Session 5.1 – Annotation

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Bacterial genome characteristics

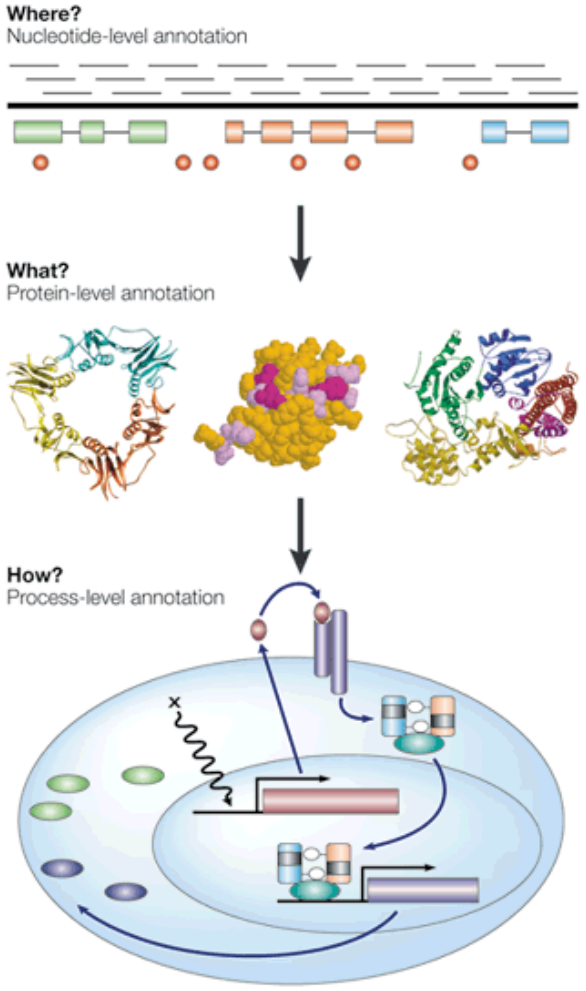
- **A bacterial genome is a single "circular" DNA molecule with several million base pairs in size**
- **Bacteria can contains plasmids (small and circular DNA molecules, that contain (usually) non-essential genes)**
- **Genomes contain a few thousand genes.**
- **"Gene density" is much higher than in humans, one million base pairs of bacterial DNA contains about 500 to 1000 genes.**
  - bacterial genes have no introns,
  - the average number of codons in bacterial genes is less than in human genes
  - neighboring genes are very close together throughout the genome

Annotation

Genome annotation is the process of **attaching biological (and positional) information to sequences.** It consists of three main steps:

- identifying portions of the genome that **do not code for proteins**
- Identifying coding elements on the genome, a process called **gene prediction**
- attaching **biological information** to these elements

https://galaxyproject.github.io/training-material/topics/genome-annotation/tutorials/genome-annotation/tutorial.html



Lincoln Stein, 2001. Nat.Review Nature R

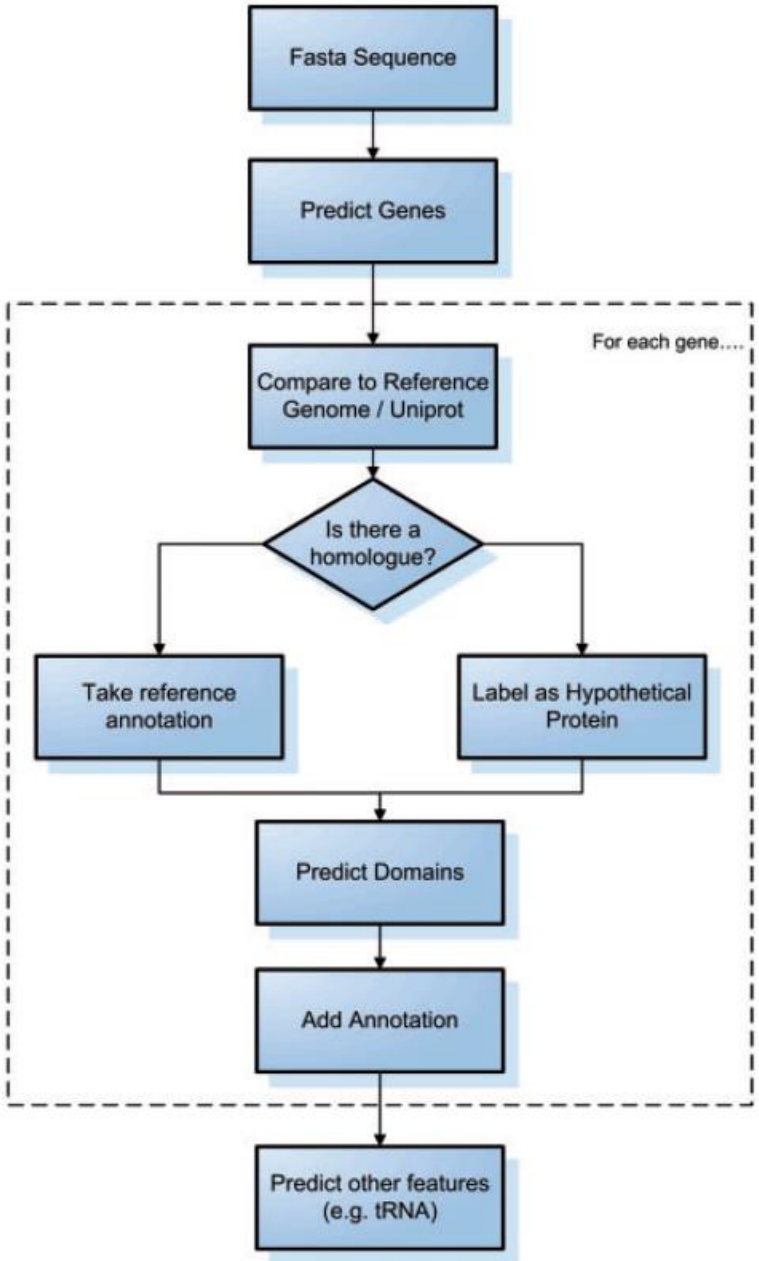
Main categories

- **Structural annotation** – Finding genes and other biologically relevant sites with **specific locations but unknown function**
  - ORFs
  - Coding sequences(cds)
  - Promoters and regulatory regions

- **Functional annotation** – Elements are used in database searches to attach biologically relevant information to whole sequence and individual objects

Automatic annotation

- **Exponential submission of bacterial genomes**
- **Databases**
  - Uniprot
  - RefSeq
  - Encyclopedia of DNA elements (ENCODE)
  - Entrez Gene
  - Ensembl
  - GENCODE
  - Gene Ontology Consortium
  - GeneRIF
  - Vertebrate and Genome Annotation Project (Vega)
  - Pfam
  - etc



https://www.ncbi.nlm.nih.gov/books/NBK20253/#!po=3.12500

Automatic annotation

Two strategies for identifying coding genes:

- **Sequence alignment to find known protein sequences in the contigs**
  - transfer the annotation across
    - will miss proteins not present in your database
    - may miss partial proteins
- **Ab initio gene finding o find candidate open reading frames:**
  - Build model of ribosome binding sites
  - predict coding regions
    - may choose the incorrect start codon
    - may miss atypical genes, overpredict small genes

Automatic annotation

- **tRNA:** easy to find and annotate: anti-codon
- **rRNA:** easy to find and annotate: 5s 16s 23s
- **CDS:** straightforward to find candidates
  - false positives are often small ORFs
  - wrong start codon o partial genes
  - Pseudogenes
  - assigning function is the bulk of the workload

Automatic annotation: limitations

- If sequence homologous are found, may **not be functional** homologous
- If **no homology found**- limited information can be inferred
- Incorrect annotation can be **propagated** when similarity is over part on sequence not used in annotation
  - Multidomain proteins (HMM)
- Inconsistent annotation (**Different names, same protein**)
- Same **gene name, different product** name
- Spelling mistakes
- Looking for **new genes**, not present in DDBB
- Expression experiments / Manual annotation needed

