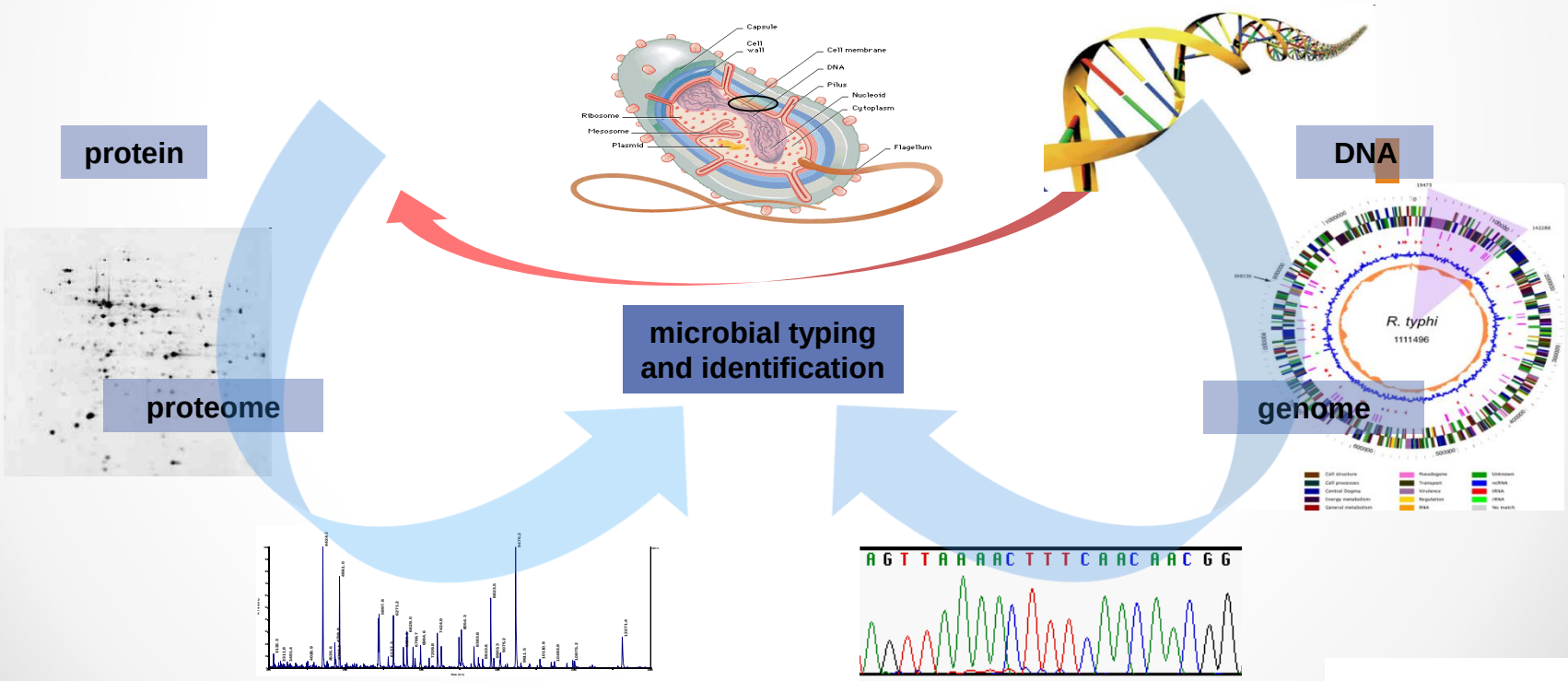
Diagnostics of Infectious Bacteria is Dependent upon a Reliable and Comprehensive Identification and Taxonomy Framework

E. R. B. Moore *et al*.

CCUG, Department of Clinical Microbiology, Sahlgrenska University Hospital
Department of Infectious Disease, Sahlgrenska Academy, University of Gothenburg, Sweden



VÄSTRA GÖTALANDSREGIONEN
SAHLGRENSKA UNIVERSITETSSJUKHUSET

Culture Collection, University of Gothenburg (CCUG)
www.ccug.se

Established 1968 (Curator: E. Falsen, 1968 – 2004; E. Moore, 2004 - ?)

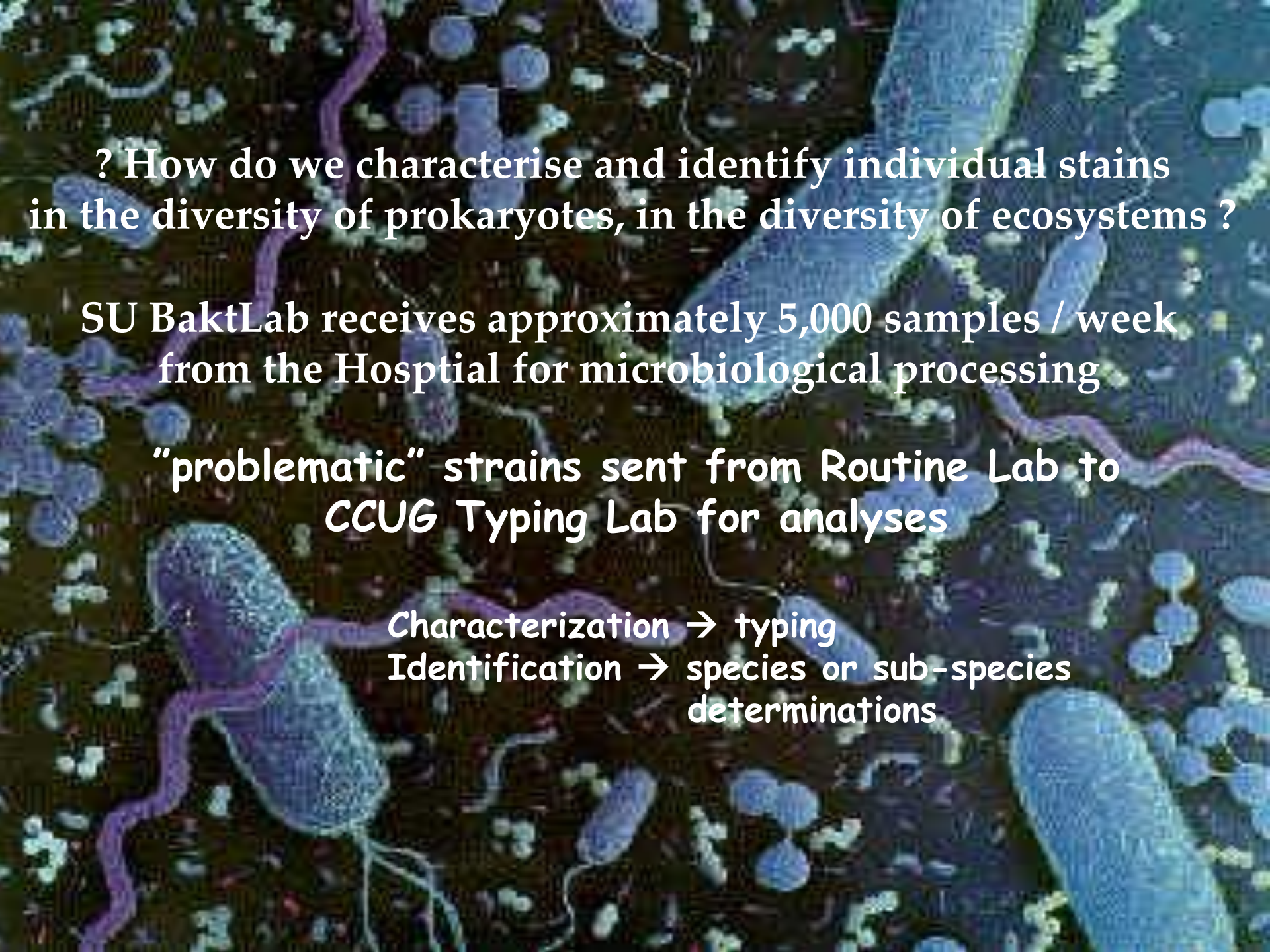
Elisabeth Inganäs, BMA (1991), Maria Ohlén, BMA (1992),
Susanne Jensie-Markopolous, BMA (2002), Sofia Cardew, BMA (2007)
Kent Molin, Chemist (2001), Liselott Svensson-Stadler, Enhetschef (2012)

Created as a unit within the Bacteriological Laboratory of the Dept Clinical Bacteriology, Sahlgrenska University-Hospital (SU)
SU is a teaching and research hospital

Associated with Univ Gothenburg through Sahlgrenska Academy

Repository for reference strains (> 40,000) of microorganisms and typing lab for the identification of bacteria from clinical samples from SU and other hospitals and clinics in Scandinavia

1. ***Typing and identification*: *phenotyping and genotyping***
2. ***Archiving, storing, distributing bacterial strains* (some few *yeast*, *fungi*)**
3. ***Research*: *systematics / diagnostics / antibiotic resistance***


? How do we characterise and identify individual stains
in the diversity of prokaryotes, in the diversity of ecosystems ?
SU BaktLab receives approximately 5,000 samples / week
from the Hosptial for microbiological processing
"problematic" strains sent from Routine Lab to
CCUG Typing Lab for analyses
Characterization → typing
Identification → species or sub-species
determinations

What are the "problematic" organisms?

- bacterial strains that are not differentiated from similar and closely related bacteria.

- bacterial strains that are "new", *i.e.*, they do not fit a database profile;

→ in the CCUG, as many as 150 new 'cases' per year!

Coming from clinical samples!!

→ need effective, reliable characterisation and identification!!!!!

Genotypic/Phylogenetic emphasis in bacterial systematics

- The *Bergey's Manuals for Systematic Bacteriology* and *The Prokaryotes* based on 16S rRNA gene sequence-based phylogenies.
- 16S rRNA gene sequence determination and analysis the only single test required for the description of all new bacterial species.
- Genomic DNA-DNA similarities have been the genotypic criterion for defining bacterial species

→ "Gold Standard " ?

[1] "... the complete deoxyribonucleic acid (DNA) sequence would be the reference standard to determine phylogeny and ... phylogeny should determine taxonomy."

