Plant Pathogenic Bacteria
A Basic Guide to Identification

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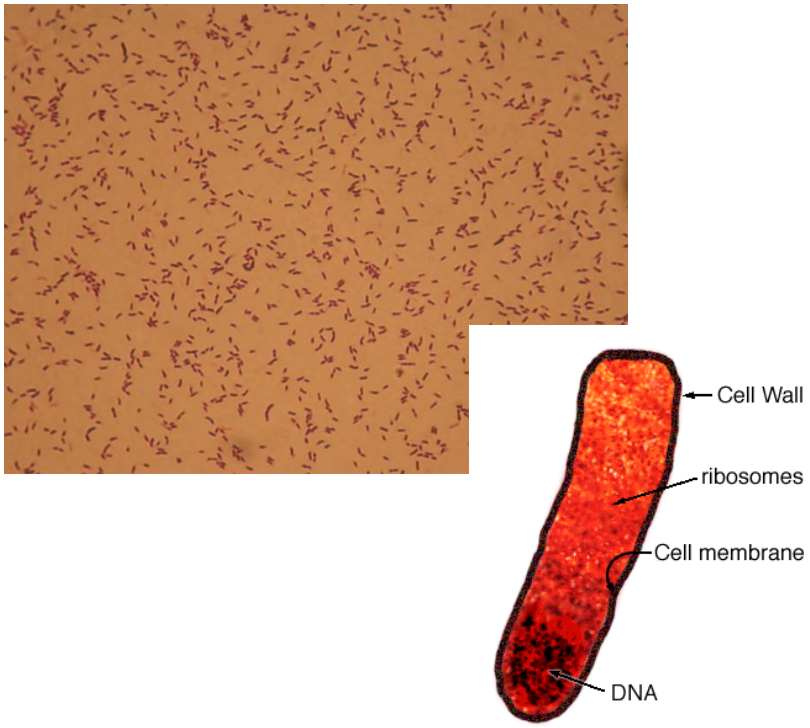
Module 1