Richardson and Watson. Briefings in Bioinformatics. 2012

Automatic annotation: limitations

Inconsistent annotation

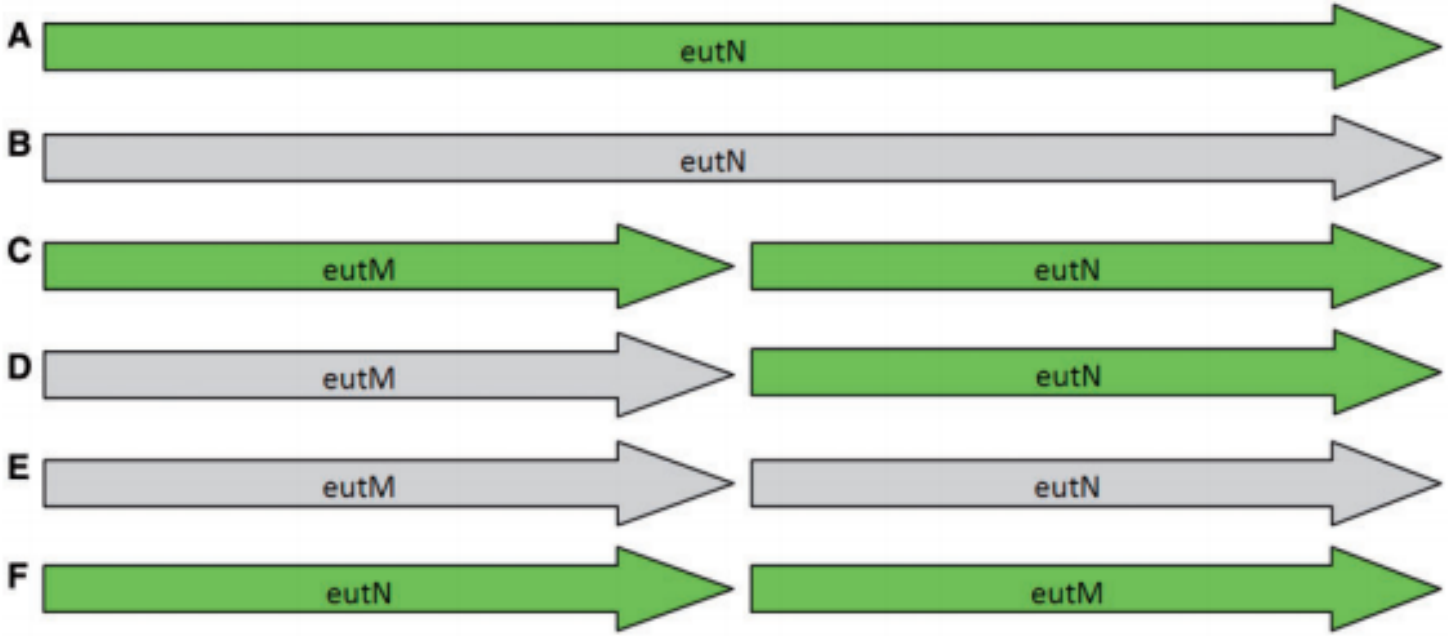


Figure 2: The six different models present across 17 RefSeq entries for Salmonella species for the eutM/eutN locus. Green indicates normal gene/CDS features, lighter grey indicates gene features annotated as pseudogenes. (A) A single intact gene of 690 bp; (B) a single pseudogene of 690 bp; (C) two short intact genes ~300 bp in length; (D) one pseudogene and one intact gene, each ~300 bp in length; (E) two pseudogenes, each 300 bp in length; and (F) two intact genes with the order reversed.

Richardson and Watson. Briefings in Bioinformatics. 2012

Automatic annotation: limitations

Inconsistent annotation

These two regions are more than 97% identical at the nucleotide level; however, the annotation differs considerably.

While E. coliK12MG1655 contains features with gene names araA, araB and araC, the equivalent features in E. coli 0157:H7 Sakai do not have those gene names and have been assigned uninformative locus tags

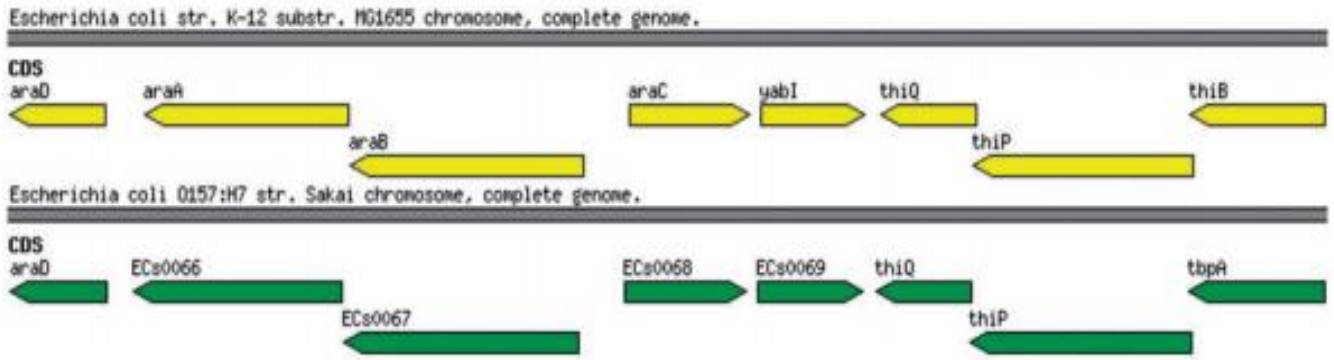


Figure 3: A syntenic block of genes showing inconsistent gene name annotations in *E.coli KI2 MGI655* and *E. coli 0157:H7 Sakai*.

Richardson and Watson. Briefings in Bioinformatics. 2012

Automatic annotation: limitations

- **Spelling mistakes**
  - There are 128 proteins in UniProt that contain the word ‘syntase’, an incorrect spelling of the word ‘synthase’
  - If a user was to visit any of these databases and search for ‘dihydrofolate synthase’ the misspelled entries would be omitted from the search results

Richardson and Watson. Briefings in Bioinformatics. 2012

Automatic annotation: limitations

- **‘Same gene name, different product name’**
  - The NCBI validation software specifically highlights when this occurs intra-genomically with the description ‘Same gene name, different product name’

Table I: Different product names assigned to features with the gene name ‘*int*’ across 17 different RefSeq entries for *Salmonella* species

Gene name	Product name	Accession
int	bacteriophage integrase	NC.003198, NC.004631, NC.015761
int	Gifsy-1 prophage Int	NC.006905
int	hypothetical protein	NC.006905
int	Integrase	NC.003198, NC.004631, NC.006511, NC.012125
int	integrase (fragment)	NC.003198
int	phage integrase family site specific recombinase	NC.006905
int	putative cytoplasmic protein	NC.006905
Int	Putative integrase	NC.003384
int	putative integrase protein	NC.006905
int	putative P4-type integrase	NC.006905
int	putative phage integrase protein	NC.006905
int	site-specific recombinase, phage integrase family	NC.012125

Richardson and Watson. Briefings in Bioinformatics. 2012

Automatic annotation: limitations

Hypothetical proteins

- These may be real genes with no known function or they may be artifacts of the gene prediction process.
- Often there are features which are only orthologous to other hypothetical features and do not contain any domains. These could either be regions with no functionality, a relic of the feature prediction software or the domains present have not been discovered yet
- Whether or not to include them is often a decision made by the annotation team and varies between groups
- **As experimental data becomes more ubiquitous evidence tags should play a larger role in annotation.**

Richardson and Watson. Briefings in Bioinformatics. 2012

Automatic annotation: limitations

Distinguishing orthologs from paralogs

orthologs tend to retain similar functions, whereas paralogs tend to diverge over time to perform different functions

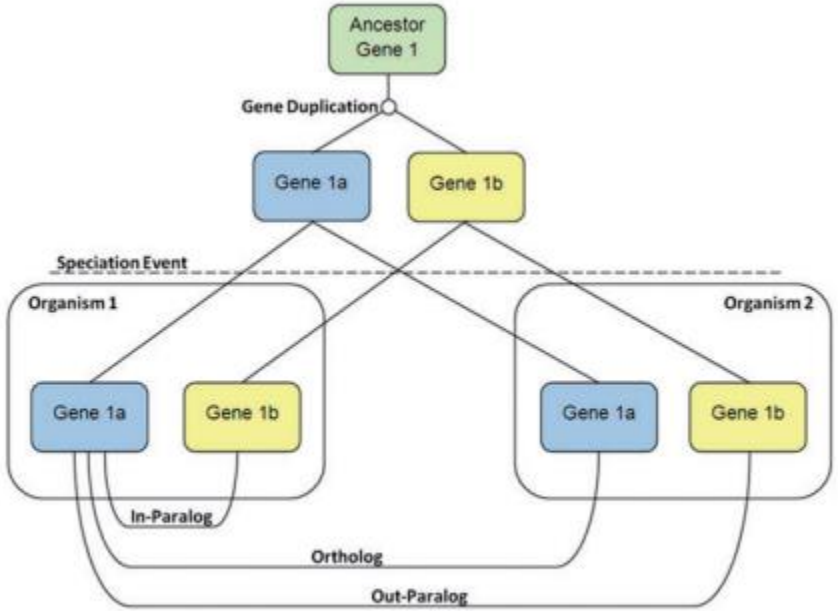
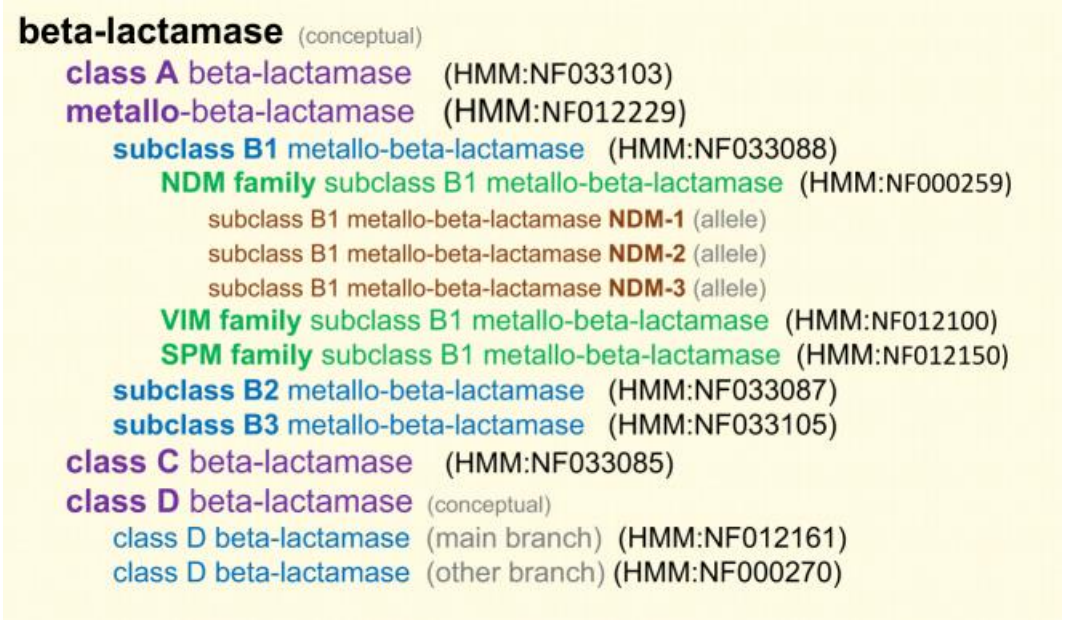


Figure 4: A diagram displaying the processes that can lead to, and define, orthologs and paralogs. Gene duplication and speciation events create complex evolutionary relationships between genes.