[1] Wayne et al. 1987, Report of the ad-hoc committee on reconcilliation of approaches to bacterial systematics, Int J Syst Bacteriol 37: 463-464

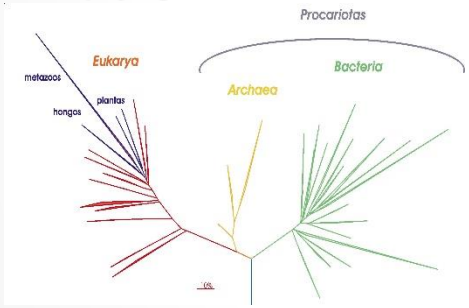
International Journal of Systematic and Evolutionary Microbiology (2010), **60**, 249–266 DOI 10.1099/ijs.0.016949-0

Taxonomic Note

Notes on the characterization of prokaryote strains for taxonomic purposes

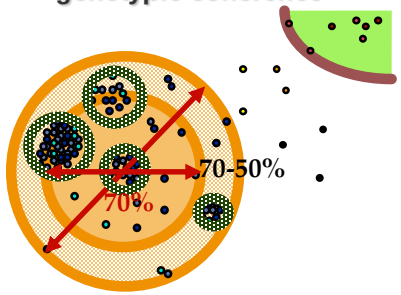
B. J. Tindall,[1] R. Rosselló-Móra,[2] H.-J. Busse,[3] W. Ludwig[4] and P. Kämpfer[5]

phylogenetic coherence



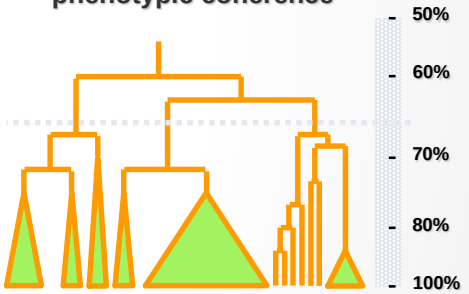
16S 23S rRNA
Functional genes (MLSA)
Genomic analyses

genotypic coherence



Reasociation DNA-DNA
G+C, PFGE, AFLP, MLSA
Genomic comparisons
(ANI; AAI)

phenotypic coherence



metabolism
chemotaxonomy
proteomics
(MALDI-TOF; LC-MS/MS)

Species descriptions: the examination should be as exhaustive as possible

from R Rosselló-Móra, IMEDEA, ES

Systematic and Applied Microbiology

Volume 38, Issue 4, June 2015, Pages 217–222

Genome sequences as the type material for taxonomic descriptions of prokaryotes

William B. Whitman

Show more

https://doi.org/10.1016/j.syapm.2015.02.003

At recent ICSP Meeting in Valencia, July 2017, this 'proposal' was discussed

Abstract

Genome sequencing of type strains promises to revolutionize prokaryotic systematics by greatly improving the identification of species, elucidating the functional properties of taxonomic groups, and resolving many of the ambiguities in the phylogeny of the higher taxa. Genome sequences could also serve as the type material for naming prokaryotic taxa, which will greatly expand the nomenclature governed by the Bacteriological Code to include many fastidious and uncultured organisms and endosymbionts of great biological interest.

Keywords

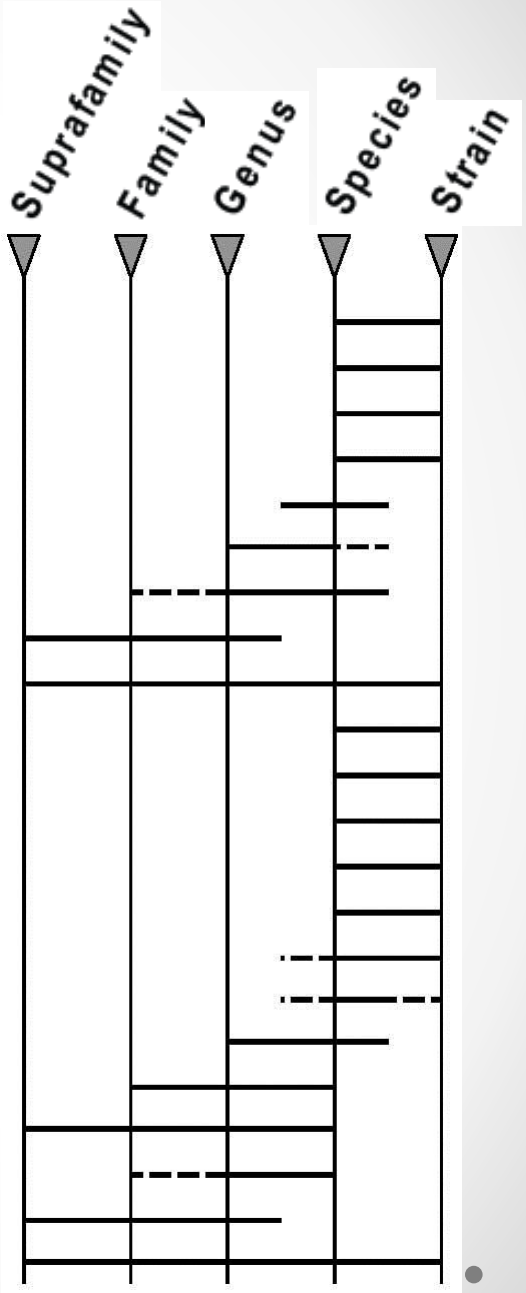
Genomics; Type strain; Bacteriological Code

Bacterial Characterisation and Identification

Methodology

phenotypic
- serotyping (monoclonal and polyclonal antisera)
- Raman spectroscopy
- protein SDS-PAGE (whole-cell or cell envelope)
- pyrolysis mass spectrometry
- Fourier-Transformation Infra-Red (FTIR) spectroscopy
- MALDI-TOF mass spectrometry
- cellular fatty acid fingerprinting (FAME)
- chemotaxonomy (polyamine, polar lipid, quinone, etc., analysis)
- phenotyping (growth, morphology, Api, BIOLOG, etc.)

genotypic
- restriction analysis of amplified fragments (PCR-RFLP)
- low-frequency restriction fragment analysis (PFGE)
- PCR-amplfication analysis (REP-, BOX-, ERIC-PCR, RAPD)
- selective amplification of restriction fragments (AFLP)
- Multi-Locus Variable-number tandem-repeat Analysis (MLVA)
- Ribotyping
- Mulit-Locus Sequence Typing (MLST)
- Multi-Locus Sequence Analysis (MLSA)
- Amplified Ribosomal DNA Restriction Analysis (ARDRA)
- rRNA gene sequence analysis
- DNA-DNA Hybridisation (DDH)
- Genomic DNA mol % G+C
- whole genome sequence analysis



CCUG: since 2013, focussed on developing proteomic-genomic methods for:

strain identification

strain sub-typing

strain characterisation

→ diagnostics of infectious disease in clinical samples without cultivation

Characterisation of infectious or clinically-relevant bacteria

- **Virulence – a measure of the degree of pathogenicity**

  virulence factors – effectively enable:

  - colonization in the host (attachment)

  - evasion of host defense mechanisms

  - potential to cause disease

  bacterial toxins, cell surface proteins, hydrolytic enzymes, …

- **Antibiotic resistance – inherent and acquired resistance**

  WHO – ”… ***one of the biggest threats to global health, food security, and development today. …we are heading for a ‘post-antibiotic era’, in which common infections and minor injuries can once again kill.”***

  resistance factors - enable:

  - survival in competitive environments

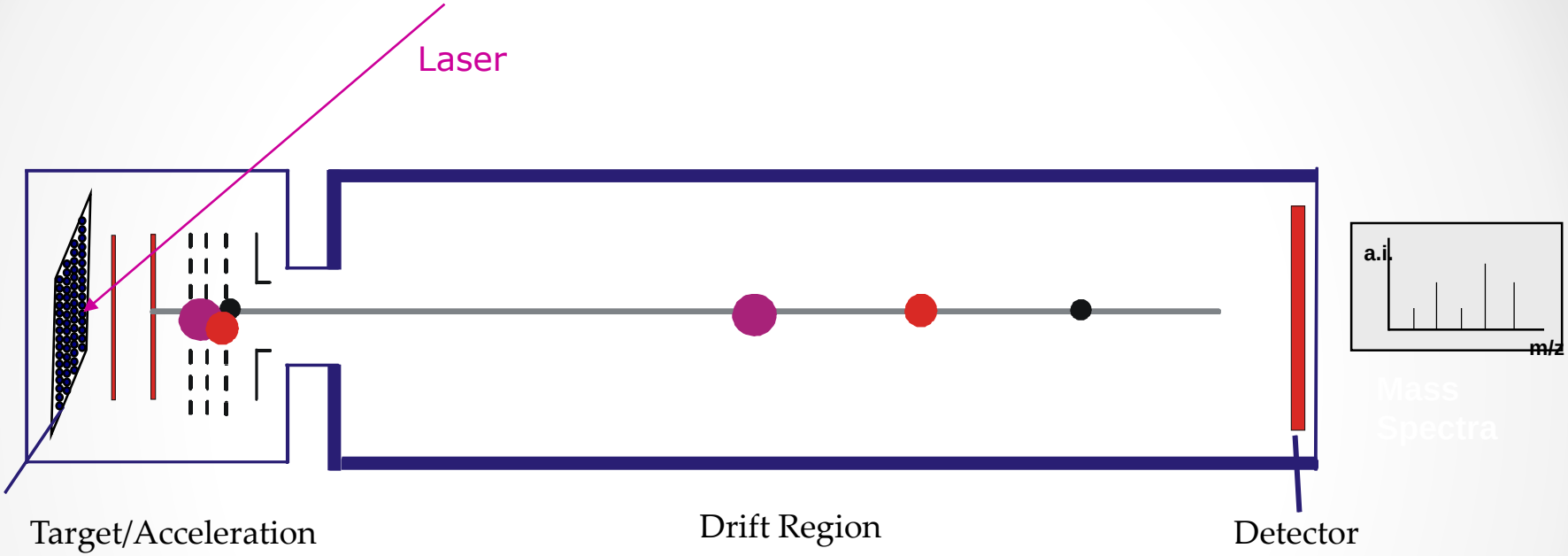
  - pathogenic bacteria to adapt to antimicrobial therapies

  gene activation/repression, binding proteins, porins,

  efflux pumps, …

Virulence Factor Database: www.mgc.ac.cn/VFs/main.htm

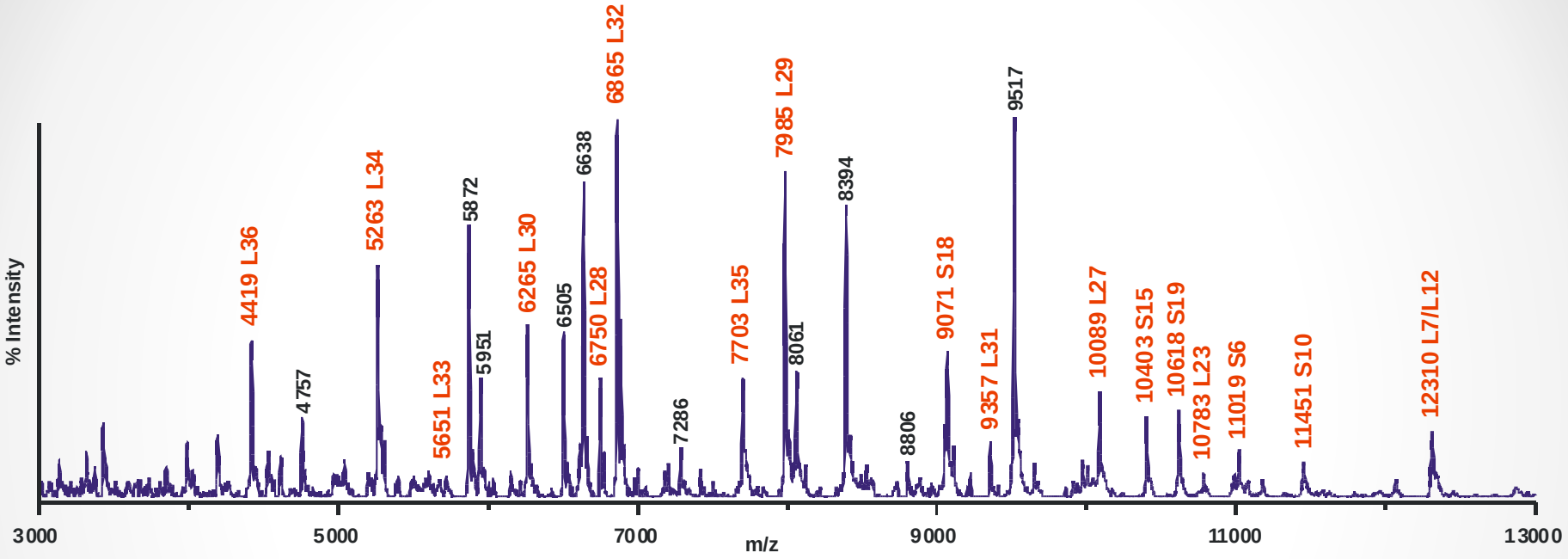
MALDI-TOF MS



Desorption/Ionisation	Time-of-Flight	Molecular Mass

Modified from M Erhard, Anagnostec, GmbH

Streptococcus pneumoniae: identfying mass signals



mass signals matching to ribosomal proteins are marked in red

~50% of peaks represent ribosomal proteins

protein masses obtained from the Swiss-Prot/TrEMBL sequence database

"-omics" relevant for identification of proteins

modified from M Welker, Anagnostec, GmbH

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Review

Proteotyping: Proteomic characterization, classification and identification of microorganisms – A prospectus

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ARTICLE INFO

Keywords:
Proteotyping
Proteomics
Mass spectrometry
Microbial systematics

ABSTRACT

Modern microbial systematics requires a range of methodologies for the comprehensive characterization, classification and identification of microorganisms. While whole-genome sequences provide the ultimate reference for defining microbial phylogeny and taxonomy, selected biomarker-based strategies continue to provide the means for the bulk of microbial systematic studies. Proteomics, the study of the expression of genes, as well as the structure and function of the resulting proteins, offers indirect measures of genome sequence data. Recent developments in applications of proteomics for analyzing

Can tandem Mass Spectrometry and proteomics – 'Proteotyping', be applied for the direct analyses of clinical samples för rapid and reliable diagnostics of infectious bacteria?

