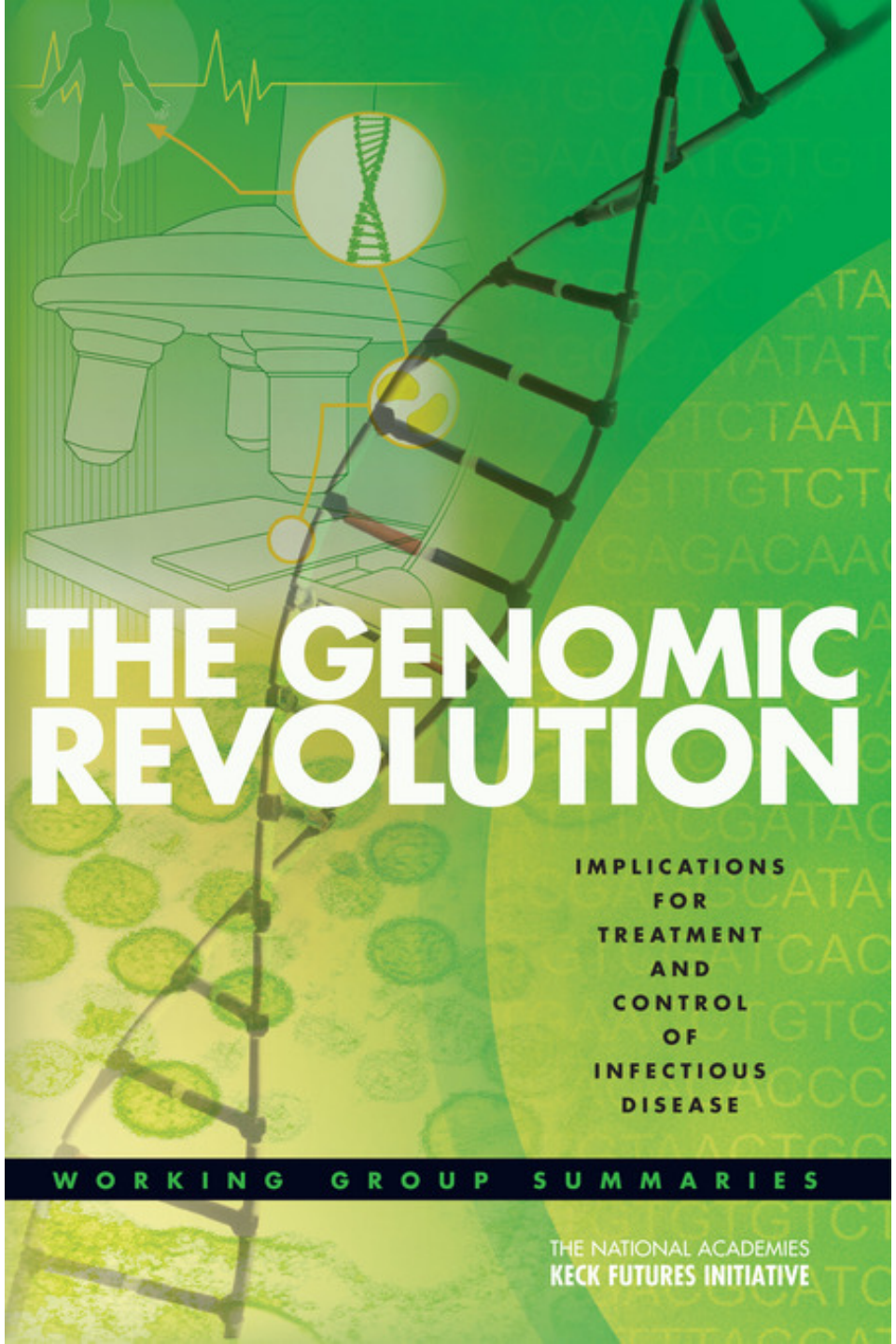

Platform	Read length (bp)	Throughput	Reads	Runtime	Error profile	Instrument cost (US$)	Cost per Gb (US$, approx.)
Sequencing by ligation							
SOLiD 5500 Wildfire	50 (SE)	80 Gb	~700 M*	6 d*	≤0.1%, AT bias‡	NA§	$130‡
	75 (SE)	120 Gb					
	50 (SE)*	160 Gb*					
SOLiD 5500 xl	50 (SE)	160 Gb	~1.4 B*	10 d*	≤0.1%, AT bias‡	$251,000‡	$70‡
	75 (SE)	240 Gb					
	50 (SE)*	320 Gb*					
BGISEQ-500 FCS[155]	50–100 (SE/PE)*	8–40 Gb*	NA‖	24 h*	≤0.1%, AT bias‡	$250 (REF. 155)	NA‖
BGISEQ-500 FCL[155]	50–100 (SE/PE)*	40–200 Gb*	NA‖	24 h*	≤0.1%, AT bias‡	$250,000 (REF. 155)	NA‖
Sequencing by synthesis: CRT							
Illumina MiniSeq Mid output	150 (SE)*	2.1–2.4 Gb*	14–16 M*	17 h*	<1%, substitution‡	$50,000 (REF. 118)	$200–300 (REF. 118)
Illumina MiniSeq High output	75 (SE)	1.6–1.8 Gb	22–25 M (SE)*	7 h	<1%, substitution‡	$50,000 (REF. 118)	$200–300 (REF. 118)
	75 (PE)	3.3–3.7 Gb	44–50 M (PE)*	13 h			
	150 (PE)*	6.6–7.5 Gb*		24 h*			
Illumina MiSeq v2	36 (SE)	540–610 Mb	12–15 M (SE)	4 h	0.1%, substitution‡	$99,000‡	~$1,000
	25 (PE)	750–850 Mb	24–30 M (PE)*	5.5 h			$996
	150 (PE)	4.5–5.1 Gb		24 h			$212
	250 (PE)*	7.5–8.5 Gb*		39 h*			$142‡
Illumina MiSeq v3	75 (PE)	3.3–3.8 Gb	44–50 M (PE)*	21–56 h*	0.1%, substitution‡	$99,000‡	$250
	300 (PE)*	13.2–15 Gb*					$110‡
Illumina NextSeq 500/550 Mid output	75 (PE)	16–20 Gb	Up to 260 M (PE)*	15 h	<1%, substitution‡	$250‡	$42
	150 (PE)*	32–40 Gb*		26 h*			$40‡
Illumina NextSeq 500/550 High output	75 (SE)	25–30 Gb	400 M (SE)*	11 h	<1%, substitution‡	$250‡	$43
	75 (PE)	50–60 Gb	800 M (PE)*	18 h			$41
	150 (PE)*	100–120 Gb*		29 h*			$33‡
Illumina HiSeq2500 v2 Rapid run	36 (SE)	9–11Gb	300 M (SE)*	7 h	0.1%, substitution‡	$690‡	$230
	50 (PE)	25–30 Gb	600 M (PE)*	16 h			$90
	100 (PE)	50–60 Gb		27 h			$52
	150 (PE)	75–90 Gb		40 h			$45
	250 (PE)*	125–150 Gb*		60 h*			$40‡
Illumina HiSeq2500 v3	36 (SE)	47–52 Gb	1.5 B (SE)	2 d	0.1%, substitution‡	$690‡	$180
	50 (PE)	135–150 Gb	3 B (PE)*	5.5 d			$78
	100 (PE)*	270–300 Gb		11 d*			$45‡
Illumina HiSeq2500 v4	36 (SE)	64–72 Gb	2 B (SE)	29 h	0.1%, substitution‡	$690‡	$150
	50 (PE)	180–200 Gb	4 B (PE)*	2.5 d			$58
	100 (PE)	360–400 Gb		5 d			$45
	125 (PE)*	450–500 Gb*		6 d*			$30‡
Illumina HiSeq3000/4000	50 (SE)	105–125 Gb	2.5 B (SE)*	1–3.5 d*	0.1%, substitution‡	$740/$900 (REF. 156)	$50
	75 (PE)	325–375 Gb					$31
	150 (PE)*	650–750 Gb*					$22 (REF. 157)

Platform	Read length (bp)	Throughput	Reads	Runtime	Error profile	Instrument cost (US$)	Cost per Gb (US$, approx.)
Sequencing by synthesis: SNA (cont.)							
Illumina HiSeq X	150 (PE)*	800–900 Gb per flow cell*	2.6–3 B (PE)*	<3 d*	0.1%, substitution‡	$1,000‡,¶	$7.0‡
Qiagen GeneReader	NA‖	12 genes; 1,250 mutations[22]	NA‖	Several days[22]	Similar to other SBS systems[22]	NA‖	$400–$600 per panel[22]
Sequencing by synthesis: SNA							
454 GS Junior	Up to 600; 400 average (SE, PE)*	35 Mb*	~0.1 M*	10 h*	1%, indel‡	NA§	$40,000‡
454 GS Junior+	Up to 1,000; 700 average (SE, PE)*	70 Mb*	~0.1 M*	18 h*	1%, indel‡	$108,000‡	$19,500‡
454 GS FLX Titanium XLR70	Up to 600; 450 mode (SE, PE)*	450 Mb*	~1 M*	10 h*	1%, indel‡	NA§	$15,500‡
454 GS FLX Titanium XL+	Up to 1,000; 700 mode (SE, PE)*	700 Mb*	~1 M*	23 h*	1%, indel‡	$450,000‡	$9,500‡
Ion PGM 314	200 (SE)	30–50	400,000–550,000*	23 h	1%, indel‡	$49‡	$25–3,500‡
	400 (SE)	60–100 Mb*		3.7 h*			
Ion PGM 316	200 (SE)	300–500 Mb	2–3 M*	3 h	1%, indel‡	$49‡	$700–1,000‡
	400 (SE)*	600 Mb–1 Gb*		4.9 h*			
Ion PGM 318	200 (SE)	600 Mb–1 Gb	4–5.5 M*	4 h	1%, indel‡	$49‡	$450–800‡
	400 (SE)*	1–2 Gb*		7.3 h*			
Ion Proton	Up to 200 (SE)	Up to 10 Gb*	60–80 M*	2–4 h*	1%, indel‡	$224‡	$80‡
Ion S5 520	200 (SE)	600 Mb–1 Gb	3–5 M*	2.5 h	1%, indel‡	$65 (REF. 158)	$2,400*
	400 (SE)*	1.2–2 Gb*		4 h*			$1,200*
Ion S5 530	200 (SE)	3–4 Gb	15–20 M*	2.5 h	1%, indel‡	$65 (REF. 158)	$950*
	400 (SE)*	6–8 Gb*		4 h*			$475*
Ion S5 540	200 (SE)*	10–15 Gb*	60–80 M*	2.5 h*	1%, indel‡	$65 (REF. 158)	$300*
Single-molecule real-time long reads							
Pacific BioSciences RS II	~20 Kb	500 Mb–1 Gb*	~55,000*	4 h*	13% single pass, ≤1% circular consensus read, indel‡	$695‡	$1,000‡
Pacific Biosciences Sequel	8–12 Kb[69]	3.5–7 Gb*	~350,000*	0.5–6 h*	NA‖	$350 (REF. 69)	NA‖
Oxford Nanopore MK 1 MinION	Up to 200 Kb[159]	Up to 1.5 Gb[159]	>100,000 (REF. 159)	Up to 48 h[160]	~12%, indel[159]	$1,000*	$750*
Oxford Nanopore PromethION	NA‖	Up to 4 Tb*	NA‖	NA‖	NA‖	$75*	NA‖
Synthetic long reads							
Illumina Synthetic Long-Read	~100 Kb synthetic length*	See HiSeq 2500	See HiSeq 2500	See HiSeq 2500	See HiSeq 2500 (possible barcoding and partitioning errors)	No additional instrument required	~$1,000*
10X Genomics	Up to 100 Kb synthetic length*	See HiSeq 2500	See HiSeq 2500	See HiSeq 2500	See HiSeq 2500 (possible barcoding and partitioning errors)	$75 (REFS 72,161)	See HiSeq 2500 +$500 per sample[161]