Richardson and Watson. Briefings in Bioinformatics. 2012

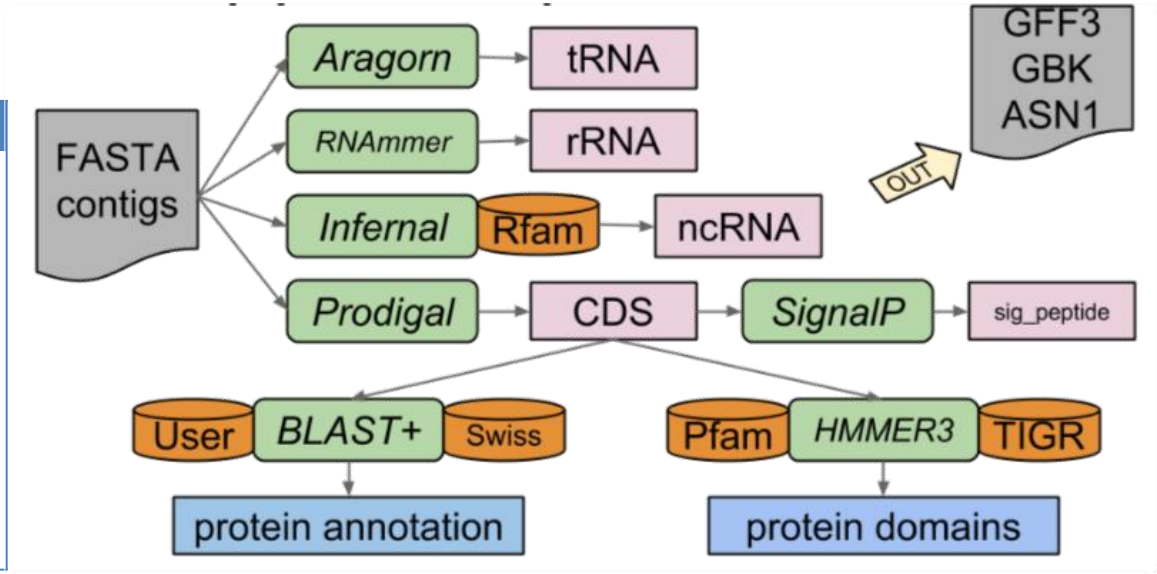
Automatic annotation: limitations

- **RefSeq is one attempt to standardize and improve the quality of genome annotation**
  - WP_ prefix. All identical proteins regardless of species
  - Standard classification



Automatic annotation: Prokka

Tool (reference)	Features predicted
Prodigal (Hyatt 2010)	Coding sequence (CDS)
RNAmmer (Lagesen et al. , 2007)	Ribosomal RNA genes (rRNA)
Aragorn (Laslett and Canback, 2004)	Transfer RNA genes
SignalP (Petersen et al. , 2011)	Signal leader peptides
Infernal (Kolbe and Eddy, 2011)	Non-coding RNA
BLAST+ (Camacho *et al.* , 2009)	Specific function or name
	Personal database



- Optional **user-provided** set of annotated proteins
- All bacterial proteins in **UniProt**
- All proteins from finished bacterial genomes in **RefSeq**
- Hidden Markov model profile databases, **Pfam and TIGRFAMs**
- Hypothetical protein

https://galaxyproject.github.io/training-material/topics/genome-annotation/tutorials/annotation-with-prokka/slides.html#8

Prokka: Sequence databases

I'll just BLAST against the non-redundant database. -- Anonymous

- Which one?
  - nucleotide (nt) or protein (nr)
  - It's actually quite redundant o only eliminates exact matching sequences
  - It's not picky o nearly anything is admitted, garbage in garbage out
- It's too big o searching takes too long

Automatic annotation: Prokka

- **Facts**
  - searching against smaller databases is faster
  - searching against similar sequences is faster
- **Idea**
  - start with small set of close proteins
  - advance to larger sets of more distant proteins
- **Prokka**
  - your own custom "trusted" set (optional)
  - core bacterial proteome (default)
  - genus specific proteome (optional)
  - whole protein HMMs: PRK clusters, TIGRfams
  - protein domain HMMs: Pfam

Prokka uses this method, but in a hierarchical manner, starting with a **smaller trustworthy database**, moving to medium sized but **domain-specific databases**, and finally to **curated models of protein families**

Automatic annotation: Prokka

Core Bacterial proteome

- **Many bacterial proteins are conserved**
  - experimentally validated o small number of them
  - good annotations
- **Prokka provides this database**
  - derived from UniProt-Swissprot
  - only bacterial proteins
  - only accept evidence level 1 (aa) or 2 (RNA)
  - reject "Fragment" entries
  - extract /gene /EC_number /product /db_xref ●
- **First step gets ~50% of the genes**
  - BLAST+ blastp, multi-threading to use all CPUs

Automatic annotation: Prokka

- **Prokka has genus specific databases**
  - aim to capture "genus specific" naming conventions
  - derived from proteins in completed genomes
  - proteins are clustered and majority annotation wins
  - some annotations are rubbish though
- **Custom model databases**
  - I took COG/PRK MSAs and made HMMs
- **Existing model databases**
  - Pfam, TIGRfams are well curated
- **And if all else fails**
  - we always have our friend "hypothetical protein"

Automatic annotation: Prokka output

Suffix	Description of file contents
.fna	FASTA file of original input contigs (nucleotide)
.faa	FASTA file of translated coding genes (protein)
.ffn	FASTA file of all genomic features (nucleotide)
.fsa	Contig sequences for submission (nucleotide)
.tbl	Feature table for submission
.sqn	**Sequin** editable file for submission
.gbk	Genbank file containing sequences and annotations
.gff	GFF v3 file containing sequences and annotations
.log	Log file of Prokka processing output
.txt	Annotation summary statistics

Annotation format: gff3

1. Seqid - name
2. Source - program
3. Type – term or SOFA sequence ontology
4. Start
5. End
6. Score
7. Strand – (+/-)
8. Phase – (0/1/2)
9. Attributes
   - Name
   - Alias
   - Parent
   - Target
   - Gap
   - Derives_from
   - Note
   - Dbxref
   - Ontology_term

```
##gff-version 3.2.1
##sequence-region ctg123 1 1497228
ctg123 . gene            1000  9000  .  +  .  ID=gene00001;Name=EDEN
ctg123 . TF_binding_site 1000  1012  .  +  .  ID=tfbs00001;Parent=gene00001
ctg123 . mRNA            1050  9000  .  +  .  ID=mRNA00001;Parent=gene00001;Name=EDEN.1
ctg123 . mRNA            1050  9000  .  +  .  ID=mRNA00002;Parent=gene00001;Name=EDEN.2
ctg123 . mRNA            1300  9000  .  +  .  ID=mRNA00003;Parent=gene00001;Name=EDEN.3
ctg123 . exon            1300  1500  .  +  .  ID=exon00001;Parent=mRNA00003
ctg123 . exon            1050  1500  .  +  .  ID=exon00002;Parent=mRNA00001,mRNA00002
ctg123 . exon            3000  3902  .  +  .  ID=exon00003;Parent=mRNA00001,mRNA00003
ctg123 . exon            5000  5500  .  +  .  ID=exon00004;Parent=mRNA00001,mRNA00002,mRNA00003
ctg123 . exon            7000  9000  .  +  .  ID=exon00005;Parent=mRNA00001,mRNA00002,mRNA00003
ctg123 . CDS             1201  1500  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1
ctg123 . CDS             3000  3902  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1
ctg123 . CDS             5000  5500  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1
ctg123 . CDS             7000  7600  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1
ctg123 . CDS             1201  1500  .  +  0  ID=cds00002;Parent=mRNA00002;Name=edenprotein.2
ctg123 . CDS             5000  5500  .  +  0  ID=cds00002;Parent=mRNA00002;Name=edenprotein.2
ctg123 . CDS             7000  7600  .  +  0  ID=cds00002;Parent=mRNA00002;Name=edenprotein.2
ctg123 . CDS             3301  3902  .  +  0  ID=cds00003;Parent=mRNA00003;Name=edenprotein.3
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ctg123 . CDS             7000  7600  .  +  1  ID=cds00003;Parent=mRNA00003;Name=edenprotein.3
ctg123 . CDS             3391  3902  .  +  0  ID=cds00004;Parent=mRNA00003;Name=edenprotein.4
ctg123 . CDS             5000  5500  .  +  1  ID=cds00004;Parent=mRNA00003;Name=edenprotein.4
ctg123 . CDS             7000  7600  .  +  1  ID=cds00004;Parent=mRNA00003;Name=edenprotein.4
```