What would be the advantages over traditional diagnostic methods or NGS-based methods?

1) **Respiratory infections: detection and identification, virulence and antibiotic resistance**

   S. pneumoniae, S. aureus, P. aeruginosa, K. pneumoniae, S. pyogenes, H. influenzae, M. catarrhalis, M. pneumoniae

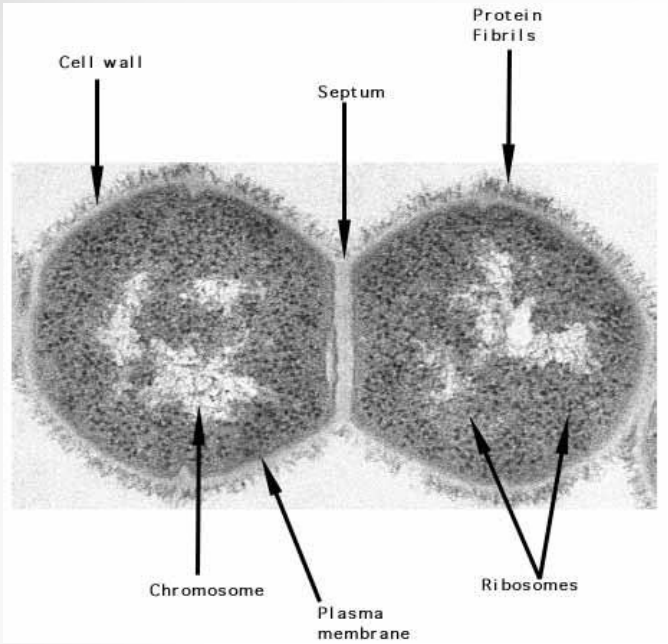
2) **Urinary-tract infections: detection and identification, virulence and antibiotic resistance**

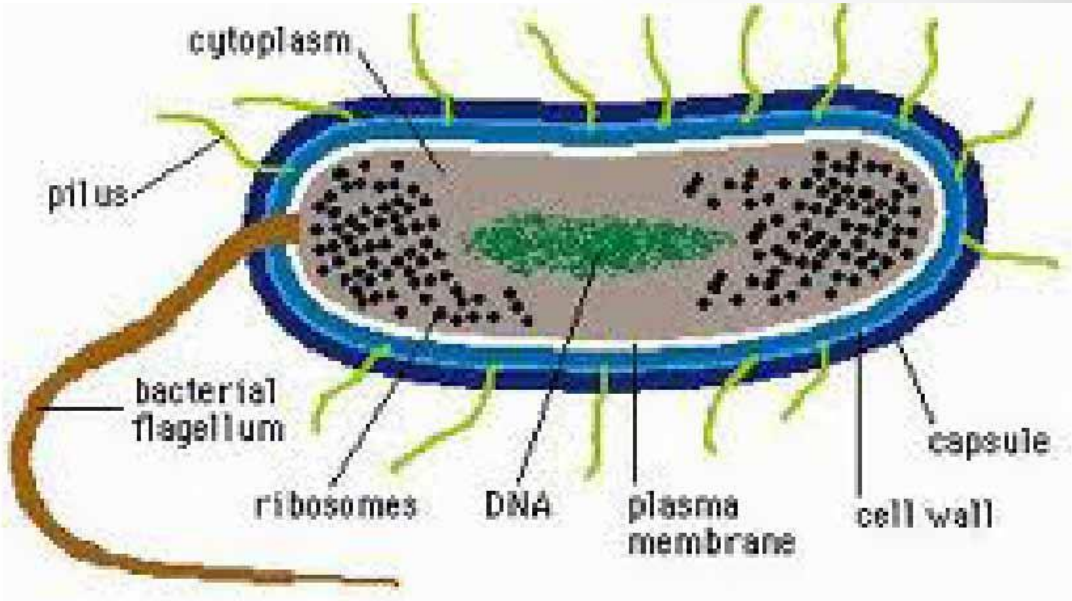
   E. coli, K. pneumoniae, Enterobacteriaceae

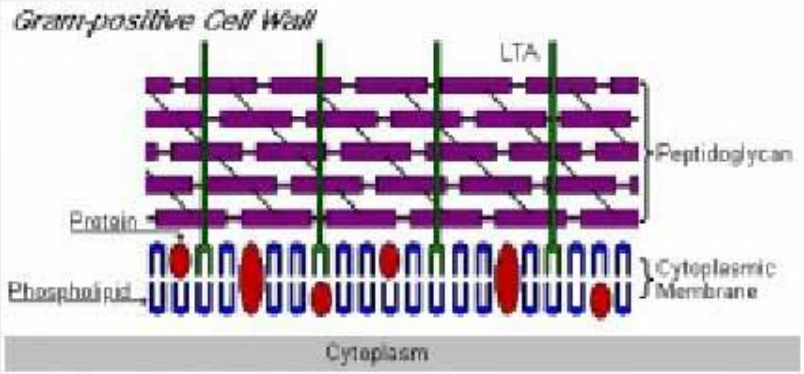
3) **Septicemia and Sepsis: detection and identification, virulence and antibiotic resistance**

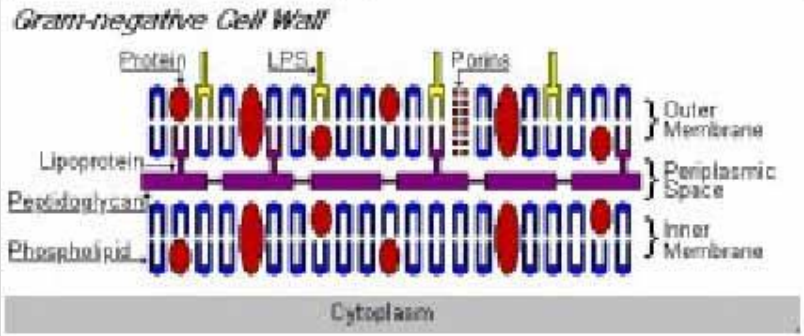
   E. coli, S. aureus, S. pyogenes

Bacterial Cell Structure









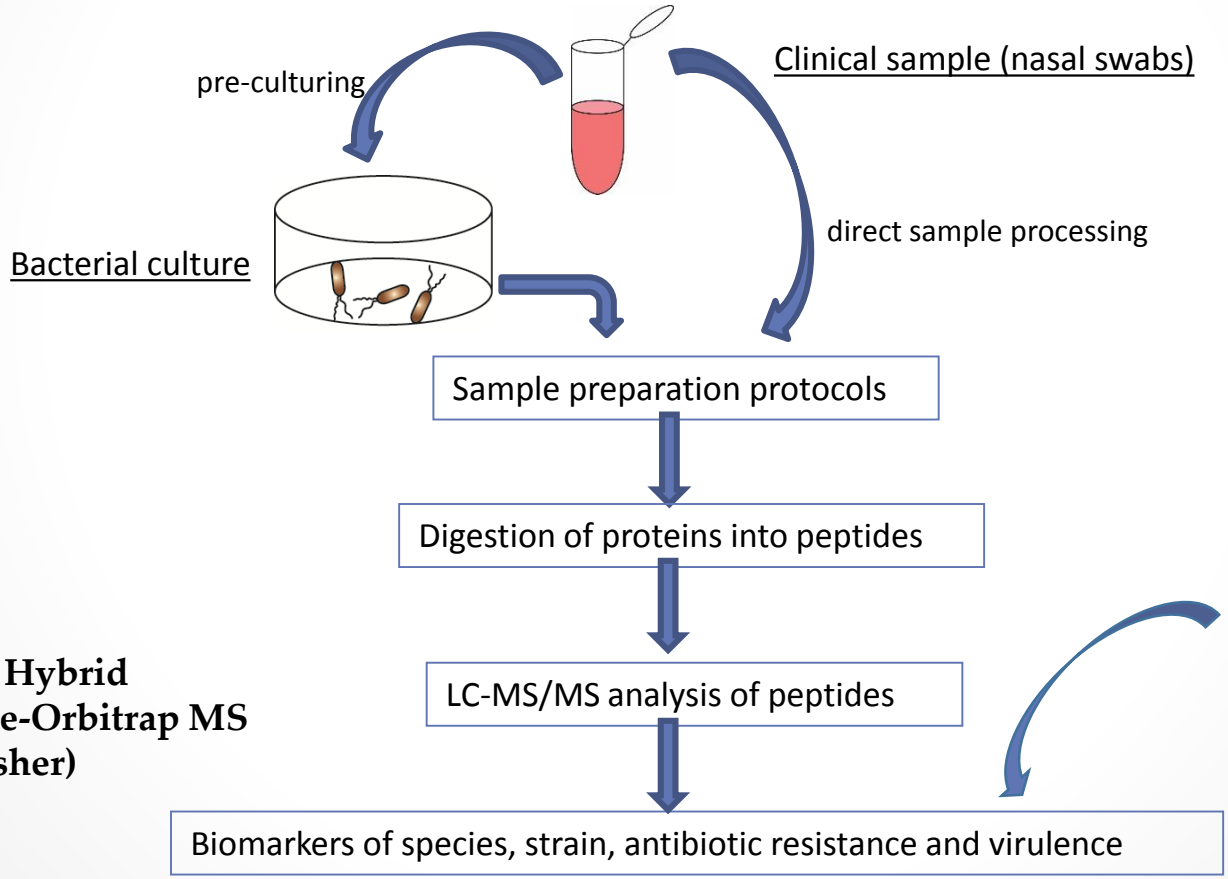
Optimisation of MS-proteomics

Different protein targets in different cell compartments.

* optimised fractionation of Gram-positive and Gram-negative cells.