Goodwin, McPherson and McCombie
NATURE REVIEWS | GENETICS VOLUME 17 | JUNE 2016

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SOLiD 5500 Wildfire	50 (SE)	80 Gb	~700 M*	6 d*	≤0.1%, AT bias‡	NA§	$130‡
	75 (SE)	120 Gb					
	50 (SE)*	160 Gb*					
SOLiD 5500 xl	50 (SE)	160 Gb	~1.4 B*	10 d*	≤0.1%, AT bias‡	$251,000‡	$70‡
	75 (SE)	240 Gb					
	50 (SE)*	320 Gb*					
BGISEQ-500 FCS[155]	50–100 (SE/PE)*	8–40 Gb*	NA‖	24 h*	≤0.1%, AT bias‡	$250 (REF. 155)	NA‖
BGISEQ-500 FCL[155]	50–100 (SE/PE)*	40–200 Gb*					
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Illumina MiSeq v2	36 (SE)	540–610 Mb					
	25 (PE)	750–850 Mb					
	150 (PE)	4.5–5.1 Gb					
	250 (PE)*	7.5–8.5 Gb*					
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Illumina NextSeq 500/550 High output	75 (SE)	25–30 Gb					
	75 (PE)	50–60 Gb					
	150 (PE)*	100–120 Gb*					
Illumina HiSeq2500 v2 Rapid run	36 (SE)	9–11 Gb					
	50 (PE)	25–30 Gb					
	100 (PE)	50–60 Gb					
	150 (PE)	75–90 Gb					
	250 (PE)*	125–150 Gb*		60 h*			$40‡
Illumina HiSeq2500 v3	36 (SE)	47–52 Gb	1.5 B (SE)	2 d	0.1%, substitution‡	$690‡	$180
	50 (PE)	135–150 Gb	3 B (PE)*	5.5 d			$78
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	50 (PE)	180–200 Gb	4 B (PE)*	2.5 d			$58
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	125 (PE)*	450–500 Gb*		6 d*			$30‡
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454 GS Junior+	Up to 1,000; 700 average (SE, PE)*	70 Mb*	~0.1 M*	18 h*	1%, indel‡	$108,000‡	$19,500‡
				10 h*	1%, indel‡	NA§	$15,500‡
				23 h*	1%, indel‡	$450,000‡	$9,500‡
				23 h	1%, indel‡	$49‡	$25–3,500‡
				8.7 h*			
				8 h	1%, indel‡	$49‡	$700–1,000‡
				4.9 h*			
				4 h	1%, indel‡	$49‡	$450–800‡
				7.3 h*			
				2–4 h*	1%, indel‡	$224‡	$80‡
				2.5 h	1%, indel‡	$65 (REF. 158)	$2,400*
				4 h*			$1,200*
				2.5 h	1%, indel‡	$65 (REF. 158)	$950*
				4 h*			$475*
				2.5 h*	1%, indel‡	$65 (REF. 158)	$300*
				4 h*	13% single pass, ≤1% circular consensus read, indel‡	$695‡	$1,000‡
				0.5–6 h*	NA‖	$350 (REF. 69)	NA‖
Oxford Nanopore MK 1 MinION	Up to 200 Kb[159]	Up to 1.5 Gb[159]	>100,000 (REF. 159)	Up to 48 h[160]	~12%, indel[159]	$1,000*	$750*
Oxford Nanopore PromethION	NA‖	Up to 4 Tb*	NA‖	NA‖	NA‖	$75*	NA‖
Synthetic long reads							
Illumina Synthetic Long-Read	~100 Kb synthetic length*	See HiSeq 2500	See HiSeq 2500	See HiSeq 2500	See HiSeq 2500 (possible barcoding and partitioning errors)	No additional instrument required	~$1,000*
10X Genomics	Up to 100 Kb synthetic length*	See HiSeq 2500	See HiSeq 2500	See HiSeq 2500	See HiSeq 2500 (possible barcoding and partitioning errors)	$75 (REFS 72,161)	See HiSeq 2500 +$500 per sample[161]

UP TO 900Gb per run
COST ≥7 €/Gb
ERROR PROFILE <0.1%

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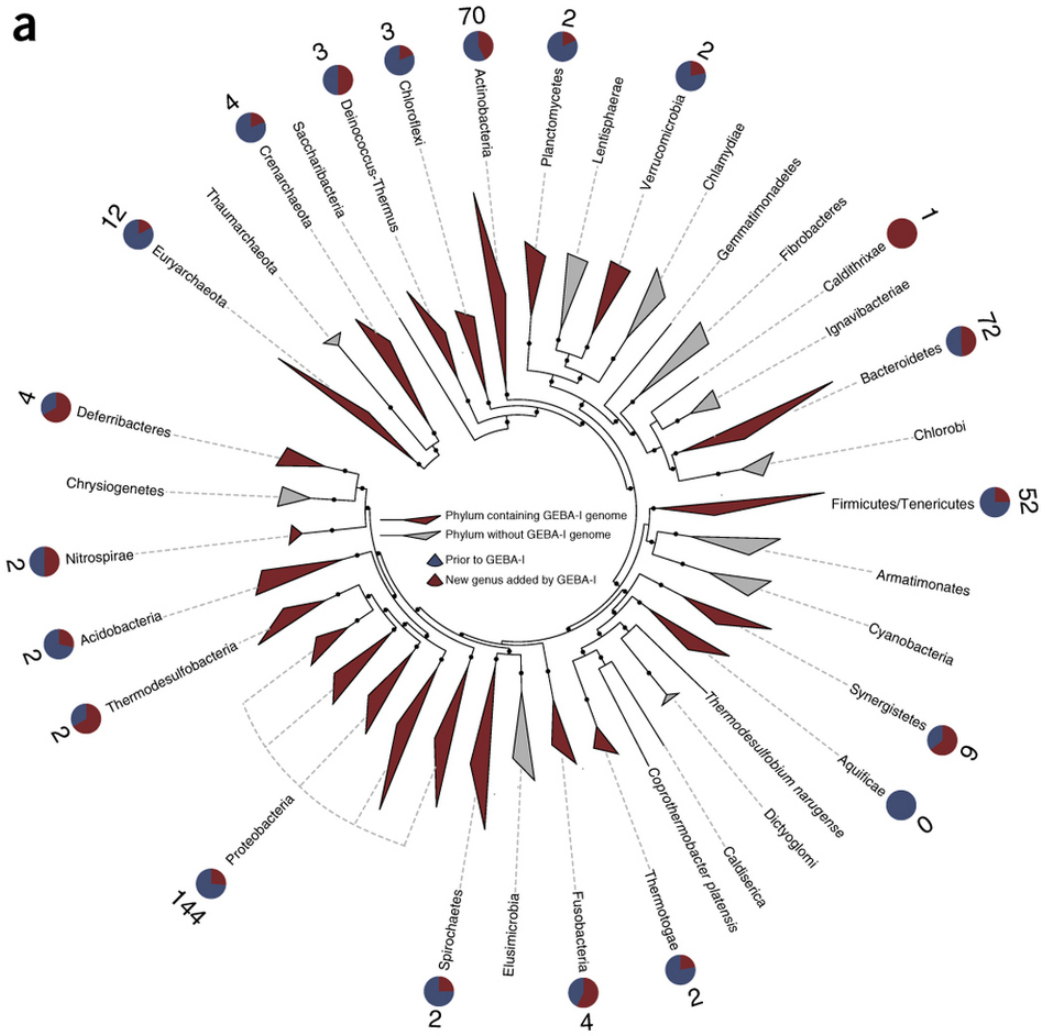
nature biotechnology

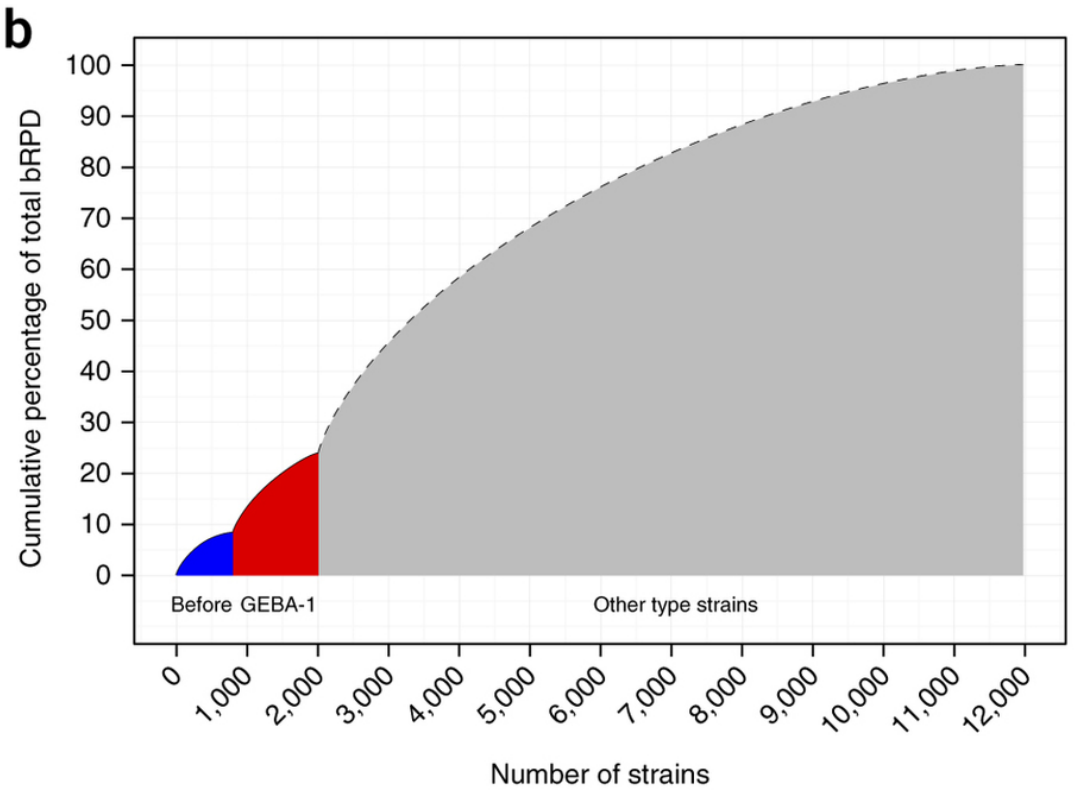
OPEN

Genomic Encyclopedia of Bacteria and Archaea (GEBA)