Annotation format: gbk

- LOCUS – Annotated sequence
- DEFINITION
- ACCESION
- FEATURES
  - source
  - gene
  - CDS
    - Locus tag
    - function
    - Product
    - protein_id
    - Translation (sequence)

```
LOCUS       AF068625                200 bp    mRNA    linear   ROD 06-DEC-1999
DEFINITION  Mus musculus DNA cytosine-5 methyltransferase 3A (Dnmt3a) mRNA,
            complete cds.
ACCESSION   AF068625 REGION: 1..200
VERSION     AF068625.2  GI:6449467
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia;
            Sciurognathi; Muroidea; Muridae; Murinae; Mus.
REFERENCE   1  (bases 1 to 200)
  AUTHORS   Okano,M., Xie,S. and Li,E.
  TITLE     Cloning and characterization of a family of novel mammalian DNA
            (cytosine-5) methyltransferases
  JOURNAL   Nat. Genet. 19 (3), 219-220 (1998)
   PUBMED   9662389
REFERENCE   2  (bases 1 to 200)
  AUTHORS   Xie,S., Okano,M. and Li,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (28-MAY-1998) CVRC, Mass. Gen. Hospital, 149 13th Street,
            Charlestown, MA 02129, USA
REFERENCE   3  (bases 1 to 200)
  AUTHORS   Okano,M., Chijiwa,T., Sasaki,H. and Li,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (04-NOV-1999) CVRC, Mass. Gen. Hospital, 149 13th Street,
            Charlestown, MA 02129, USA
  REMARK    Sequence update by submitter
COMMENT     On Nov 18, 1999 this sequence version replaced gi:3327977.
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                     /mol_type="mRNA"
                     /db_xref="taxon:10090"
                     /chromosome="12"
                     /map="4.0 cM"
     gene            1..>200
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ORIGIN
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       61 gcccagcgct gaggctgcac ttttccgagg gcttgacatc agggtctatg tttaagtctt
      121 agctcttgct tacaaagacc acggcaattc cttctctgaa gccctcgcag ccccacagcg
      181 ccctcgcagc cccagcctgc
//
```

Annotation format: gbk

- LOCUS – Annotated sequence
- DEFINITION
- ACCESION
- FEATURES
  - source
  - gene
  - CDS
    - Locus tag
    - function
    - Product
    - protein_id
    - Translation (sequence)

```
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /strain="SA1"
                     /sub_species="pneumoniae"
                     /db_xref="taxon:1379688"
                     /note="contig LPSB1_2557_Contig_49"
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     CDS             415..1536
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                     unknown function"
                     /codon_start=1
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                     /product="conserved hypothetical protein"
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                     MEPEVDSRLVSLKNGKWQASNGPFEHFDGLGETGWSLLAQAVNHWHMPAAELVRPFRV
                     LPDWRLDDLMISFSVPGGGVGPHIDQYDVFIIQGMGSRRWRVGDKLPMRQFCPHPALL
                     HVDPFPPIIDEDLQPGDILYIPPGFPHDGITHETALNYSVGFRGPNGRDLISSFADYV
                     LENDLGDEHYSDPDLTCREHPGRVEEYELERLRTMMIDMIRQPEDFKQWFGSFVTTPR
                     HELDIAPAEPPYEEEEVLDALLGGEKLSRLSGLRVLHIGDSFFVHSEQLDTTDAEALD
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                     unknown function"
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     CDS             complement(2128..2394)
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                     /note="Evidence 4:Homologs of previously reported genes of
                     unknown function"
```

Resistance prediction using WGS

Hendrisken et al. Frontiers in Microbiology. 2019.

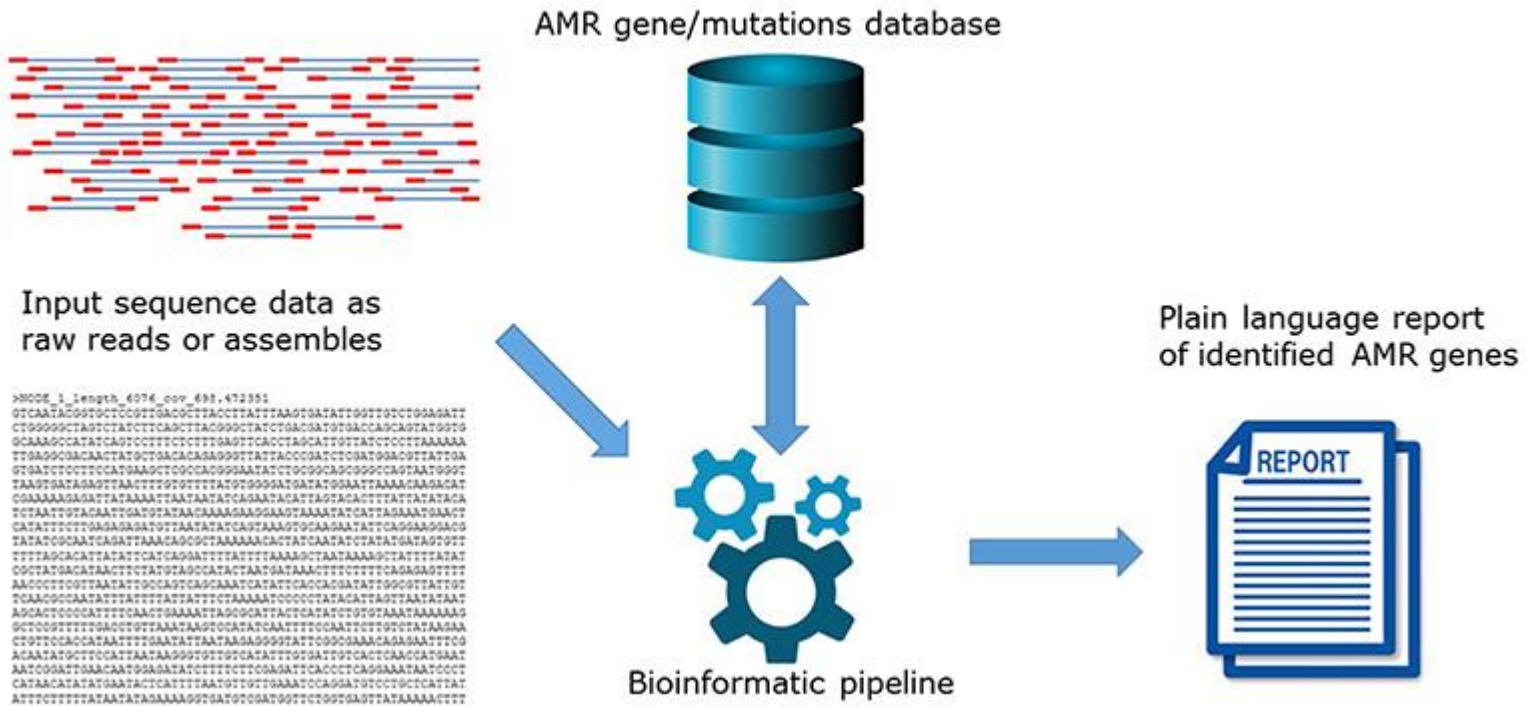
Concordance between phenotypic susceptibility testing and WGS based predicted antimicrobial resistance

	Pathogen	No. of pathogens	AST method	No. of antimicrobials	Bioinformatic tool	Sequencing data	Concordance	Sensitivity	Specificity	Comment	References
2013	*S. Typhimurium*	49	MIC	17	ResFinder	Assembled, Velvet	99.74%			Disagreement: 7 isolates including 6 *E. coli* resistent to Spec	(7)
	E. coli	48									
	E. faecalis	50		14							
	E. faecium	50									
2013	*E. coli* (ESBL)	74	DD	7	BLASTn, selected panel	Assembled, Velvet		96%	97%	VM rate: 1.2%/M rate: 2.1%	(8)
	K. pneumonia (ESBL)	69									
2014	*S. aureus*	501	DD/MIC (Vitek)	12	BLASTn, selected panel	Assembled, Velvet		97%	99%	VM rate: 0.5%/M rate: 0.7%	(9)
2016	*C. jejuni*	32	MIC	9	BLASTx	Assembled, CLC-bio	99.2%			Lower concordance to	(10)
	C. coli	82								Gen, Azi, Clin, Tel	
2016	*S. enterica*	104	MIC	14	ResFinder/ ARG-ANNOT/ CARD/BLAST	Assembled, CLC-bio	99.0%	99.2%	99.3%	Lower concordance to	(11)
		536						97.6%	98.0%	aminoglycosides/β-lactams	
2017	*E. coli*	31	MIC	4	Custom DB based on ARDB/CARD/β-lactamase allelles			87%	98%	Neg. predictive value: 97%	(12)
	K. pneumonia	24								Pos. Predictive value: 91%	
	P. aeruginosa	22									
	E. cloacae	13									
2017	*S. enterica*	50	MIC	4	ResFinder/ PointFinder	Assembled, SPAdes	98.4%			Disagreement: 2/2 C.jejuni to FQ/ERY	(13)
	E. coli	50		6							
	C. jejuni	50		4						5 *E. coli* to COL (pmrB)	
2018	*E. faecalis*	97	MIC	11	ResFinder/NCBI Pathogen DB/BLAST	Assembled, CLC-bio	96.5%				(14)
	E. faecium	100									
2018	*S. aureus*	501	DD/MIC	12	GeneFinder/ Mykrobe/ Typewriter	FASTQ/assembled, BLAST	98.3%			Disagreements: 0.7% predicted resistant	(15)
		491									
		397	MIC							0.6% predicted susceptible	
2018	*M. tuberculosis*	10,209	MGIT 960	4	Cortex	Assembled	89.5%			97.1%/99.0% predicted R/S	(16)
				4						97.5%/98.8% predicted R/S	
				4						94.6%/93.6% predicted R/S	
				4						91.3%/96.8% predicted R/S	
2019	*H. pylori*	140	MIC (E-test)	5	ARIBA	FASTQ	99%			Phenotype issues to metronidazole	(17)

1) ESBL: Extended Spectrum Beta-Lactamase, 2) MIC: Minimum Inhibitory Concentration, 3) DD: Disk diffusion, 4) VM: Very Major, 5) M: Major, 6) R/S: Resistant/Susceptible, 7) SPEC: Spectinomycin, 8) GEN: Gentamicin, 9) AZI: Azithromycin, 10) CLIN: Clindamycin, 11) TEL: Telithromycin, 12) FQ: Fluoroquinolone, 13) ERY: Erythromycin, 14) COL: colistin.