LC-MS/MS 'bottom-up' Proteomics-based bacterial identification and diagnostics

Sample and Workflow

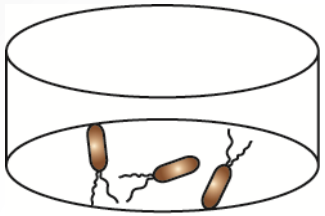


Q-Exactive Hybrid Quadrupole-Orbitrap MS (ThermoFisher)

from R Karlsson, EU TAILORED-Treatment project

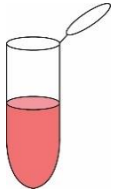
LC-MS/MS-based Proteomics

Bacterial culture
Clinical sample



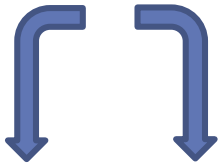
Sample preparation strategy

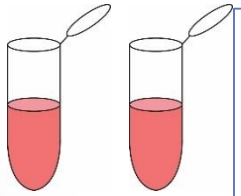
Wash cells



Intact cells - Surface shaving
Surface proteins = hotspots for genetic variation!
Virulence factors – Host-pathogen interactions

Bead beating
and centrifugation
Fractions



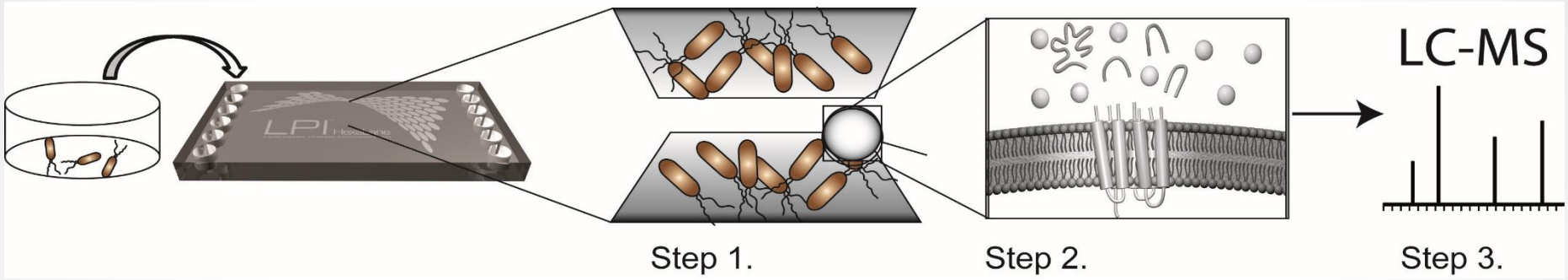


Membrane and soluble fractions
Highly abundant conserved proteins = species markers!
Antibiotic resistance markers and virulence factors

Soluble Membrane

from R Karlsson, EU TAILORED-Treatment project

Peptide generation by LPI FlowCell analysis



Step 1. Microbial cells or cell fractions are immobilized in the Hexalane Flow-Cell.

Step 2. Trypsin (digestive enzyme) is added and allowed to digest exposed proteins

Step 3. Peptides are eluted from the Flow-Cell and analyzed, using LC-MS/MS

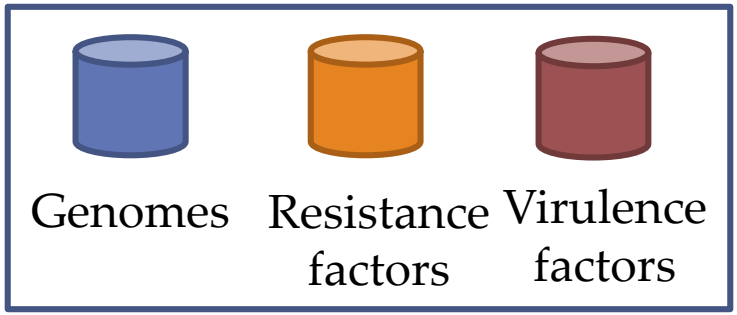
Trypsin digestion: carboxyl-side of lysine or arginine, except if followed by proline

from R Karlsson, EU TAILORED-Treatment project

Shotgun 'bottom-up' proteomics – data processing

Peptide fragments

LIEVDSQGNR
TVFFEDGNQPISK
LLEDVGSNGAR
ILEVNEEER
LLDIDQEER
AGVGEAAGRGAGVGGR



Proteome profile

Sample statistics on strain level

Sensitive matching

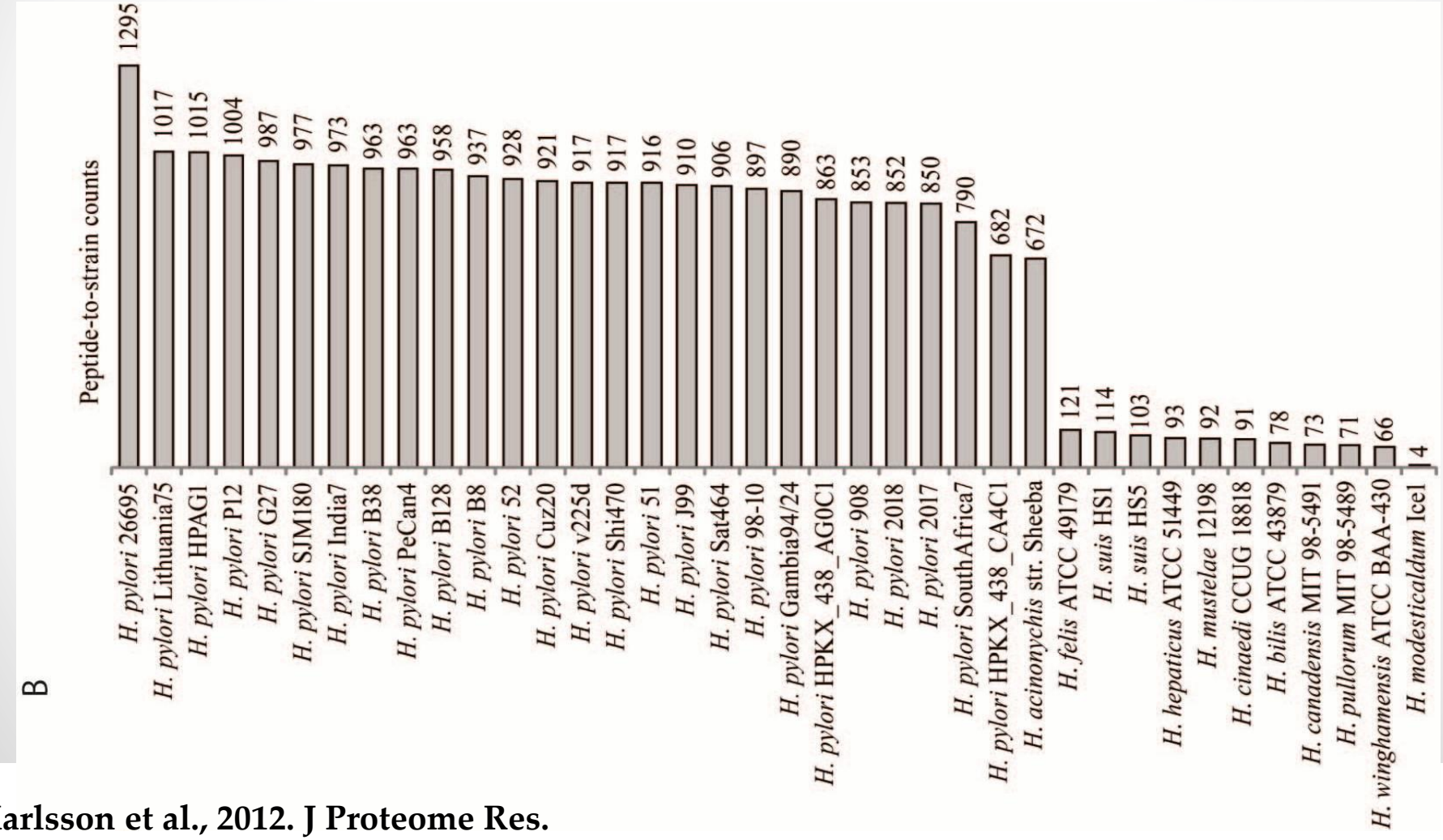
- Genome-wide alignment in all six reading-frames
- Average size of matched fragment: 16 AA (down to 6 AA)
- Database
  - well-annotated genomes
  - >5000 resistance factors (E Kristiansson, Chalmers Univ. Technology)
  - Virulence Factor Database: www.mgc.ac.cn/VFs/main.htm

→ Peptide biomarkers

from R Karlsson, EU TAILORED-Treatment project

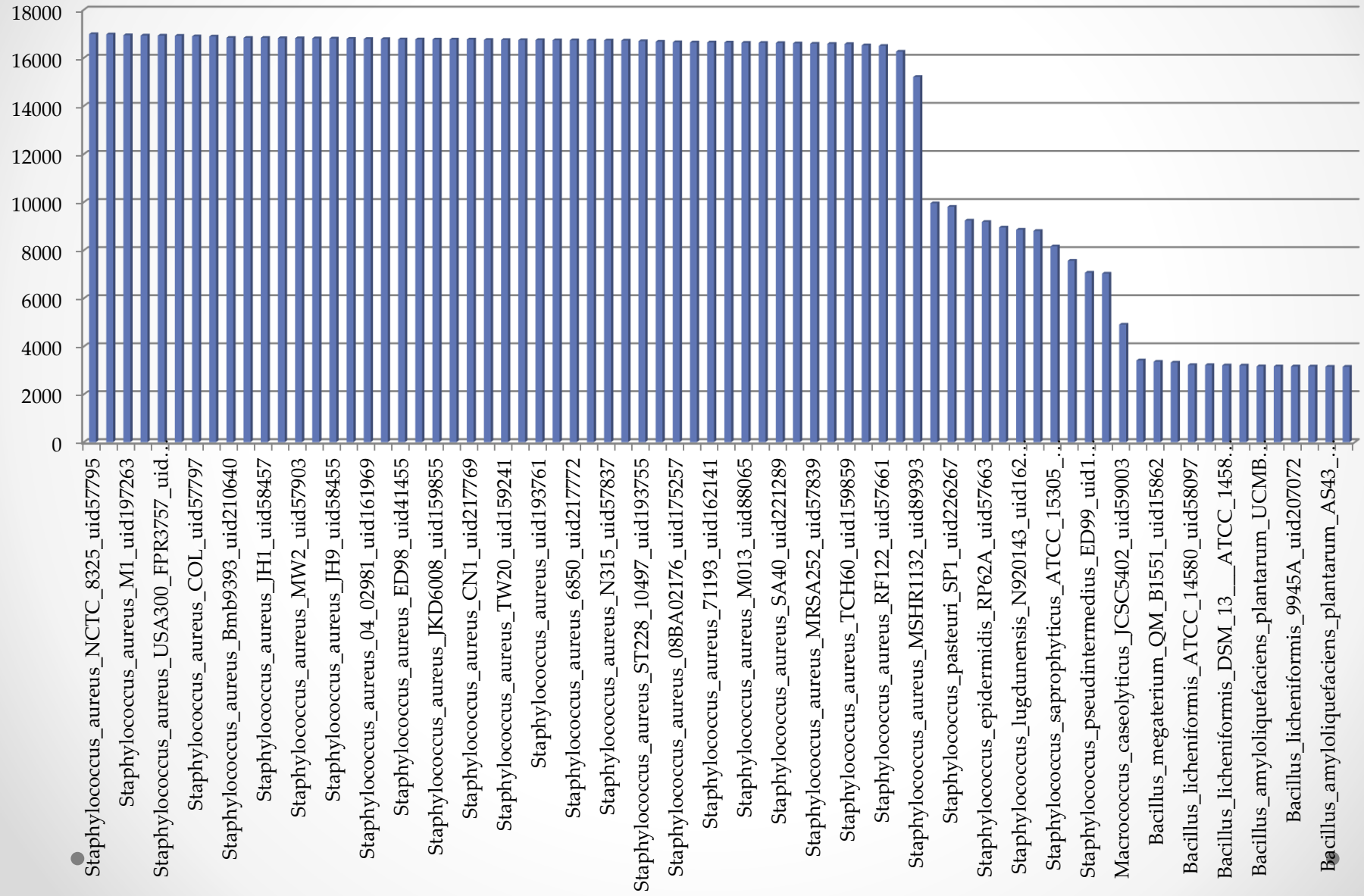
Proteotyping: *Helicobacter pylori* – ranking species matching

Analysis of the *H. pylori* strain 26695 sample. The distribution of peptide-to-strain counts for different *H. pylori* strains available in databases is shown.