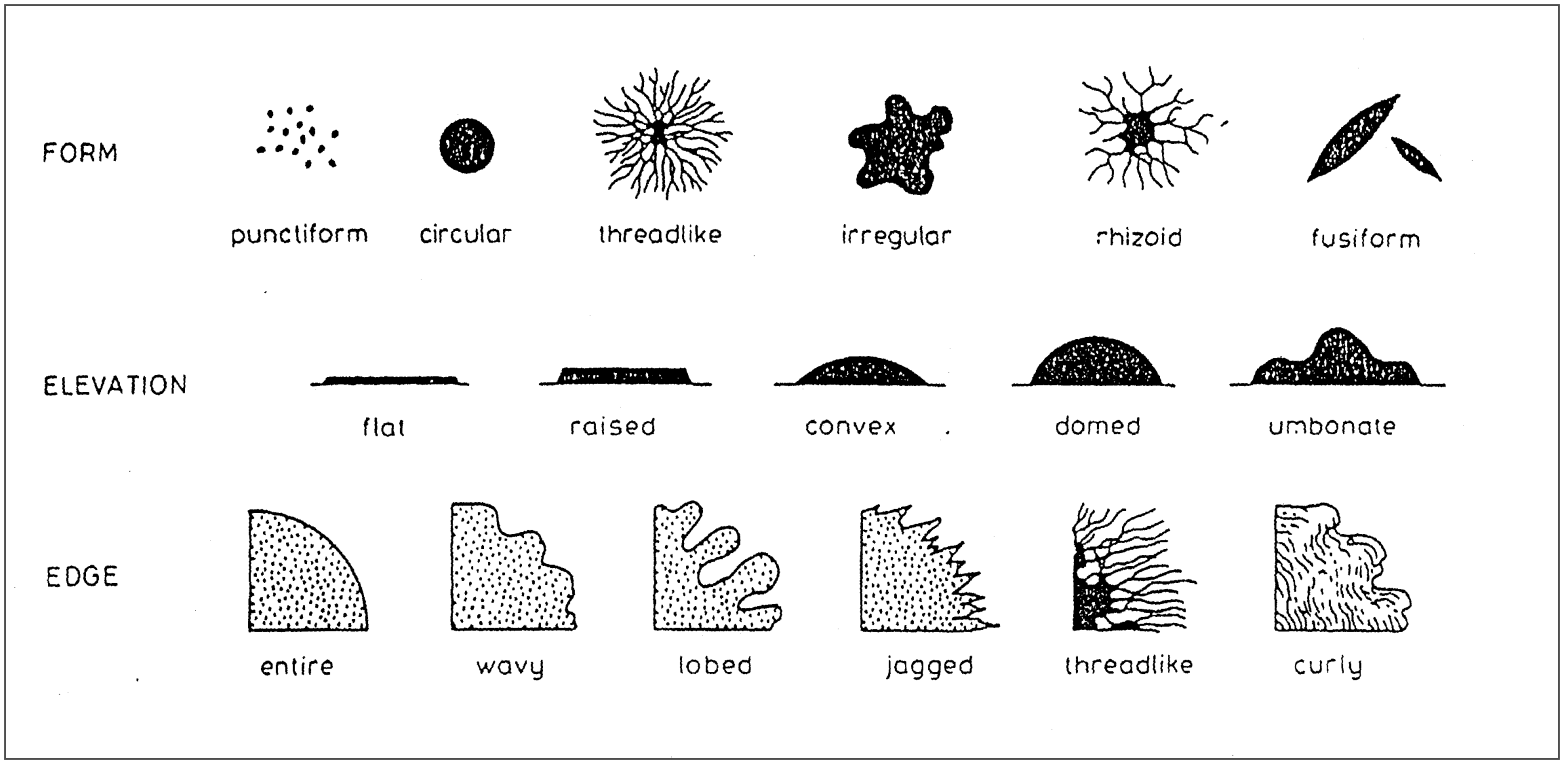
Prokaryotes

- Three invariant characteristics



- Cell membrane
- Cytoplasmic 70S ribosome
- Non-membrane-bound nucleoid

Bacterial colony morphology



- Prokaryotes are ubiquitous and physiologically diverse
- Occupy a wide range of ecological niches, active
  - as autotrophs, obtaining energy from inorganic sources or light
  - as saprophytes, obtaining energy from the breakdown of complex organic material
  - as symbionts, living co-operatively with other organisms
  - as parasites, attacking and often killing other living things. The parasites are relatively few in both kinds and numbers.

Taxonomic ranks

- **Genus:** A discrete related group of species. Strains usually have >30% DNA homology
- **Species:** A discrete taxonomic unit; several well defined phenotypic differences from related species. Strains usually have >70% DNA homology
- **Subspecies:** One or two well defined phenotypic differences from other subspecies
- **Biovar:** One or two minor phenotypic differences from other biovars
- **Pathovar**: Pathological varieties. Have distinct host specificity range for a plant species or genus, or distinct symptoms on the same host
- **Race**: Specificity / virulence for some, but not all, cultivars of a plant species. Based on host resistance genes and avirulence genes in the pathogen

Plant Bacterial Pathogens

- All within the domain Bacteria
- Occur worldwide
- Exploit all environments and affect all major plant groups, but favour warm, moist environments
- Fall within 3 major groups
  - Gram negative
  - Gram positive
  - Non-culturables

The main plant pathogenic genera

- Gram -ve
  - *Acidovorax*
  - *Agrobacterium*
  - *Brenneria*
  - *Burkholderia*
  - *Dickeya*
  - *Enterobacter*
  - *Erwinia*
  - *Pantoea*
  - *Pseudomonas*
  - *Pectobacterium*
  - *Pseudomonas*
  - *Ralstonia*
  - *Xanthomonas*

The main plant pathogenic genera

- Gram +ve
  - *Arthrobacter*
  - *Bacillus*
  - *Clavibacter*
  - *Curtobacterium*
  - *Leifsonia*
  - *Nocardia*
  - *Rathayibacter*
  - *Rhodococcus*
  - *Streptomyces*
- Non-culturables [Candidatus]
  - *Liberobacters*
  - Phytoplasmas
  - *Spiroplasma*

Characterisation and identification of bacteria

- Various methods exist
  - Host range
  - Phenotypic [biochemical] properties
  - Protein profiles
  - Fatty acid profiles
  - DNA homology and sequence data
  - DNA fingerprints