Resistance prediction using WGS

Hendrisken et al. Frontiers in Microbiology. 2019.



Resistance prediction using WGS

Hendrisken et al. Frontiers in Microbiology. 2019.

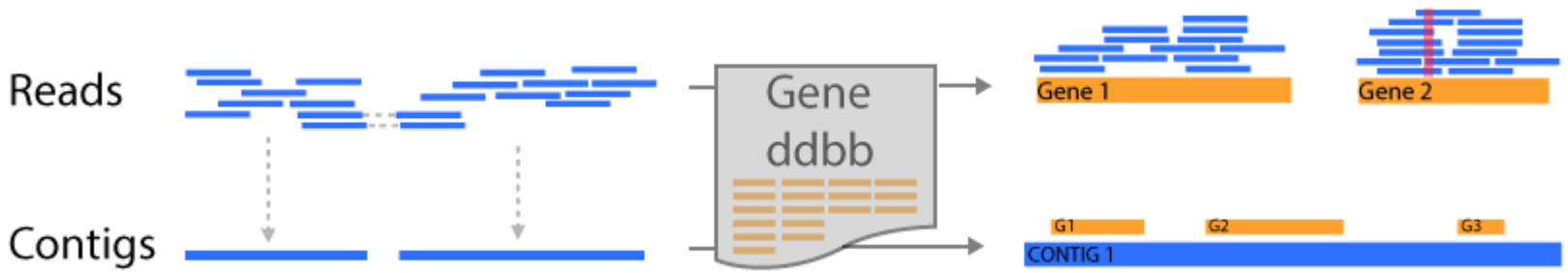
- **Huge list here: https://www.frontiersin.org/files/Articles/478239/fpubh-07-00242-HTML/image_m/fpubh-07-00242-t002.jpg**

Software	Type
SRST2	Mapping
Ariba	Mapping + assembly
ABRICATE	Assembly
ResFinder	Assembly

Mapping vs Assembly

- **Functional annotation based on mapping (srst2)**
  - Pro: more resolutive / high quality ddbb
  - Con: Unable to locate genes / no ab initio annotation

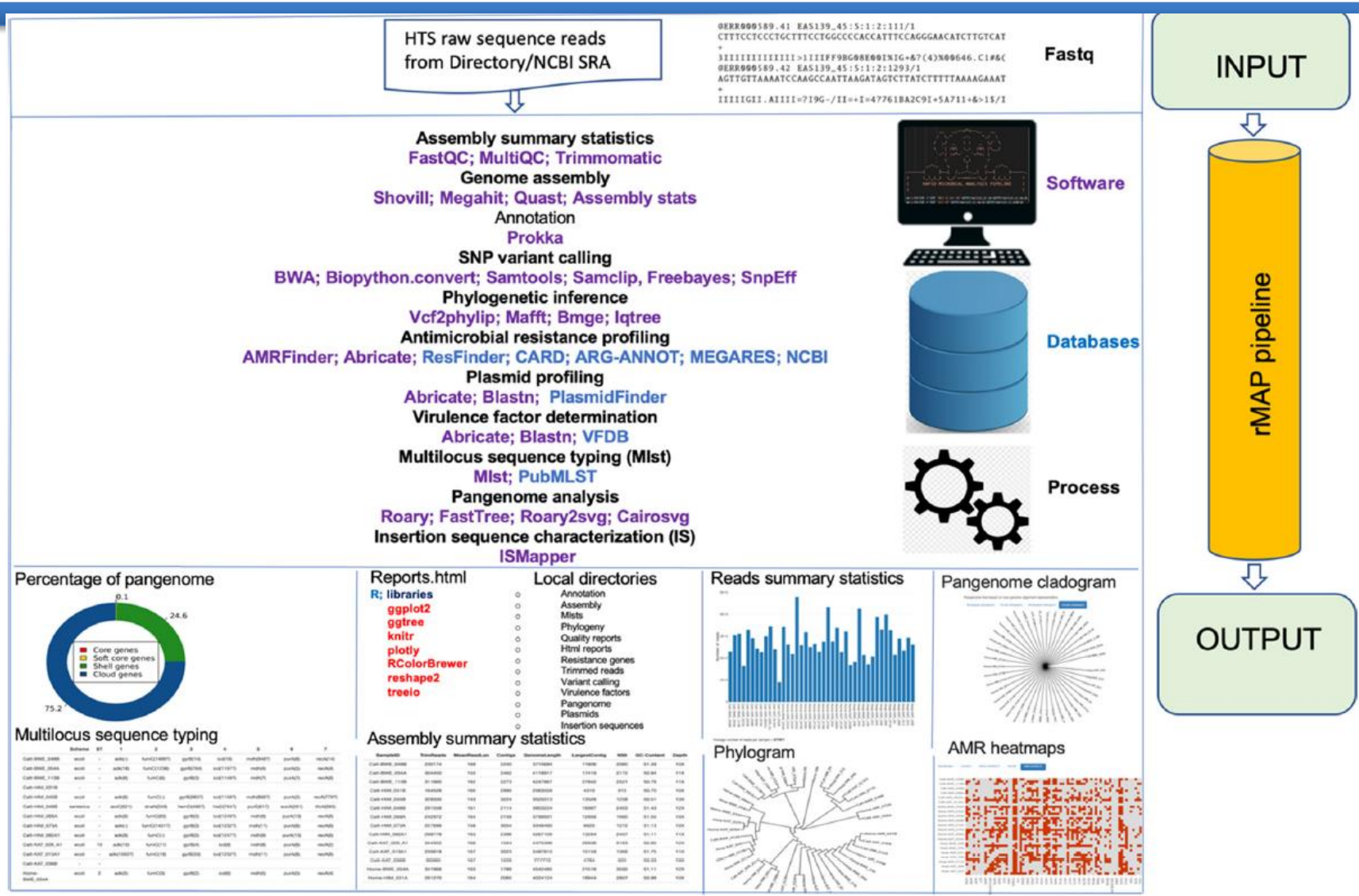
- **Functional annotation based on assembly (Resfinder)**
  - Pro: genes are located / related
  - Depend on assembly (close to repetitive regions)



rMAP - rapid microbial analysis pipeline- for ESKAPE bacterial group wgs data

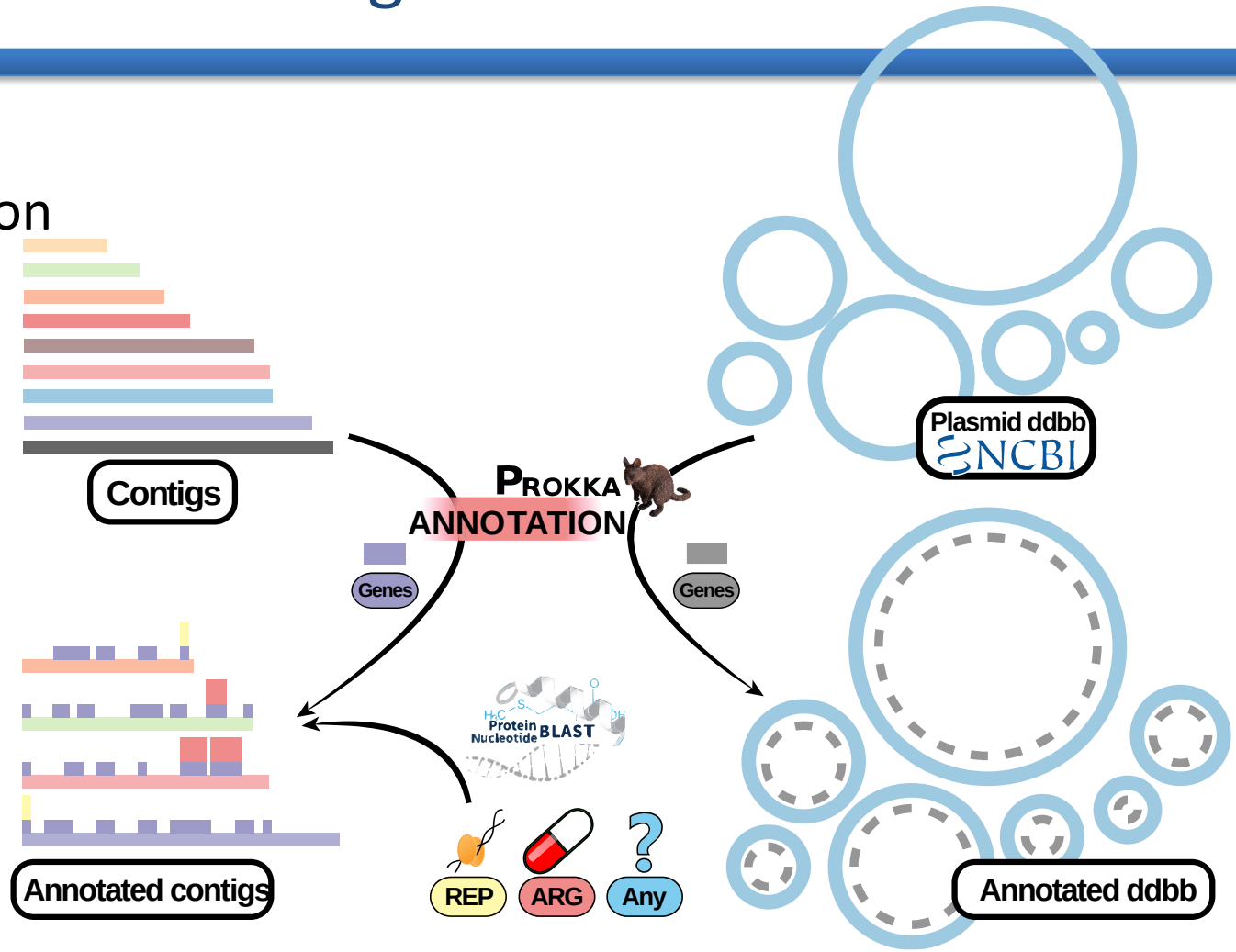
The resistomes of ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter species*)