1,003 reference genomes of bacterial and archaeal isolates expand coverage of the tree of life

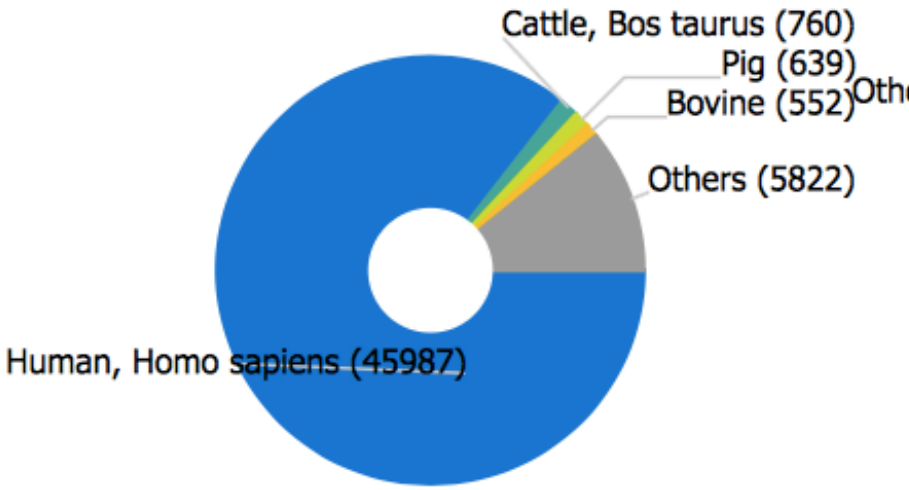
Supratim Mukherjee[1,10], Rekha Seshadri[1,10], Neha J Varghese[1], Emiley A Eloe-Fadrosh[1], Jan P Meier-Kolthoff[2], Markus Göker[2], R Cameron Coates[1,9], Michalis Hadjithomas[1], Georgios A Pavlopoulos[1], David Paez-Espino[1], Yasuo Yoshikuni[1], Axel Visel[1], William B Whitman[3], George M Garrity[4,5], Jonathan A Eisen[6], Philip Hugenholtz[7], Amrita Pati[1,9], Natalia N Ivanova[1], Tanja Woyke[1], Hans-Peter Klenk[8] & Nikos C Kyrpides[1]



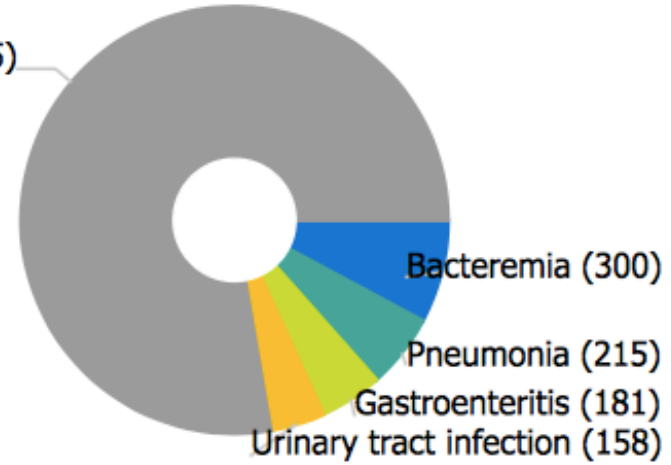


Genomes By Metadata

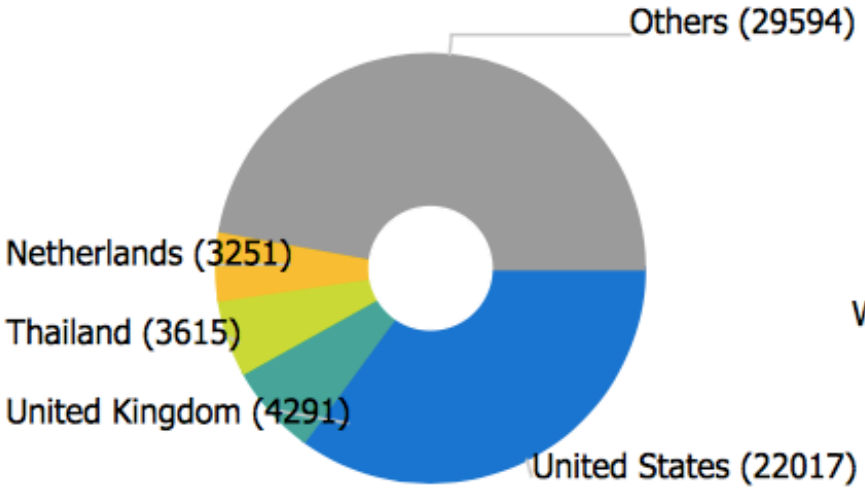
Host Name



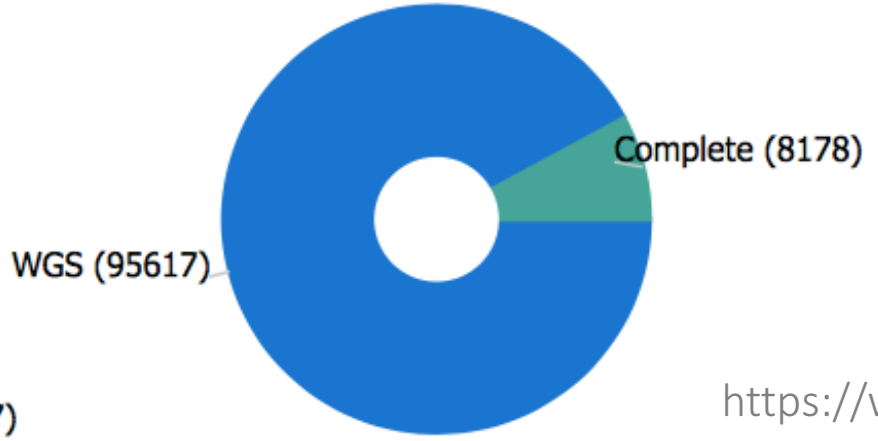
Disease



Isolation Country



Genome Status



https://www.patricbrc.org

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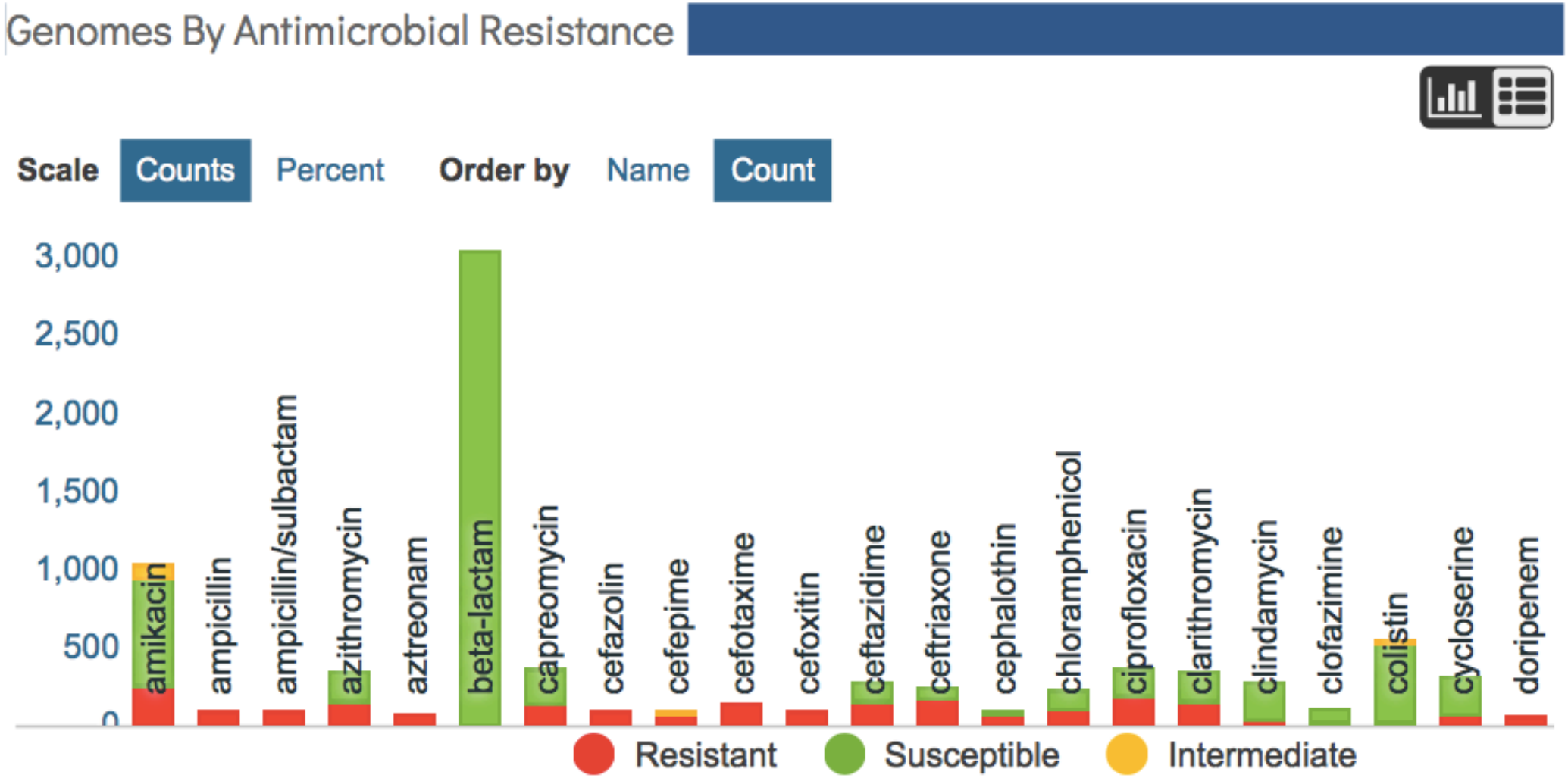
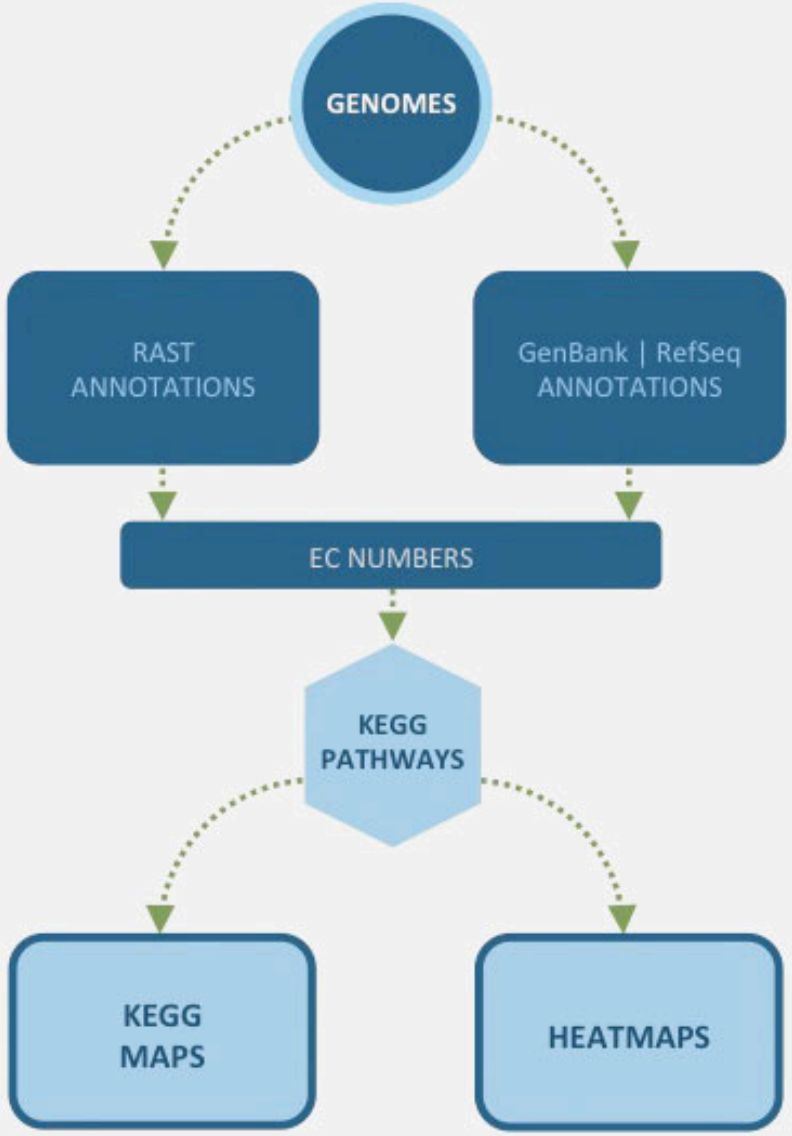