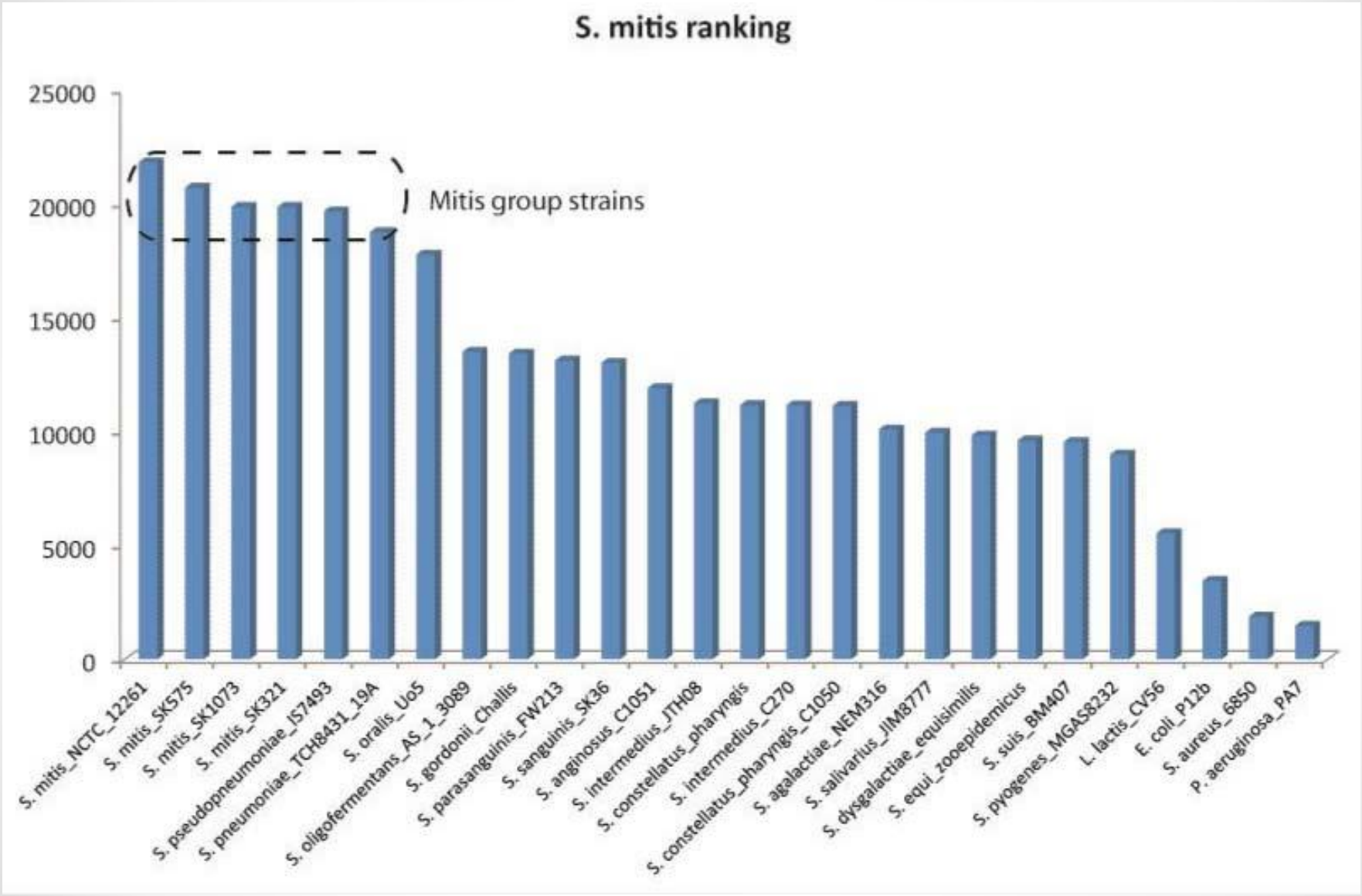


Karlsson et al., 2012. J Proteome Res.

Proteotyping: *Staphylococcus aureus* – ranking species matching

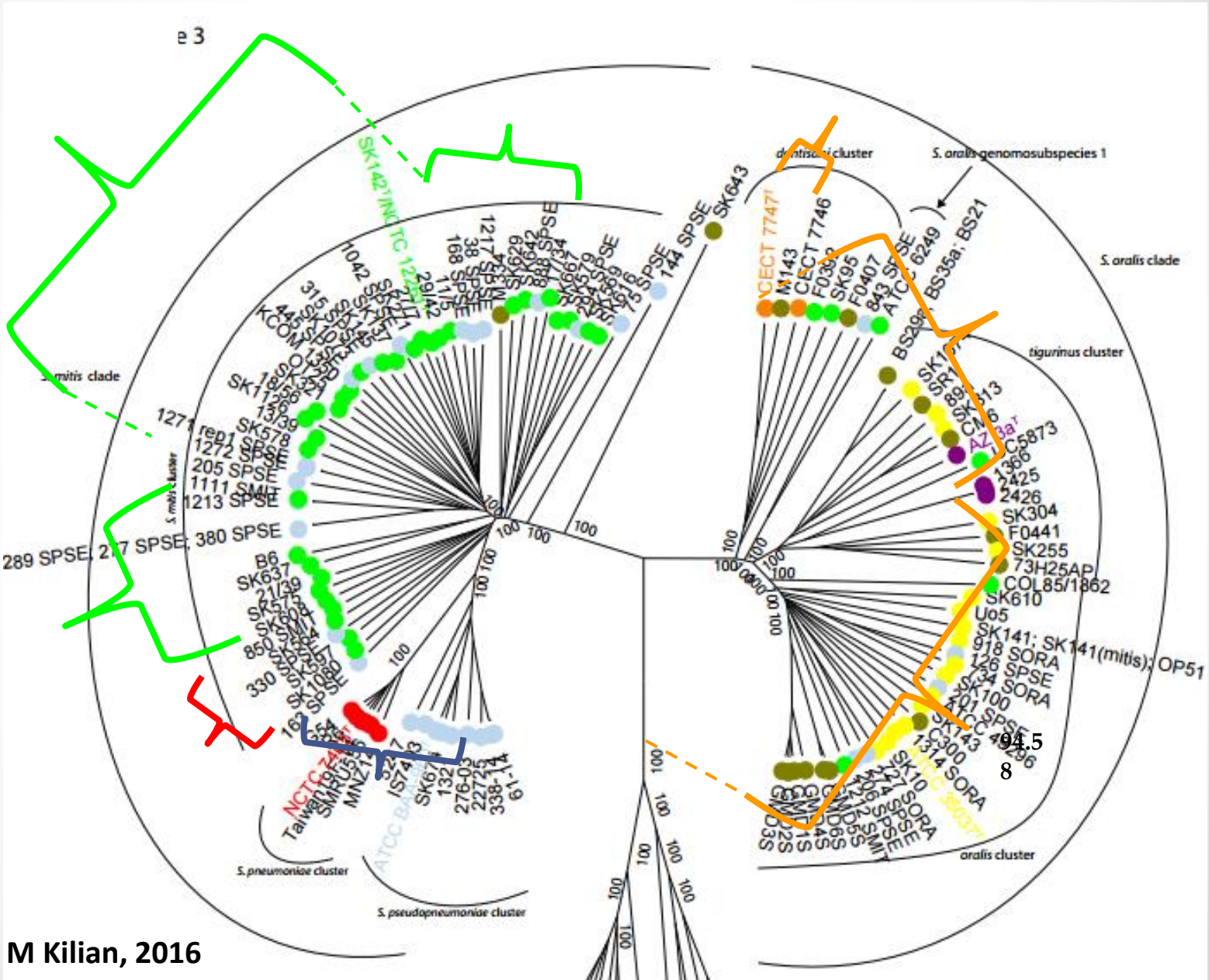


Proteotyping: *Streptococcus* spp. – ranking species matching



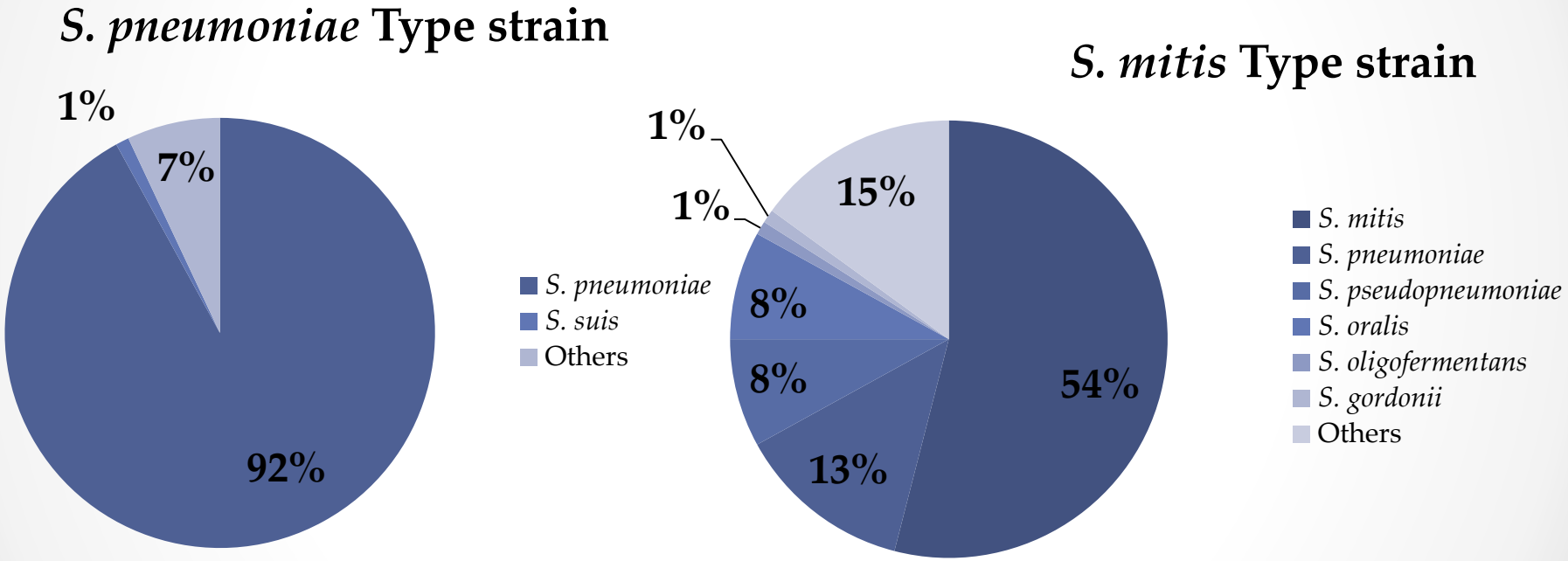
from R Karlsson, EU TAILORED-Treatment project

Streptococcus Mitis-Group: core-genome sequence clustering



modified from M Kilian, 2016

Accuracy in correct species identifications in the Mitis-Group



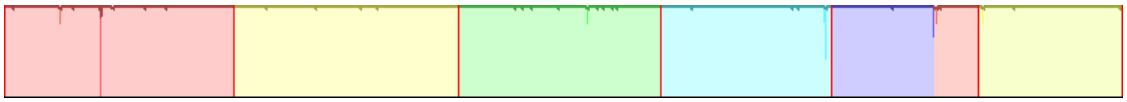
- Only 1 WGS of *S. pseudopneumoniae* and *S. mitis* available in the database
- *S. mitis* Type strain was not available – the B6 strain genome clustered far away from the Type strain

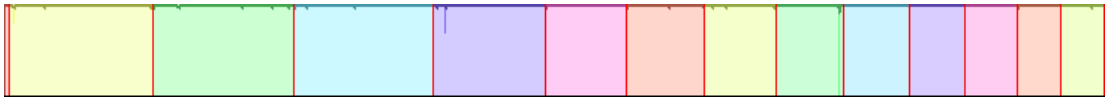
from R Karlsson, EU TAILORED-Treatment project

How can we know if a genome is correctly classified ?