Sserwadda & Mboowa, Microbiology, 2021

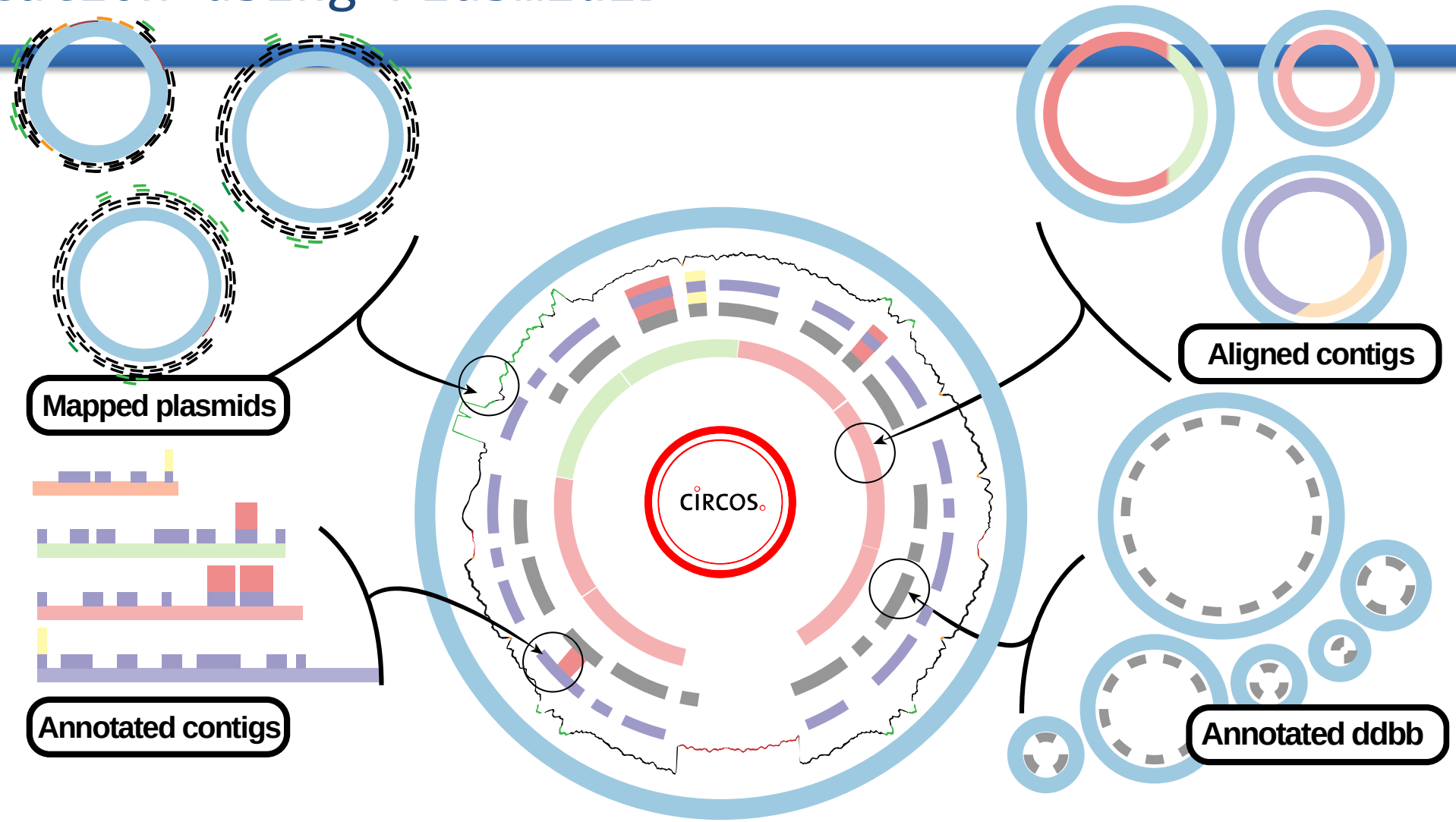


Annotation visualization using PlasmidID

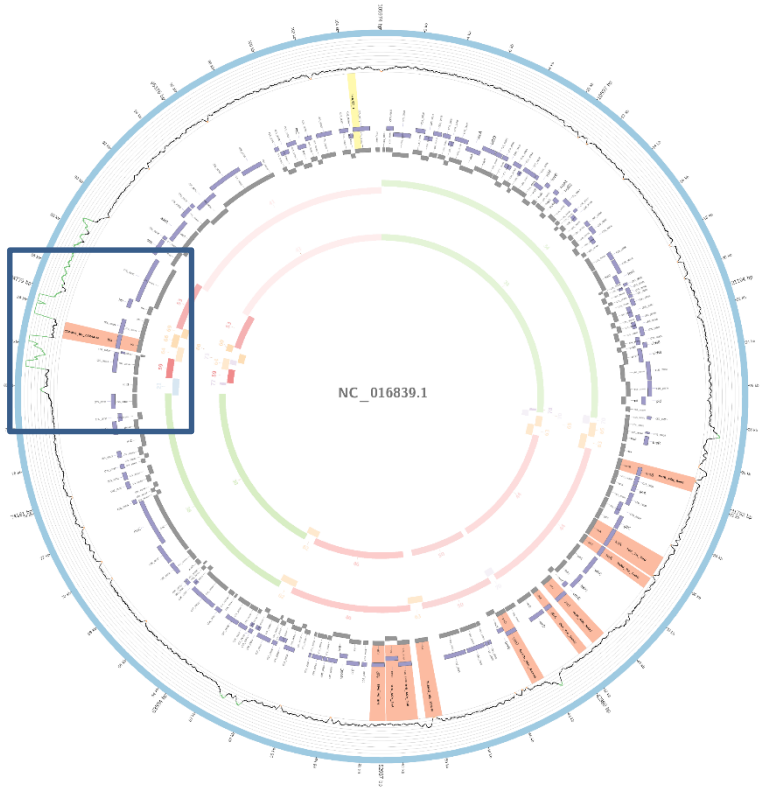
- Automatic annotation
  - Prokka
    - DDBB plasmid
    - Contigs
  - Gff to bed
- Specific annotation
  - BLAST+
  - ABR & REP
  - User input FASTA