https://www.patricbrc.org

How Do We Process and Clean Up Our Data?



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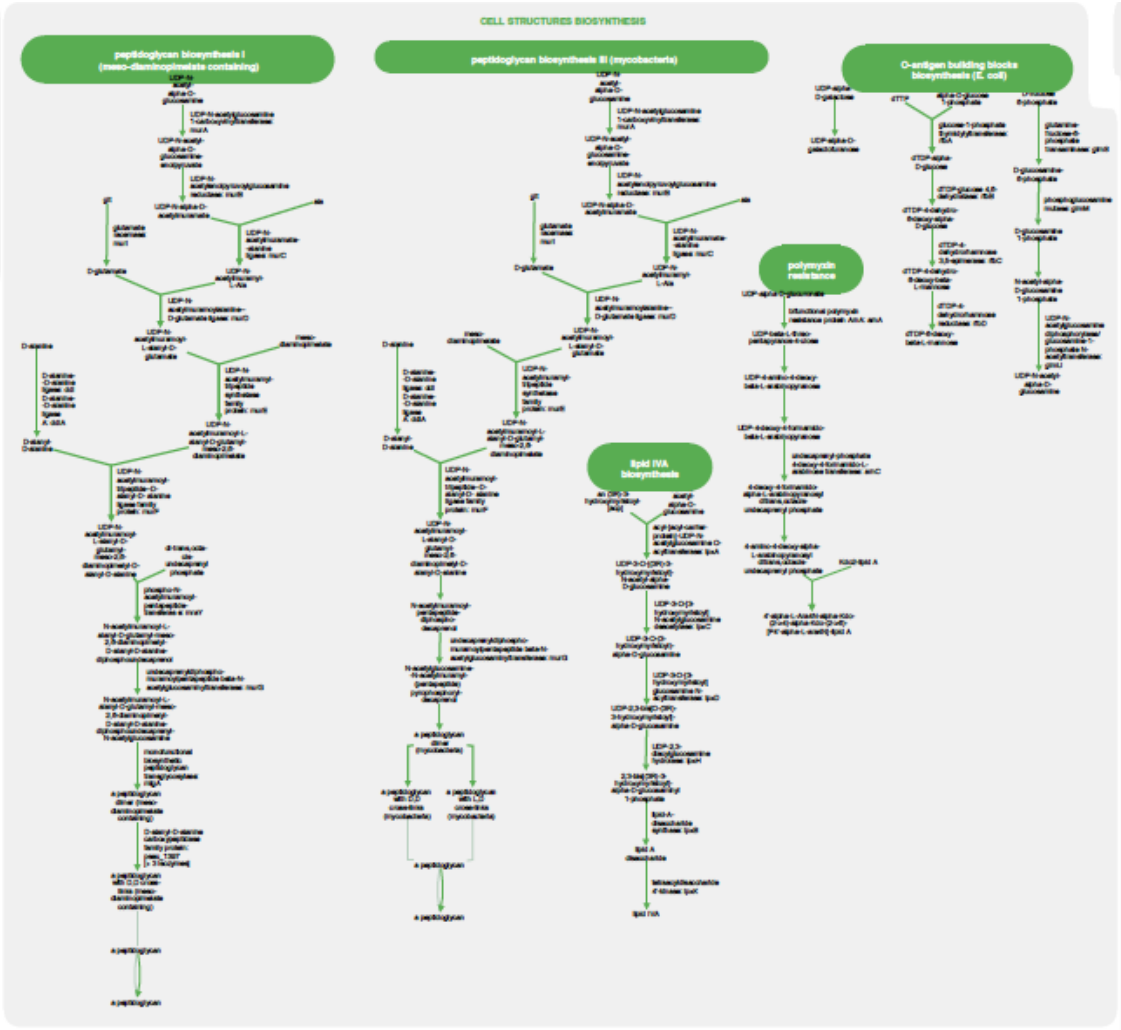