ANI: Average Nucleotide Identity

Genome sequence 1 **VS** Genome sequence 2





Fragments of the genome sequences are aligned and compared

Similarity value

ANI value between two genomes

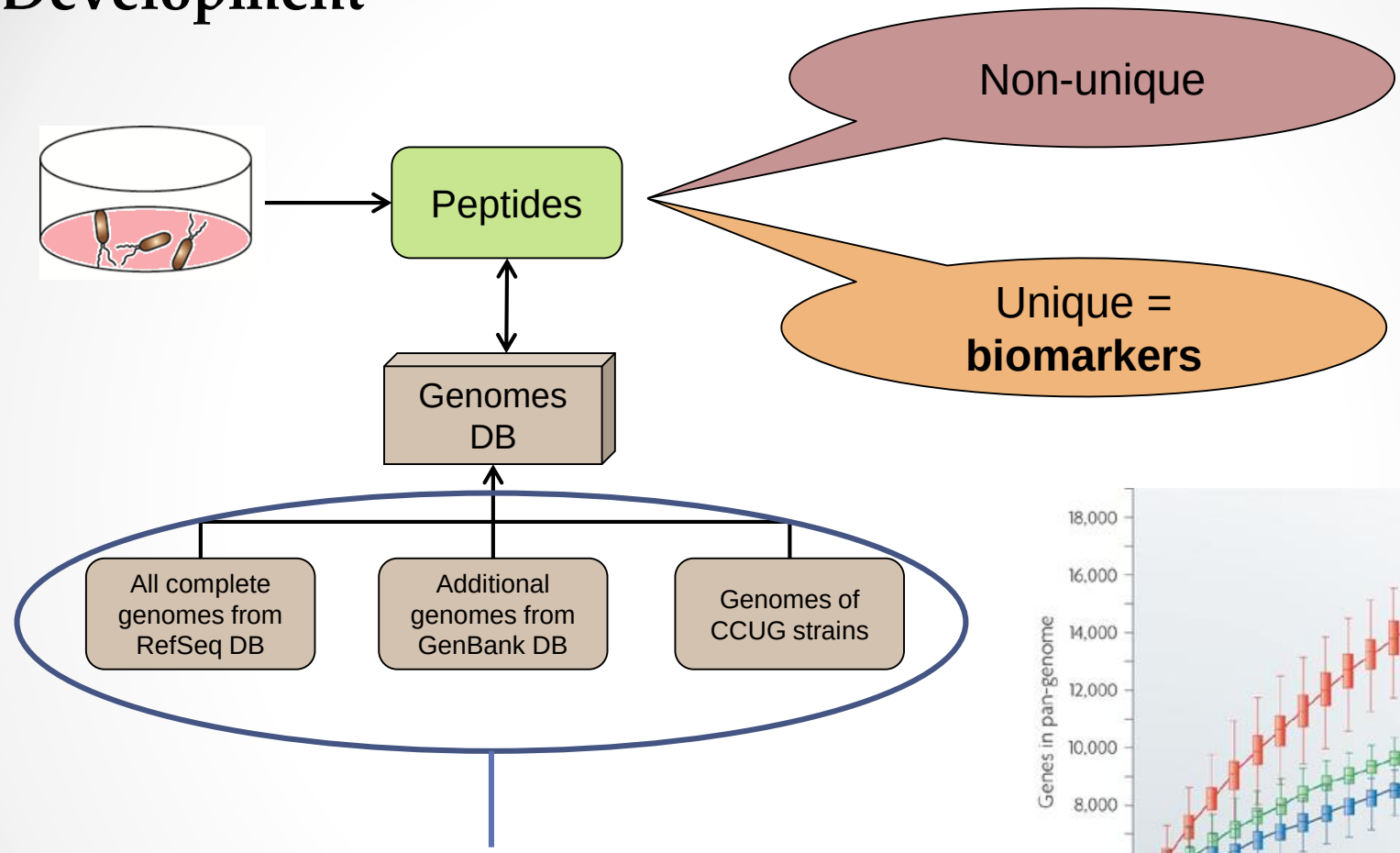
- **> 96%** = **Same species**
- **93 – 96%** = **Species boundary**
- **< 93%** = **Different species**

F Salvà Serra, CCUG

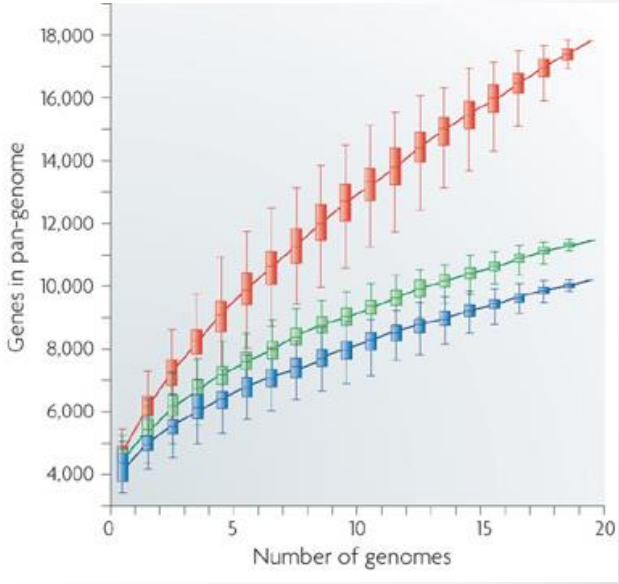
Organism	Total number of genome sequences	ANIb < 93%	ANIb 93 - 96%	ANIb ≥ 96%
Streptococcus pneumoniae	323	0	0	323
Streptococcus mitis	40	18	22	0
Streptococcus australis	0	0	0	0
Streptococcus cristatus	2	0	1	1
Streptococcus infantis	4	3	1	0
Streptococcus oligofermentans	7	7	0	0
Streptococcus oralis	11	7	4	0
Streptococcus parasanguinis	9	1	6	2
Streptococcus peroris	0	0	0	0
Streptococcus pseudopneumoniae	6	0	0	6
Streptococcus sanguinis	23	2	16	5
Streptococcus sinensis	0	0	0	0
Streptococcus tigurinus	3	0	3	0
Total	428	38	53	337
	100%	8.88%	12.38%	78.74%

F Salvà Serra, CCUG

Proteomic-Genomic analyses Development



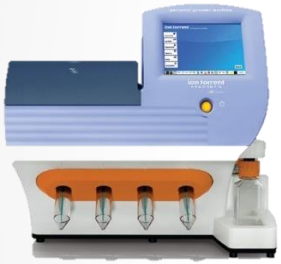
We need more genomes!!



Tenaillon *et al.* 2010

CCUG Genomics

- Started genome sequencing, January 2015
- To-date: approx 200 draft whole-genome sequences

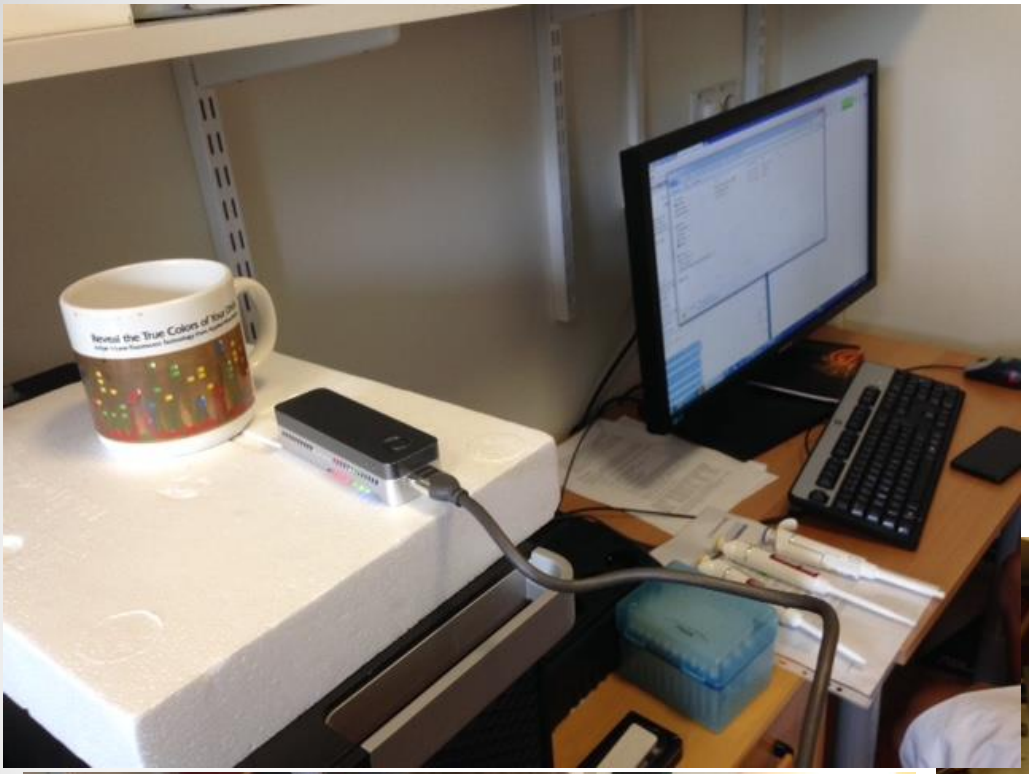


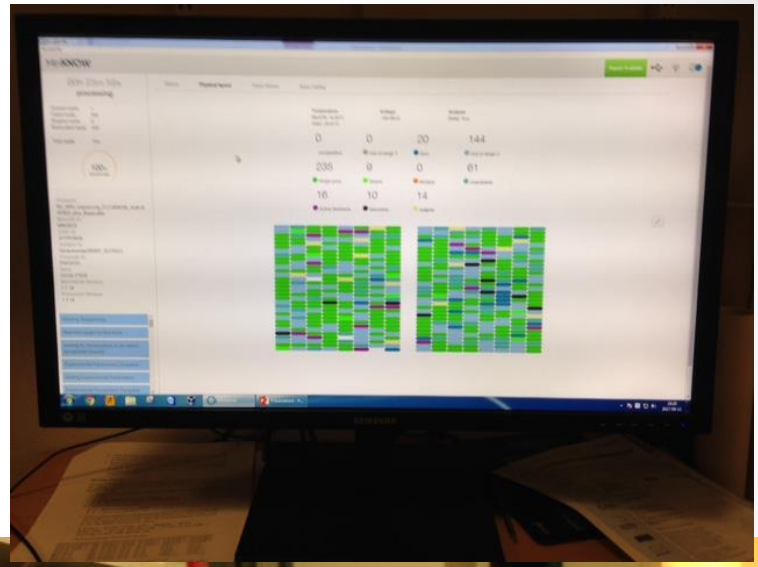
Ion-Torrent
(SU Clin Microiol)