  - [A polyphasic approach to study]

Methods, processes and end-points [2]

- Phytosanitary testing
  - Pre-described test procedure for known crop / pest combinations only
  - Limited need for wider inclusion of other pest knowledge; can be achieved by non-specialists
  - Require specific, dedicated infrastructure
  - Known outcome, with statistical confidence
  - Primarily driven by EU directives

Methods, processes and end-points [3]

- Identification of unknowns
  - Receiving of plant / pest combinations of any type
  - Need to be inclusive of all pest types [entomological, mycological, bacterial, viral]
  - Requires expert knowledge, infrastructure and access to reliable information resources
  - Driven by national demand for providing broad services in plant health as supports commercial interests

Methods, processes and end-points [4]

- Research
  - Responsive to demand
  - Need to be inclusive of all pest types [entomological, mycological, bacterial, viral]
  - Requires expert knowledge, infrastructure and access to reliable information resources
  - Driven by national demand for providing broad services in plant health as supports commercial interests

Methods for bacterial identification

- Biochemical tests – traditional methods
- Formatted biochemical tests
  - API strips
  - Biolog
- GC Fatty acid profiles – MIDI system
- Serological – immunological methods
- DNA methods
  - DNA homology
  - 16S rDNA
  - Fingerprinting

Biochemical tests

Biochemical tests – example 1

- *Pantoea stewartii*
  - Non-motile
  - Colonies do not show symplasmata or inclusion
  - Negative for production of H_2S from cysteine, acetone, phenylalanine deaminase, nitrate reductase and gelatinase
  - Acid is produced from melibiose; non-acid from dulcitol, maltose,rhamnose or starch

Biochemical tests – example 2

- *Ralstonia solanacearum*
  - Non-fluorescent pseudomonas with polar tuft flagella
  - Cells non-pigmented, but brown diffusible pigment often produced
  - PHB is accumulated
  - Levan not formed from sucrose
  - Gelatin hydrolysis weak
  - Starch and aesculin not hydrolysed
  - Nitrate reduced by nearly all strains; many produce gas [denitrifying]
  - No growth at 40C
  - Oxidase positive
  - Arginine dihydrolase negative
  - Most strains produce tyrosinase
  - Light or no growth in broth containing 2% NaCl; no growth at 40C
  - Carbon sources used for growth: acetate, aconitate, L. alanine, D-alanine, γ-aminobutyrate, asparagine, L-aspartate, benzoate, butyrate, citrate, fumarate, gluconate, D-glucose, L-glutamate, glycerol, L-histidine, β-hydroxybutyrate, α-ketoglutarate, L-malate, mucate. L-proline, proionate, pyruvate, saccharate, succinate, sucrose and trehalose

Dichotomous key – some key biochemical tests

- Gram test [Gram –ve and +ve bacterium]

- Gram –ve
  - Anaerobic growth
  - Yellow colonies on YDC
  - Fluorescent pigment
  - Urease
  - Growth at 33C and 40C
  - Growth on D1M agar
  - Utilization of arginine and betaine

- Gram +ve
  - Endospores formed
  - Anaerobic growth
  - Ariel mycelium

The LOPAT tests for fluorescent *Pseudomonads*

L	O	P	A	T	Group	Example
+	-	-	-	+	Ia	*P. syringae*
-	-	-	-	+	Ib	*P. savastanoi*
-	-	+	-	+	II	*P. viridiflava*
-	+	-	-	+	III	*P. cichorii*
+	+	+	+	-	IVa	*P. marginalis*
-	+	+	+	-	IVb	*P. fluorescens* complex
-	+	-	+	-	Va	*P. tolaasii*
+	+	-	+	-	Vb	*P. fluorescens* complex

Levan production / oxidase reaction / Potato Rot / Arginine dihydrolose production / tobacco hypersensitivity – LOPAT – p26 Phytobacteriology book