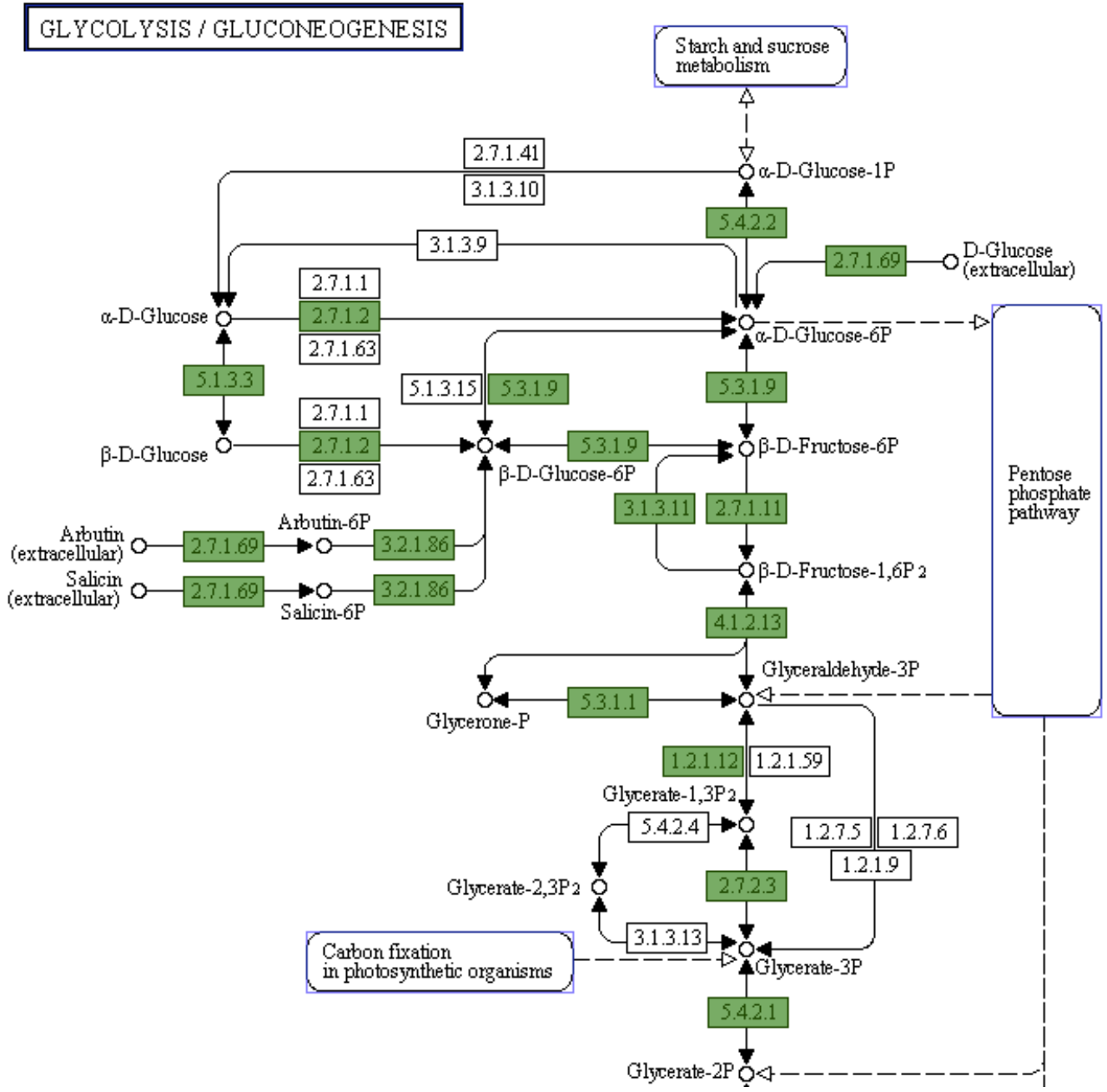

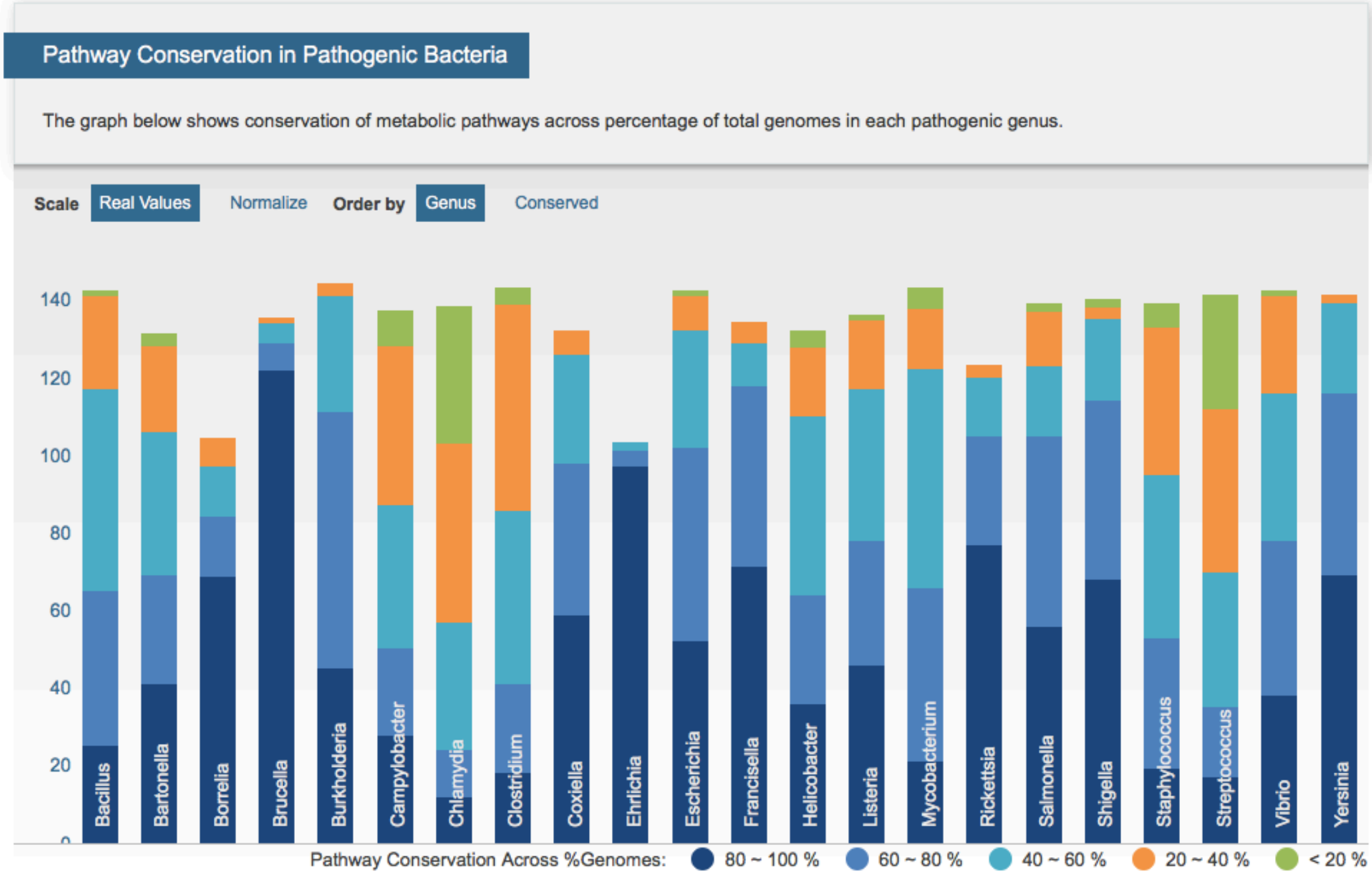

https://www.patricbrc.org

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What's needed nowadays for a whole genome sequence?

- An interesting bacterial strain
- A smart PhD student for DNA extraction
- Outsourcing the sequencing
- A PhD Student with some experience in bioinformatic

- But……

How to genomic data can be used to assess the safety of a microbial product?

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How a WGS looks like?

>g30571_seq5
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Assessment of Regulated MO

	Feed additives containing viable microorganisms		Fermentation products	
	Bacteria	Fungi - yeasts	Bacteria	Fungi - yeasts
Identification	✓	✓	✓	✓
Antimicrobial susceptibility	✓		✓	
Antimicrobial production	✓	✓	✓	✓
Toxigenicity and pathogenicity	✓	✓	✓	✓
Genetic modification			For GMMs only	For GMMs only
Absence of the production strain			✓	✓
Presence of DNA from the production strain			where relevant	where relevant

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WGS sequence in the RA of microorganims

	Feed additives containing viable microorganisms			Fermentation products		
	Bacteria	Yeasts	Fungi	Bacteria	Yeasts	Fungi
Identification	✓	✓	✓	✓	✓	✓
Antimicrobial susceptibility	✓			✓		
Antimicrobial production	✓	✓	✓	✓	✓	✓
Toxigenicity and pathogenicity	✓	✓	✓	✓	✓	✓
Genetic modification				For GMMs only	For GMMs only	For GMMs only
Absence of the production strain				✓	✓	✓
Presence of DNA from the production strain				where relevant	where relevant	where relevant

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Use of whole genome sequence for characterisation of microorganisms

- Whole genome sequence analysis (chromosome(s) and/or extra-chromosomal genetic elements e.g. plasmids) for bacterial and yeast strains intended for use either as products or production strains.
- WGS analysis is also recommended for filamentous fungi.
- WGS data provide information
  - unequivocal taxonomic identification of the strain,
  - virulence factors,
  - production of or resistance to antimicrobials of clinical relevance,
  - production of known toxic metabolites

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Quality Criteria

- the sequencing strategy and instrumentation used
- the assembly method applied (e.g., the bioinformatic approach, *de novo* or re-seq strategy)
- the statistical measure of sequence quality (e.g., number of reads, coverage, N50 and K-mer)
- the number of contigs and scaffolds required to represent the genome, their absolute length and their length relative to the genome size
- the annotation protocol used
- for fungi: information on the quality of the annotations obtained from relevant databases (e.g., BUSCO)

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Identification

- Bacteria: Data from whole genome sequence (WGS)
  - 16S rRNA gene, housekeeping genes
  - computational approach for taxonomic assignments (e.g., phylogenomics or average nucleotide identity [ANI]).
- Yeasts: Data from whole genome sequence (WGS)
  - phylogenomic analysis (e.g. using a concatenation of several conserved genes to produce a phylogeny against available related genomes).
- Filamentous fungi:
  - When WGS is available, a phylogenomic analysis comparing the genome against available related genomes.

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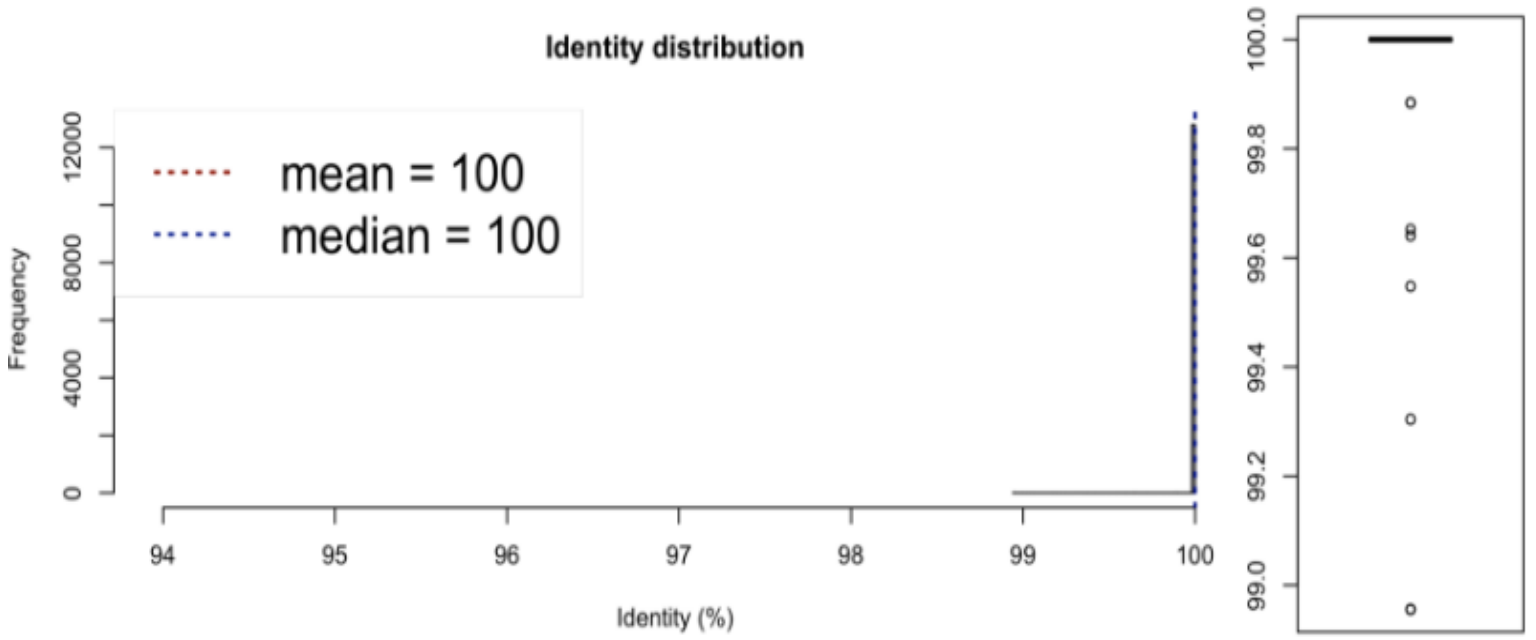
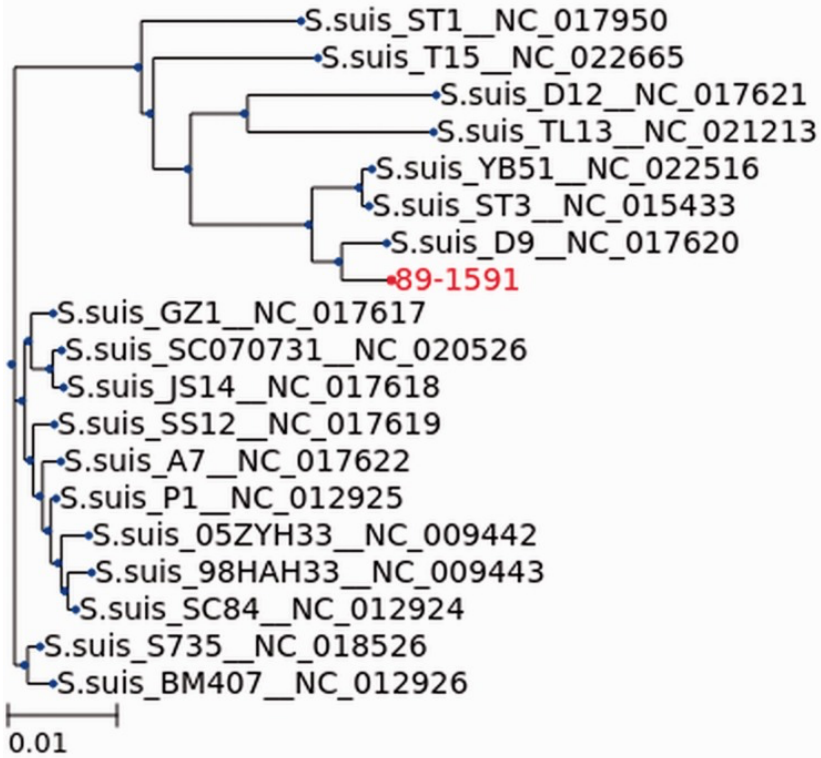
ANI: Average Nuceoltide Identity

Average nucleotide identity (ANI), calculated from pair-wise comparisons of all sequences shared between any two strains, is ciìonsidered as the new metrics for bacterial species definition and classification.