Annotation using PlasmidID



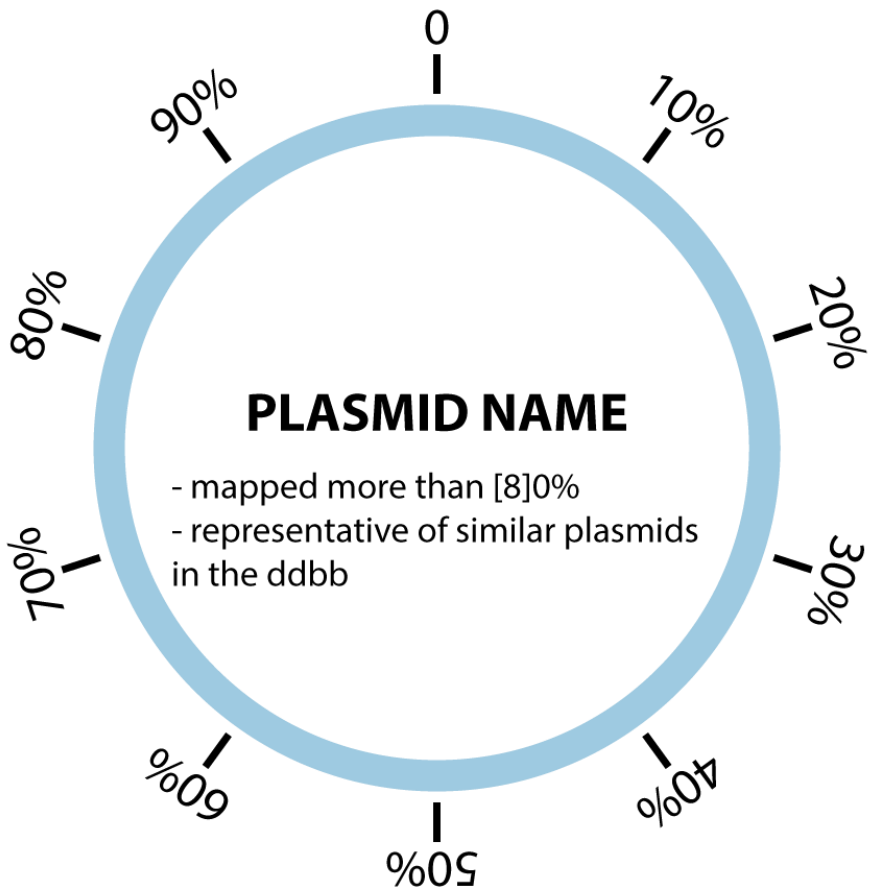
Annotation using PlasmidID



Annotation on short contigs

Understanding the image: track by track

Plasmid Track

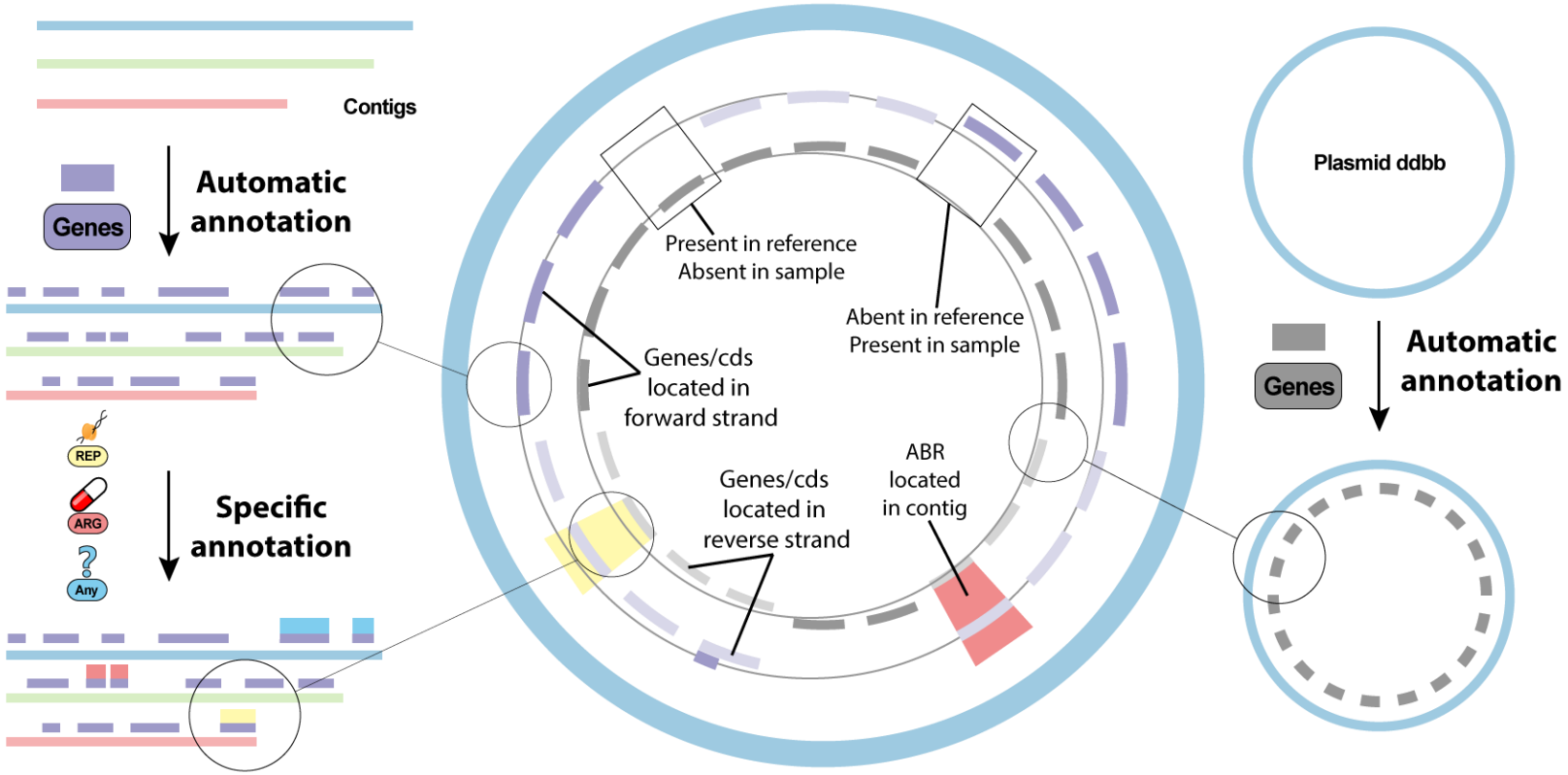


Understanding the image: track by track

Coverage Track

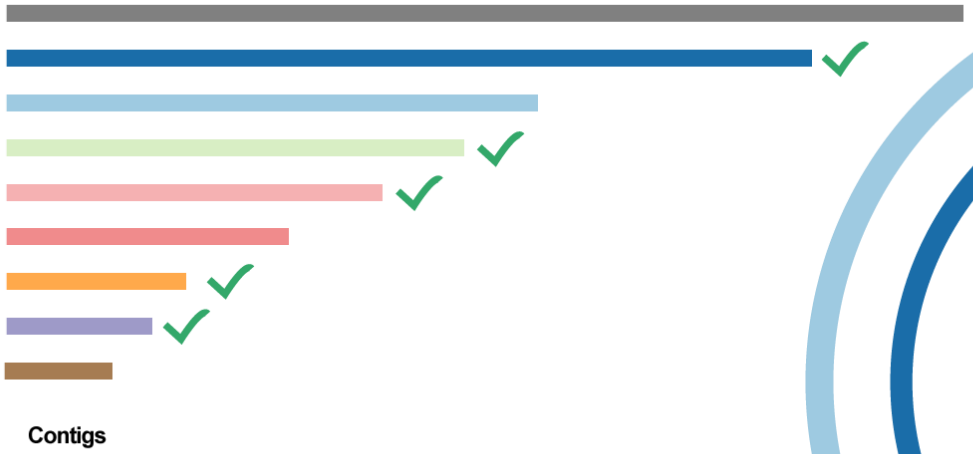
Understanding the image: track by track

Annotation Track

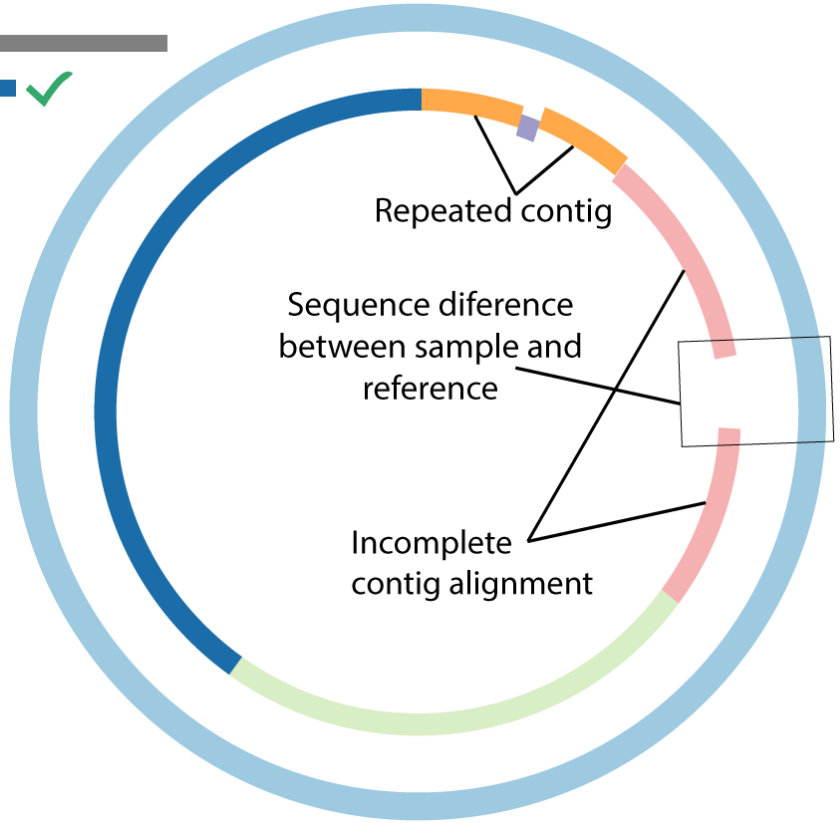


Understanding the image: track by track

Contig Track

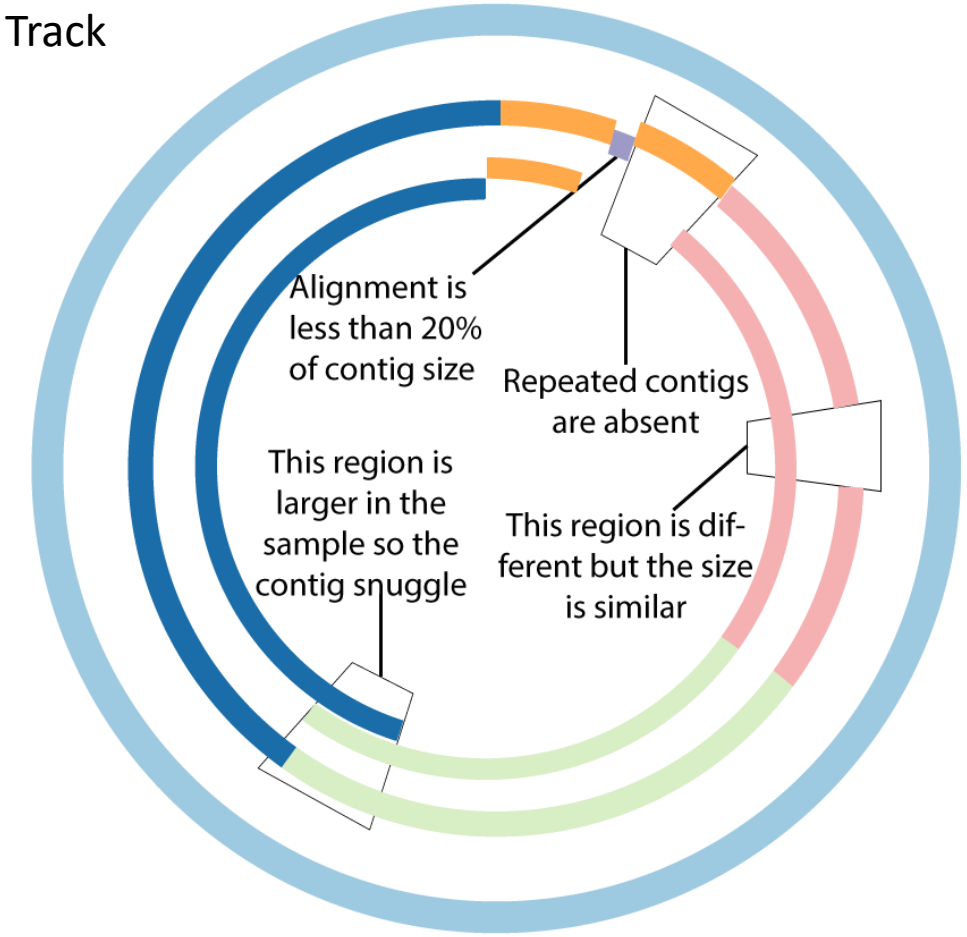


- Represent **ONLY LOCAL** alignment