Illumina
(SU Genomics Core)

PacBio
(NGI – Uppsala)

Introduction of Oxford Nanopore MinION to CCUG





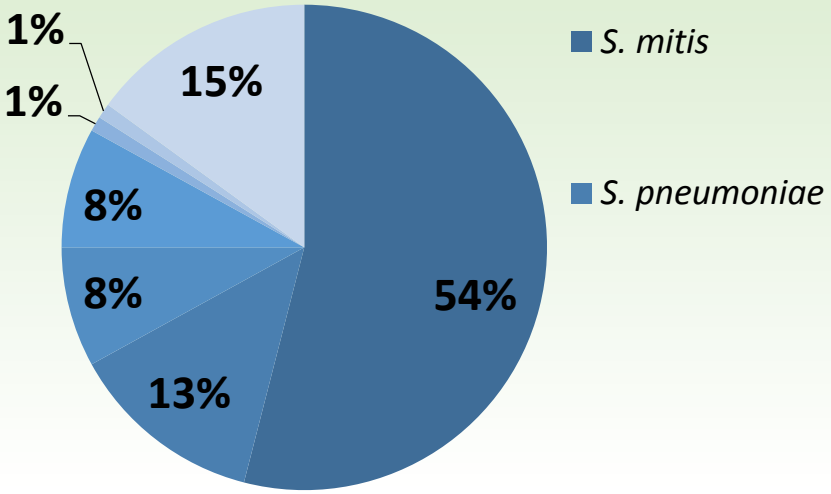
Addition of *Streptococcus* spp. genomes to the database

Organism	2015-02-10	2016-12-31
Streptococcus australis	0	1
Streptococcus cristatus	0	1
Streptococcus gordonii	1	3
Streptococcus infantis	0	1
Streptococcus mitis	1	33
Streptococcus oligofermentans	1	1
Streptococcus oralis	1	13
Streptococcus parasanguinis	2	3
Streptococcus peroris	0	1
Streptococcus pneumoniae	27	28
Streptococcus pseudopneumoniae	1	9
Streptococcus sanguinis	1	6
Streptococcus sinensis	0	1
Streptococcus tigurinus	0	4
	35	105

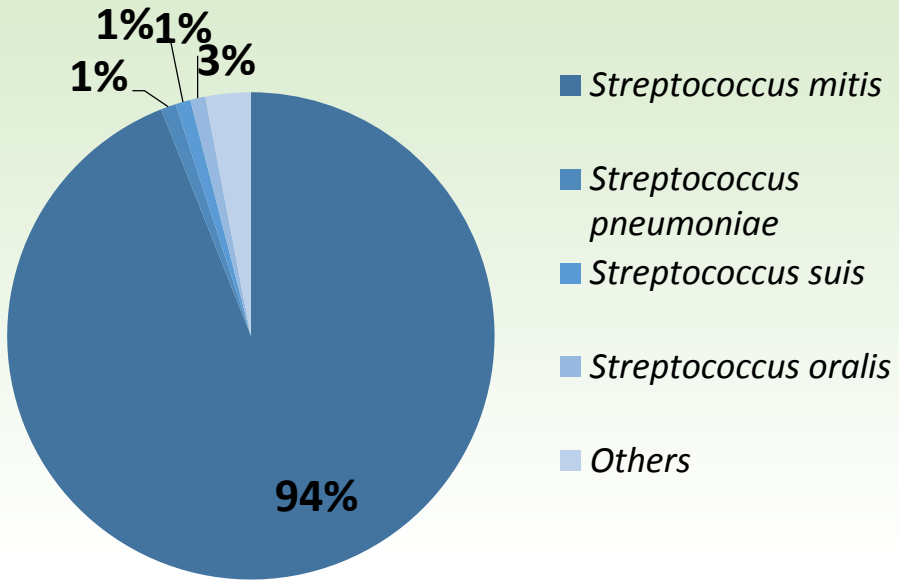
from F Salvà-Serra, EU TAILORED-Treatment project

Accuracy in finding the correct species in the S. Mitis-Group

***Streptococcus mitis* Type strain**



BEFORE
WGS database
update

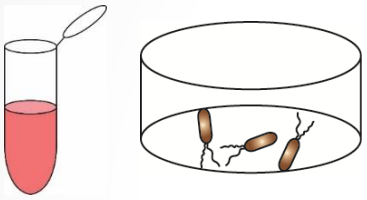


AFTER
WGS database
update

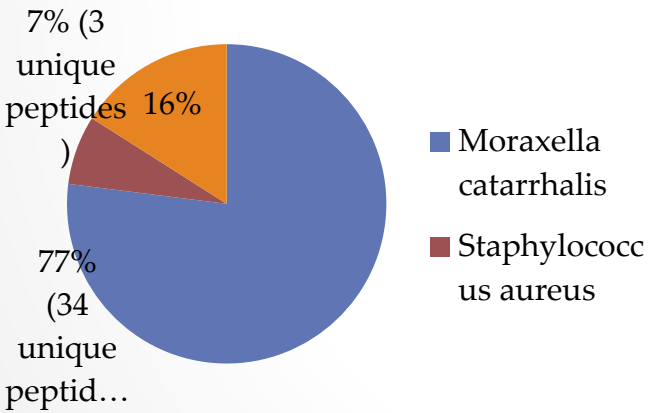
from R Karlsson, EU TAILORED-Treatment project

Proteotyping of Sahlgrenska Hospital clinical samples (nasopharyngeal) Positive samples

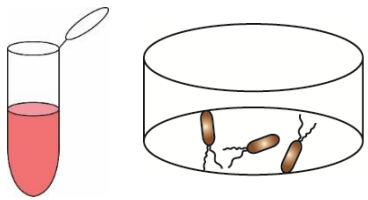
Example 1



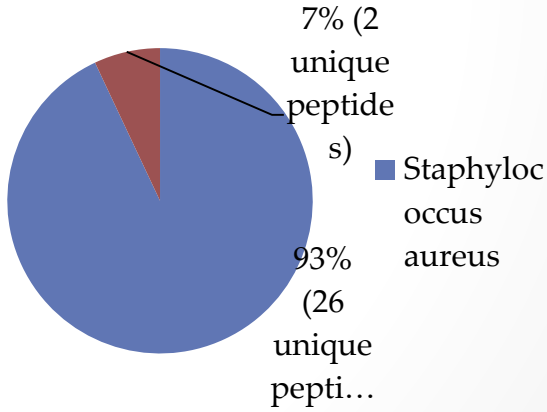
Sample **positive** for *Moraxella catarrhalis*



Example 2



Sample **positive** for *Staphylococcus aureus*

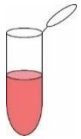


Results of MS-Proteomics peptide identification and matching

Direct MS-proteomics analysis of clinical sample
Biomarker inclusion list for *S. pneumoniae*

Samples from Utrecht Proteomic results

EU516 Clinical sample
From Utrecht Pediatrics Clinic



UM 6044

High number of bacteria

qPCR **positive** for
- ✓ *S. pneumoniae*
- ✓ *S. aureus*

Taxonomy view from Uniprot
Number of protein hits

- Bacteria (152)
  - Firmicutes (148)
    - Bacilli (144)
      - Lactobacillales (49)
        - Lactobacillus reuteri 100-23 (1)
        - Streptococcus (48)
          - Streptococcus pneumoniae (42)
          - Streptococcus pseudopneumoniae (2)
          - Streptococcus sanguinis (4)
        - Staphylococcus (95)
          - Staphylococcus aureus (91)
          - Staphylococcus hominis subsp. hominis C80 (1)
          - Staphylococcus simiae CCM 7213 (2)
          - Staphylococcus sp. M0480 (1)
      - Clostridiales (3)
        - Lachnospiraceae (2)
          - Lachnospiraceae bacterium 8_1_57FAA (1)
          - Ruminococcus torques ATCC 27756 (1)
        - Syntrophomonas wolfei subsp. wolfei (strain DSM 2245B / Goettingen) (1)
      - Mitsuokella sp. oral taxon 131 str. W9106 (1)
    - Mycobacterium vanbaalenii (strain DSM 7251 / PYR-1) (1)
    - Mycoplasma sp. CAG:472 (1)
    - Proteobacteria (2)
      - Pseudomonas fulva (strain 12-X) (1)
      - Rhodopseudomonas palustris (strain BisB5) (1)
  - Eukaryota (309)

from R Karlsson, EU TAILORED-Treatment project

Proteotyping of *S. mitis* complex

All strains analysed in triplicate
Good reproducibility
Identified peptides and proteins
Identified discriminatory peptides

Discriminatory proteins

S. pneumoniae discriminatory proteins found by proteotyping workflow

- general stress protein 24
- surface protein PspA
- rpsP 30S ribosomal protein S16
- strH beta-N-acetylhexosaminidase
- cbpA choline binding protein A
- acpP acyl carrier protein
- ugd UDP-glucose 6-dehydrogenase
- prsA foldase PrsA
- lytB endo-beta-N-acetylglucosaminidase

- **SURFACE-ASSOCIATED VIRULENCE FACTORS**

	S. pneumoniae	*S. oralis*	*S. mitis*	*S. pseudopneumoniae*	*S. pyogenes*
Number of proteins	649	564	678	622	407
Number of peptides	3556	2495	3722	2141	1197
Number of discriminative peptides	417	282	237	313	308

S. pneumoniae virulence factors

		S. pne	S. pse	S. mit
PspA	Pneumococcal surface protein A	+++	N	N
LytA	Autolysin A	++	N	N
Bac	Bacteriocin	++	++	N
StrH	B-N-acetylglucosaminidase	+++	?	?
BgaA	B-galactosidase	++	?	?
CpbA	Choline binding protein A	+++	N	N
Eno	Enolase	++	+	+
Hyl	Hyaluronate lyase	N	N	N
IgA	IgA1 protease	++	N	N
Nan	Neuraminidase	++	N	N
ChoP	Phosphorylcholine	?	?	?
PavA	Pneumococcal adhesion/virulence A	++	N	N
PlaA	Pneumococcal iron acquisition A	?	N	N
PiuA	Pneumococcal iron uptake A	?	N	N
PsaA	Pneumococcal surface antigen A	++	N	N
Ply	Pneumolysin	N	?	?