菌株名/Strain Name	菌株ID/Strain ID in NCBI	ANI value comparing with query strain
S.suis_D9	NC_017620	99.05%
S.suis_YB51	NC_022516	98.70%
S.suis_ST3	NC_015433	98.67%
S.suis_BM407	NC_012926	96.24%
S.suis_S735	NC_018526	96.21%
S.suis_SS12	NC_017619	96.20%
S.suis_P1	NC_012925	96.18%
S.suis_GZ1	NC_017617	96.13%
S.suis_A7	NC_017622	96.11%
S.suis_SC070731	NC_020526	96.06%
S.suis_SC84	NC_012924	96.05%
S.suis_JS14	NC_017618	96.04%
S.suis_ST1	NC_017950	96.00%
S.suis_98HAH33	NC_009443	95.94%
S.suis_05ZYH33	NC_009442	95.88%
S.suis_T15	NC_022665	95.87%
S.suis_D12	NC_017621	95.53%
S.suis_TL13	NC_021213	95.49%



3.进化关系树/Phylogenetic tree based on ANI matrix



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Phylogenomics

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Antimicrobial Susceptibility

- Strains of bacteria carrying an acquired resistance to antimicrobial(s) should not be used, unless it can be demonstrated that it is a result of chromosomal mutation(s).

- Antimicrobial Resistance as a food risk

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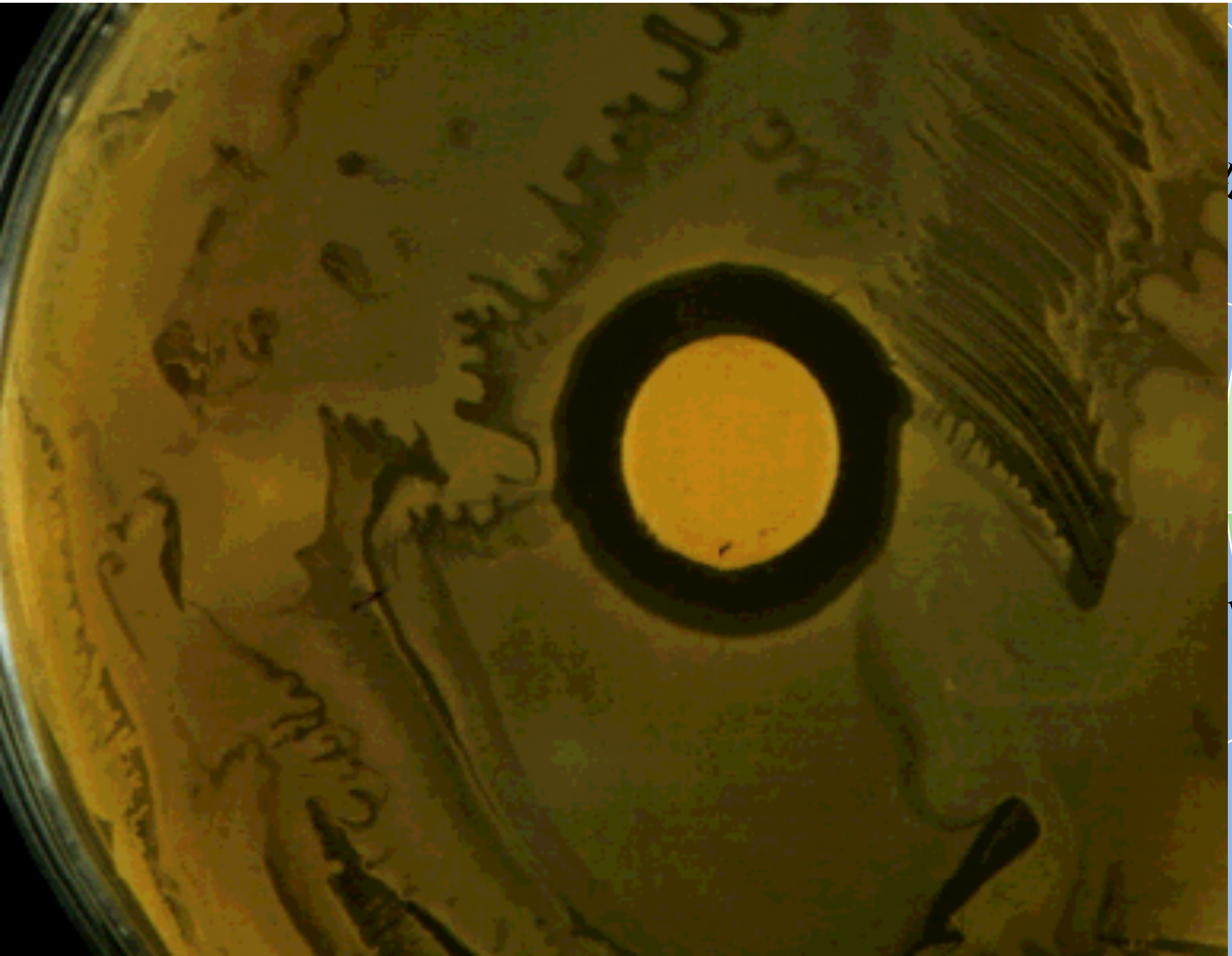
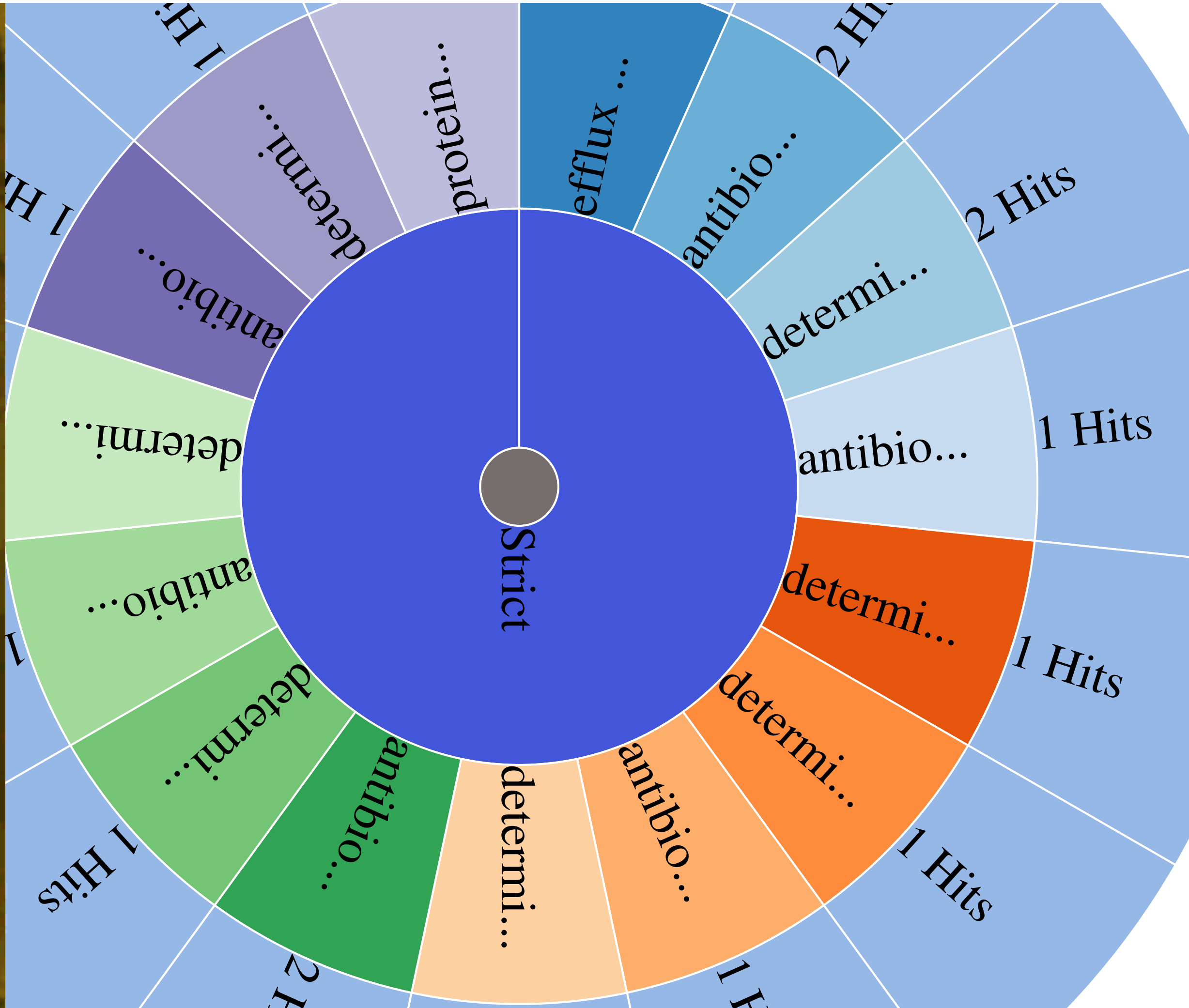
Absence of acquired antimicrobial resistances in bacteria: combined used of phenotypic and genomic data





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AM susceptibility by WGS interrogation

- Presence of genes coding for or contributing to resistance to antimicrobials relevant to their use in humans and animals (CIAs or HIAs).
- Data focusing on complete genes coding for resistance to antimicrobials.
- Data should include at least the gene identification, function of the encoded protein, percentage of identity and e-value.

https://card.mcmaster.ca/

http://en.mediterranee-infection.com/article.php?laref=283%26titre=arg-annot

https://cge.cbs.dtu.dk/services/ResFinder/

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Antimicrobial Resistance Gene Search