- Does not specify if the contig is inverted neither the total length of the contig



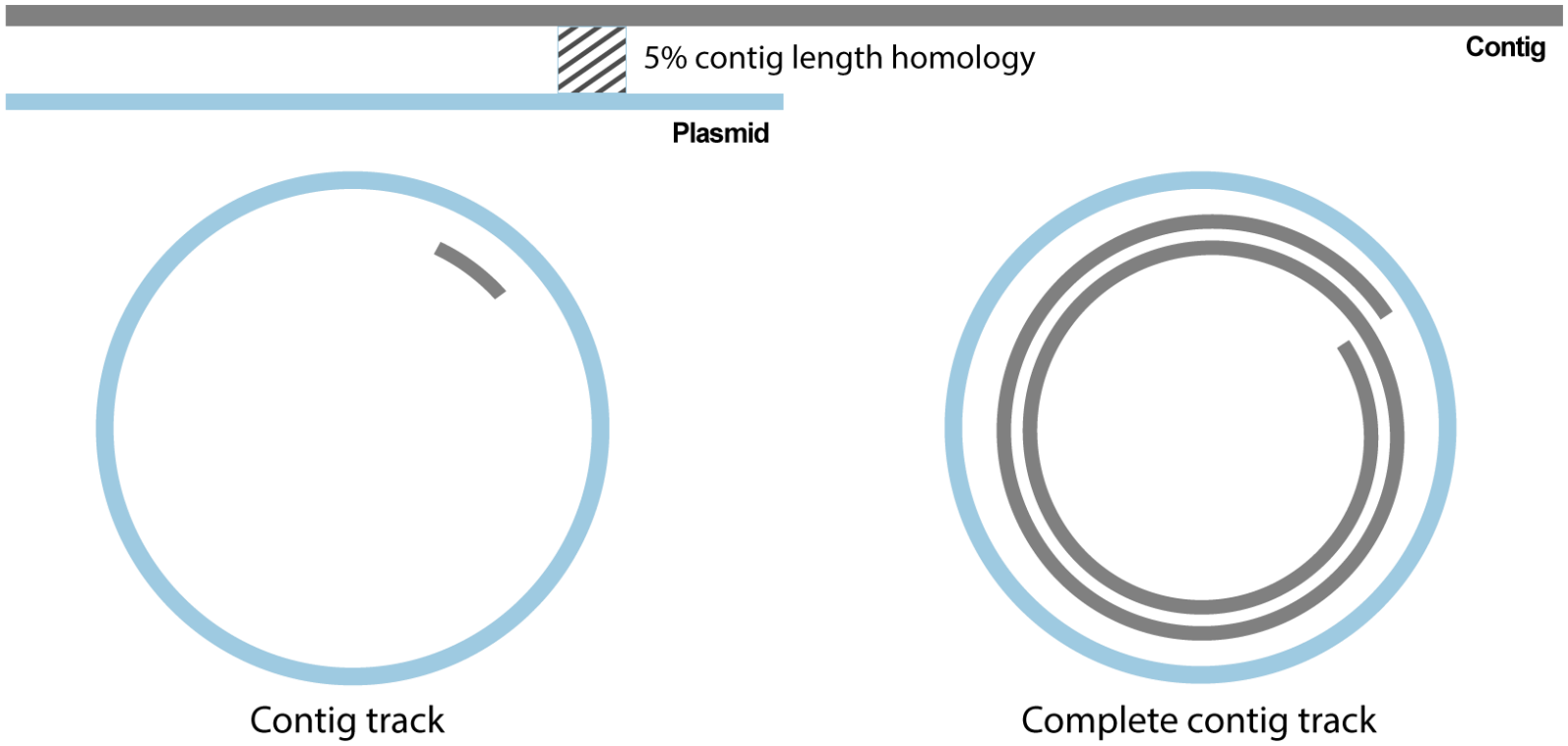
Understanding the image: track by track

Complete contig Track



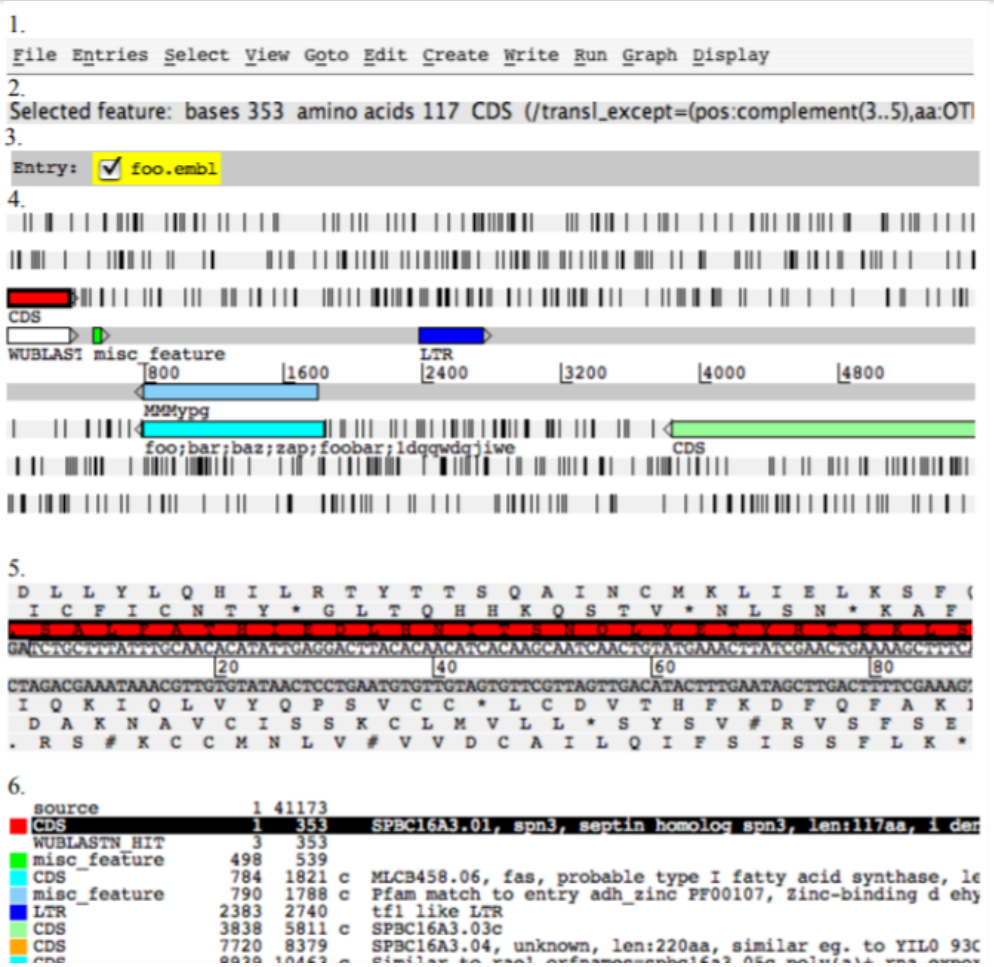
Understanding the image: track by track

Complete contig Track



Manual annotation: Artemis

Artemis is a DNA sequence viewer and annotation tool that allows visualisation of sequence features and the results of analyses within the context of the sequence, and its six-frame translation.



Thanks for your attention!