Biomarker inclusion list
S. pneumoniae

Workflow

Selection of potential biomarker peptides

- Diluted cell cultures
  - Analysis with inclusion lists to set retention time window
    - Targeted MSMS of diluted cell cultures
      - Analysis of negative clinical samples spiked with cell culture
        - Analysis of positive clinical samples

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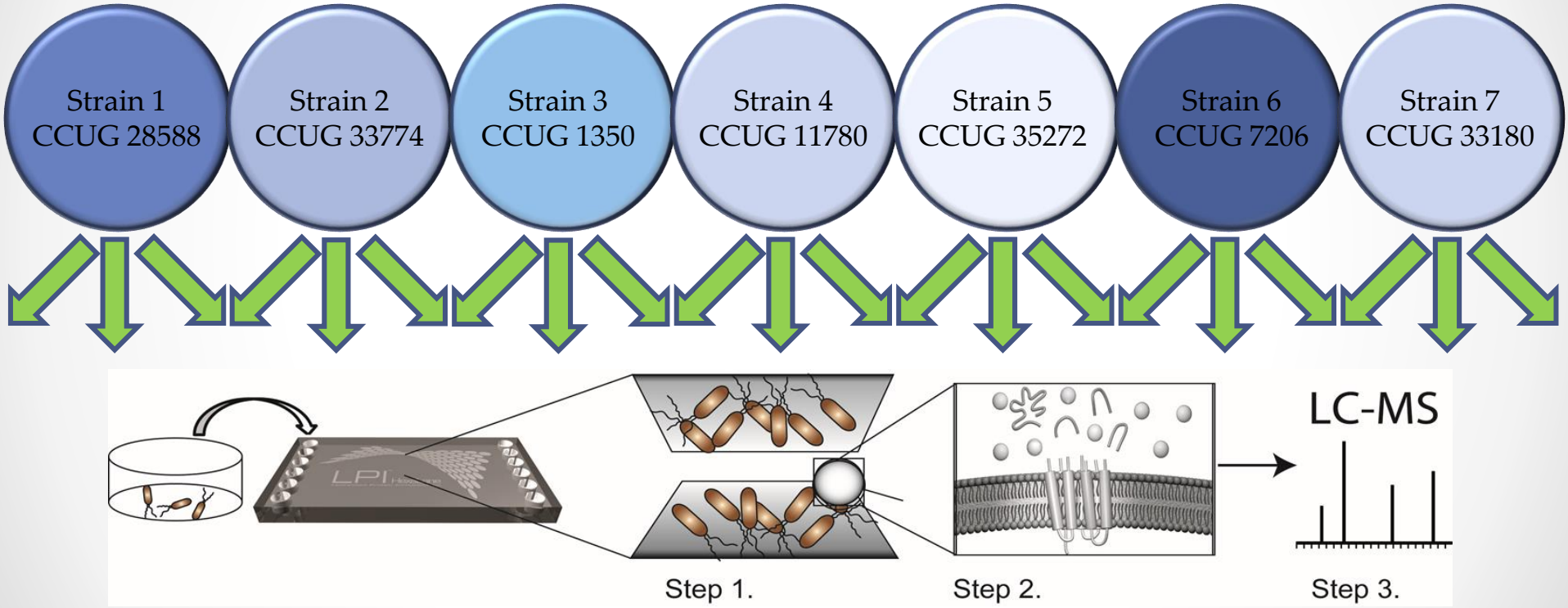
Biomarker inclusion list for *S. pneumoniae*

7 strains – including Type strain



All strains run in triplicate = 21 MS runs

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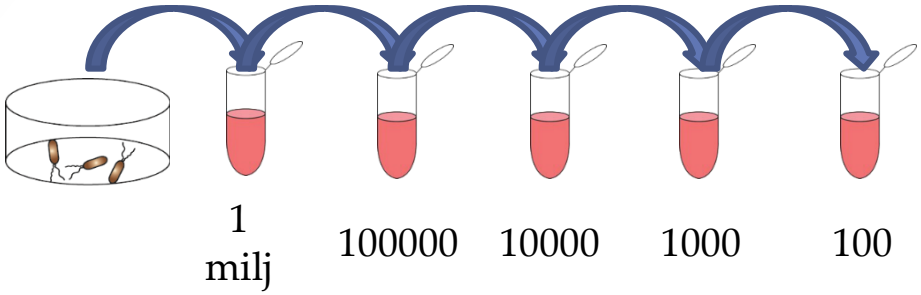
Biomarker inclusion list for *S. pneumoniae*



	Inclusion list				Normal non-targeted			
Sequence	100	1000	100000	1 milj	100	1000	100000	1 milj
VSDVAESTGEFTSEQFEK	High	High	High	High			High	High
DAELLFAGIVGDTGR		Low	High					
YTLLAGETPAVAAAIR		High	High	High				High
IDTFGTGTVAESQLEK		High	High	High			High	High
NGNYETAEGSEETSSEVK		High	High	High			High	High
AHIYFINSEEPSQLNDLQAFR		High	High	High				High
ANYGVSADK			High	High				High
AVSAILGELNGNEGK			High	High			High	High
LMDVAQPELAIVFGR			High	High				

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Analysis of positive clinical samples – respiratory tract samples
Sputum from CVID patients

	EU sample	BACT LAB IDENTIFICATION	TCUP - open	Targeted	
Clinical samples	1257	Negative	S. mitis	S. pneumoniae M. catarrhalis	
	1258	Negative	-	-	CORRECT
	1259	Negative	-	M. catarrhalis??	
	1260	Negative	-	-	CORRECT
	1261	Negative	S. mitis	S. pneumoniae M. catarrhalis	
	1262	Negative	-	-	CORRECT
	1263	H. influenzae - isolate analyzed as 1266	-	-	
	1264	H. influenzae - isolate analyzed as 1268 S. pneumoniae - isolate analyzed as 1267	H. influenzae S. pneumoniae	H. influenzae S. pneumoniae	CORRECT
	1264	H. influenzae - isolate analyzed as 1268 S. pneumoniae - isolate analyzed as 1267	H. influenzae S. pneumoniae	H. influenzae S. pneumoniae	CORRECT
	1265	Negative	-	M. catarrhalis	
Isolates from clinical samples	1266	H. influenzae	-	H. influenzae	CORRECT
	1267	S. pneumoniae	S. pneumoniae	S. pneumoniae	CORRECT
	1268	H. influenzae	H. influenzae	H. influenzae	CORRECT

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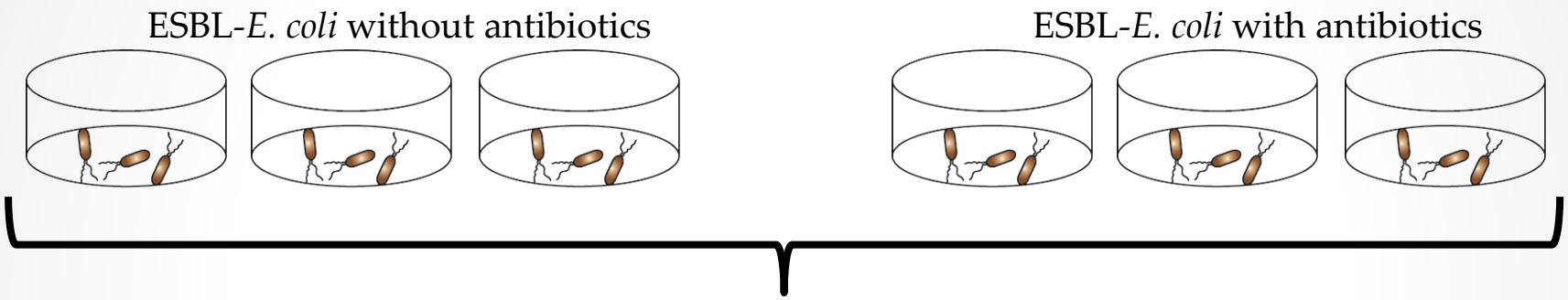
Proteotyping - Detection of anti-microbial resistance (AMR)

- WGS of urinary tract infectious bacteria
  - Type strains
  - Model strains
  - Multiresistant strains (CCUG, Sahlgrenska, Rotger)
- Inclusions lists
- Pan-genome analyses

Detection of anti-microbial resistance (AMR)



Sample preparation through bead beating

↓

Digestion of proteins using trypsin

↓

Analysis using LC-MS/MS

↓

Database search against known resistance genes

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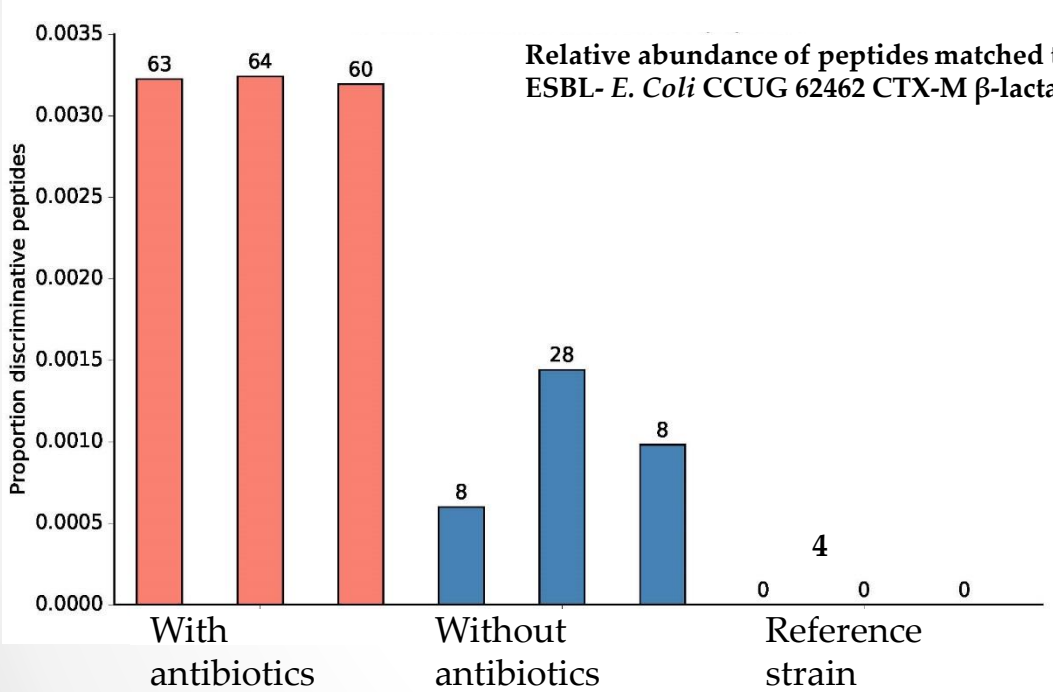
Detection of anti-microbial resistance (AMR)



- The number of peptides detected that match to CTX-M β-lactamase are significantly higher with exposure to antibiotic than without.
- These data were compared with those from analyses of a non-ESBL-*E. coli* Reference strain.
- All analyses were done in triplicate.

Experiments:
Resistance factors detected
In WGS

from R Karlsson, EU TAILORED-Treatment project

Conclusions

- Proteotyping provides high-resolution characterisation of bacteria;
- Proteotyping is able to detect virulence factors;
- Proteotyping is able to detect AMR factors;
- Proteotyping is applicable to clinical samples, without cultivation;
- Proteotyping requires comprehensive and accurate wgs database;
- Proteotyping requires a stable taxonomy;
- Bacteria do not cooperate with microbial systematists;
- There is more interesting work to be done

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EU – TAILORED-Treatment Project (www.tailored-treatment.eu)

Vetenskapsrådet

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VÄSTRA GÖTALANDSREGIONEN

Strama

ALF-Medel / Regionala FoU