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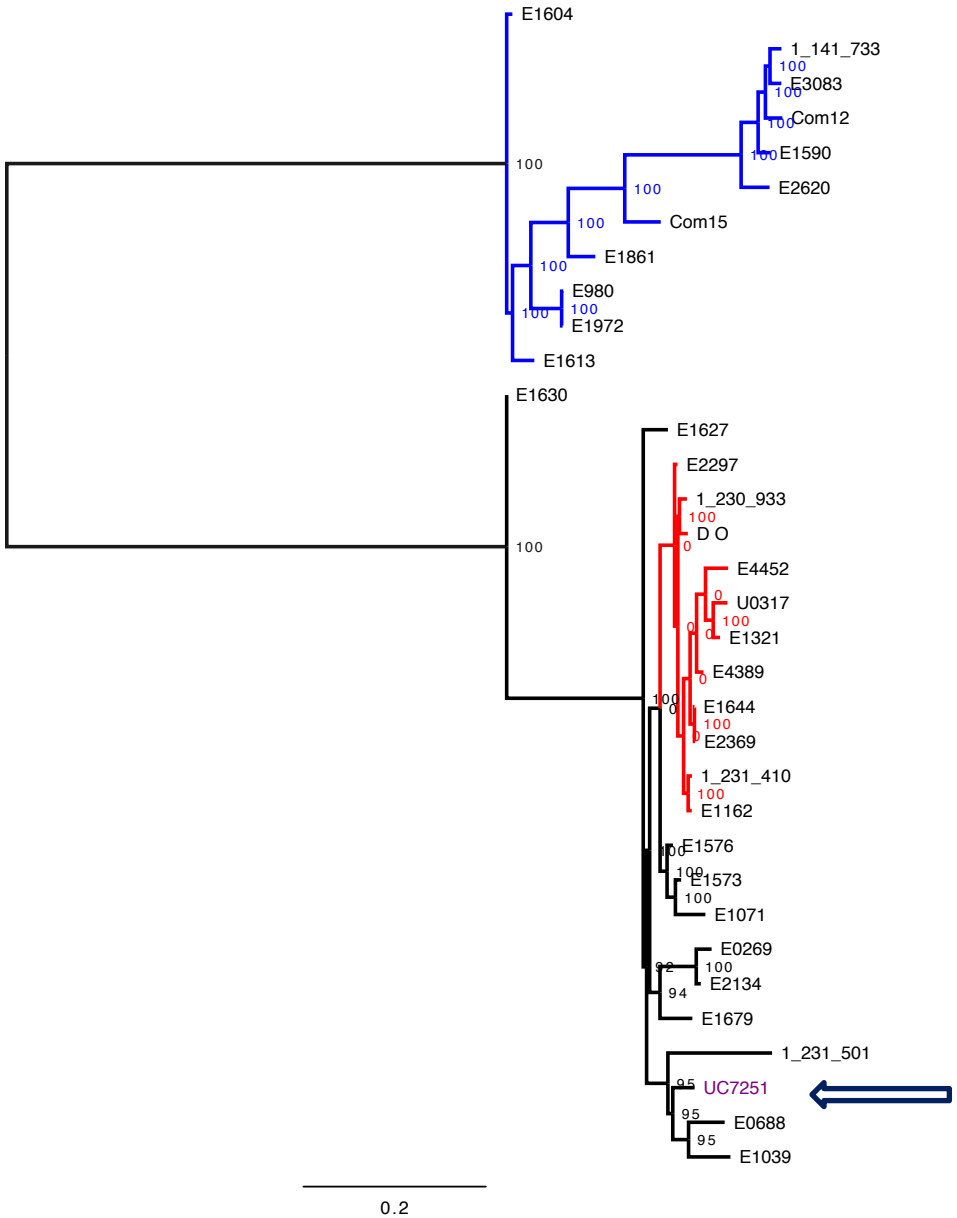
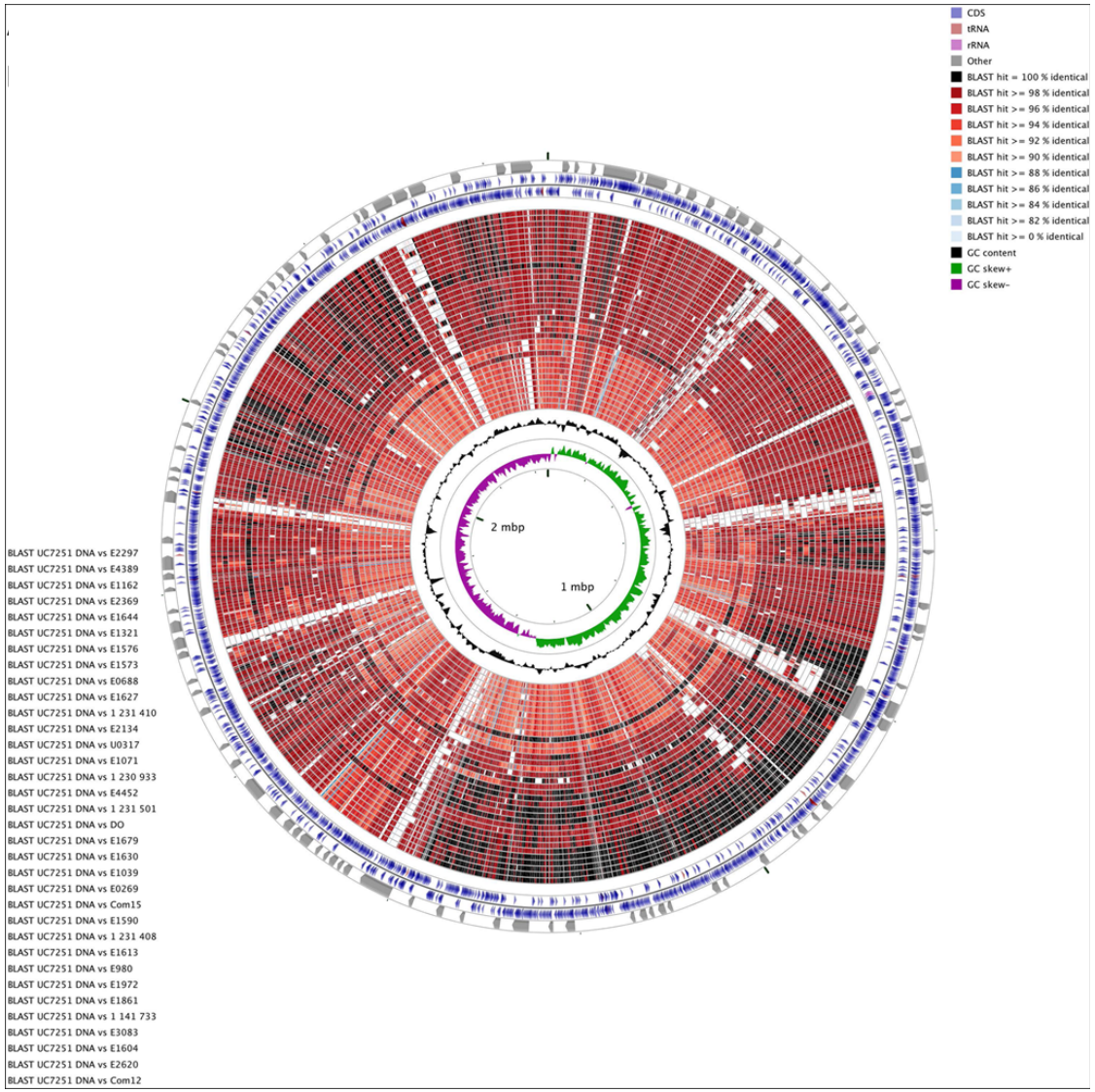
Genome and phylogeny





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Virulence

- WGS analysis should be used to identify genes coding for known virulence factors. For this purpose, comparison against specific up-to-date databases (e.g. VFDB, PAI DB, MvirDB) should be performed.
  - Virulence factors
  - Toxins and secondary metabolites

http://www.mgc.ac.cn/VFs/main.htm

http://www.paidb.re.kr/about_paidb.php

http://mvirdb.llnl.gov

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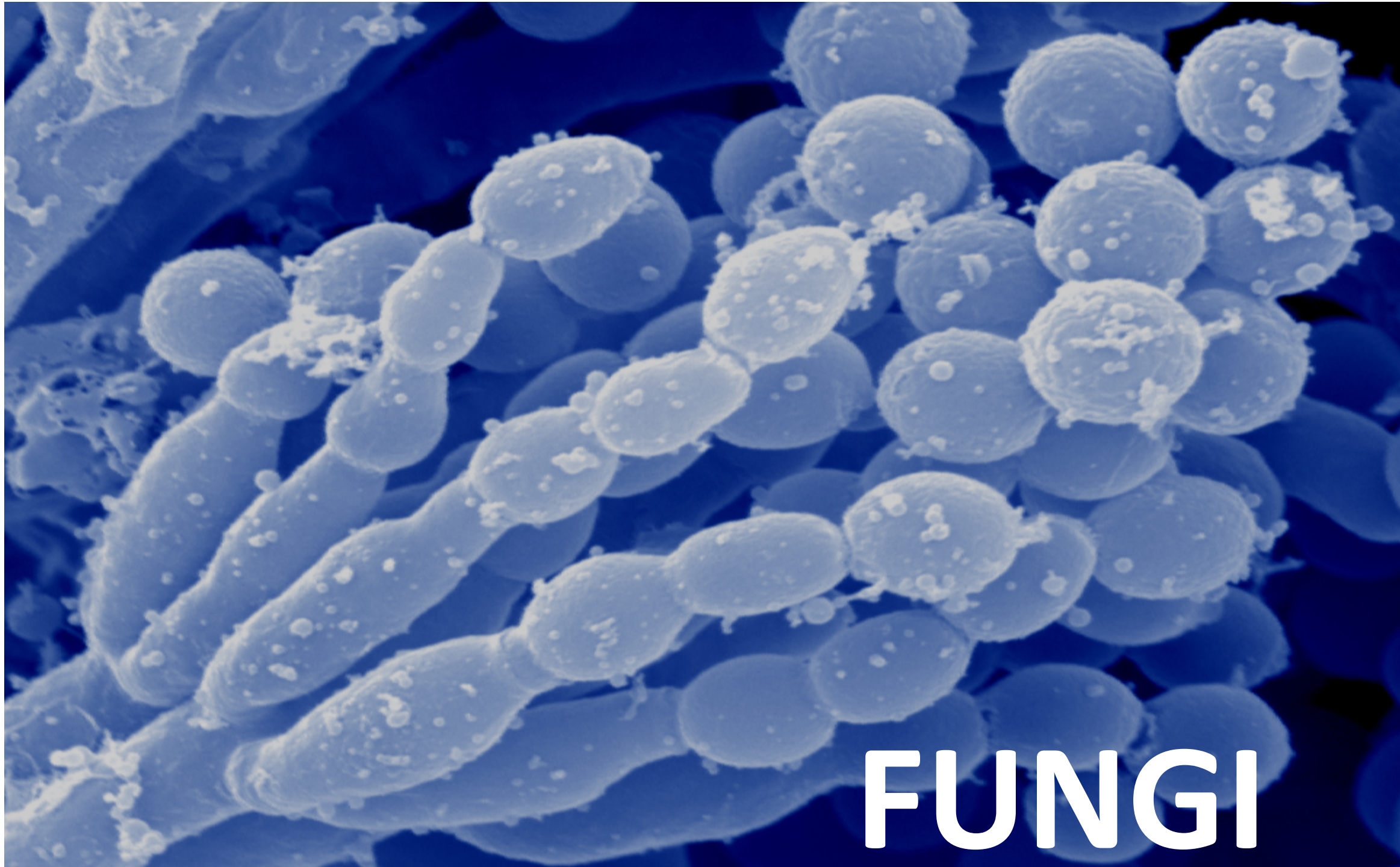
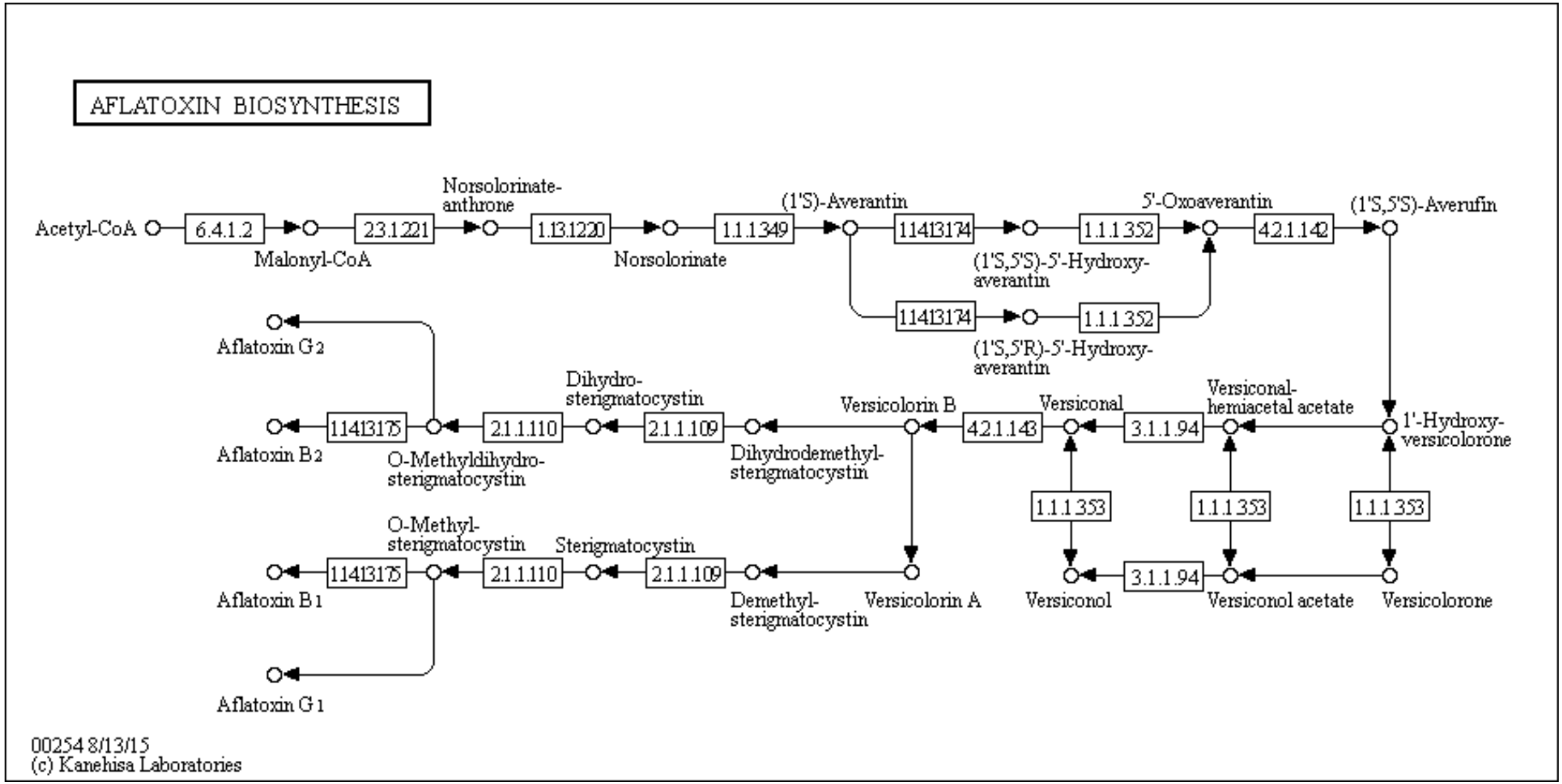
Pathways reconstruction


FUNGI


AFLATOXIN BIOSYNTHESIS
Acetyl-CoA
6.4.1.2
Malonyl-CoA
2.3.1.221
Norsolorinate-anthrone
1.13.1220
Norsolorinate
1.1.1.349
(1'S)-Averantin
1.1.1.352
(1'S,5'S)-5'-Hydroxy-averantin
5'-Oxoaverantin
4.2.1.142
(1'S,5'S)-Averufin
1.1.1.352
(1'S,5'R)-5'-Hydroxy-averantin
Aflatoxin G2
Dihydro-sterigmatocystin
2.1.1.109
Versicolorin B
4.2.1.143
Versiconal
3.1.1.94
Versiconal-hemiacetal acetate
1'-Hydroxy-versicolorone
1.1.1.353
1.1.1.353
1.1.1.353
Aflatoxin B2
2.1.1.110
O-Methyldihydro-sterigmatocystin
Dihydrodemethyl-sterigmatocystin
O-Methyl-sterigmatocystin
Sterigmatocystin
2.1.1.109
Demethyl-sterigmatocystin
Versicolorin A
Versiconol
3.1.1.94
Versiconol acetate
Versicolorone
Aflatoxin B1
2.1.1.110
Aflatoxin G1
00254 8/13/15
(c) Kanehisa Laboratories

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WGS and MICs

- MIC > cut-off value for one or more antimicrobials requires further investigation using genomic data :
  - If no known AMR gene is identified that can be linked to the phenotype: OK
  - If the phenotypic resistance related to the presence of a known AMR gene: HAZARD
- If the genetic analysis reveals AMR genes for antimicrobials considered to be CIAs or HIAs (WHO, 2016), the MIC values should be determined and compared with values in the literature:
  - If MIC≤ (reference values), the likelihood of the AMR gene to become active should be assessed (e.g. based on sequence comparison with active genes)
  - If MIC> (reference values), this is considered as a hazard.

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GMM: assessment of genetic modification by WGS analysis

- Detailed information, including a map or graphic presentation of all genomic regions (chromosome, contig or plasmid) harbouring genetic modifications, indicating:
- the open reading frames (ORF) actually inserted, modified or deleted. the non-coding sequence(s) inserted/deleted/modified. The role and function of these sequences (e.g. promoters, terminators) should be indicated
- Comparison the WGS of the GMM with that of the non-modified parental or recipient strain.

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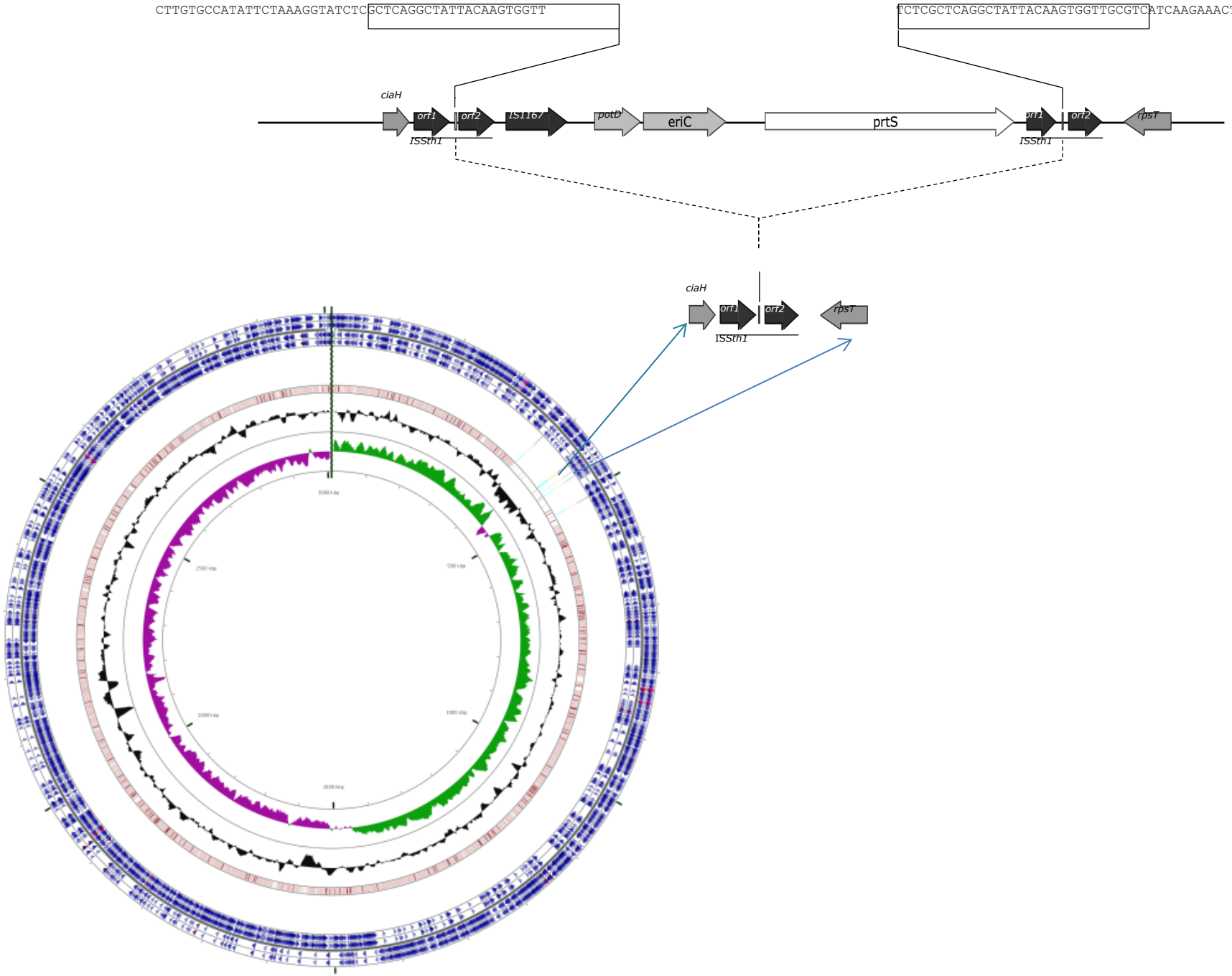

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Conclusions