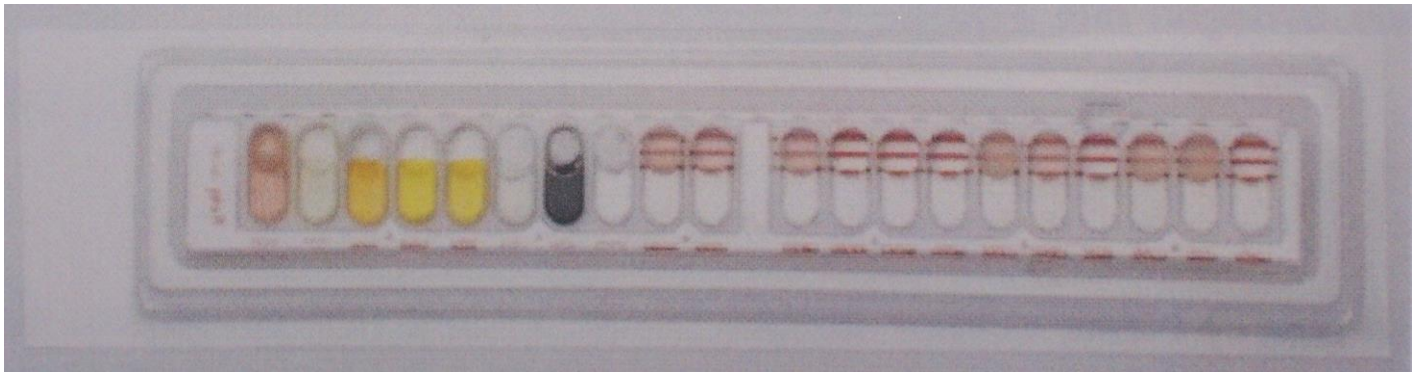
Biochemical tests

- Advantages
  - Is not requiring of expensive equipment and searchable data bases
- Limitations
  - Methods are many, some sequential and time consuming
  - Reagent list for tests is extensive and prepared media is to be aliquoted into many different formats
  - Many tests give variable strain specific results and some tests are unreliable
  - Technicians need to be very familiar with methods and competent in their use

Biochemical formatted platforms

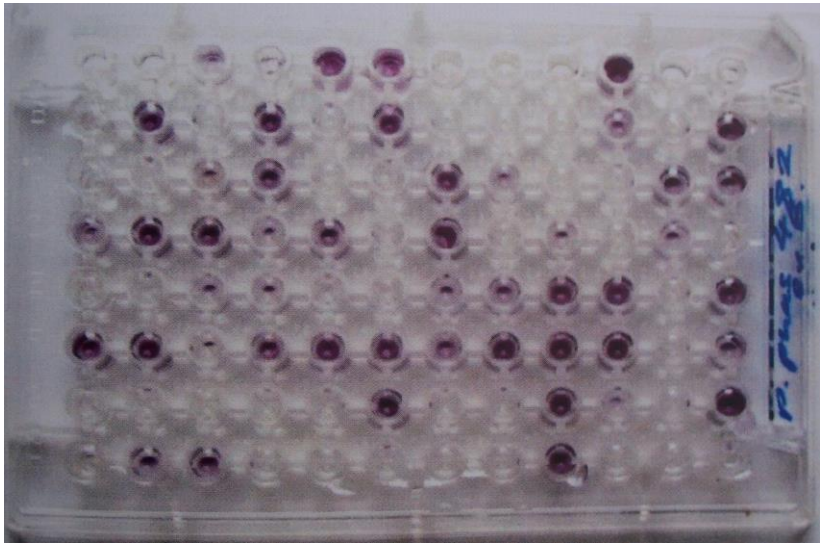
- Takes the biochemical tests and places them on a more convenient format
- Two main commercial products
  - API strips – [http://industry.biomerieux-usa.com/industry/food/api/index.htm]
  - Biolog – [http://www.biolog.com/main.html]
- Results achieved within 48hrs
- Results [+ & -ve data] fed into library of described strains
- Similarity values on most likely identification
- Requires judgement over identifications presented

API



- Each well contains a different substrate
- Results are recorded as either a substrate colour change or as growth

The Biolog system



- The Biolog system presents an extended array of biochemical tests
- A positive result is seen as a purple colour change
- The plate can be read by eye or by a plate reader

A1 Water	A2 α-cyclodextrin	A3 dextrin	A4 glycogen	A5 tween 40	A6 tween 80	A7 N-acetyl-D-galactosamine	A8 N-acetyl-D-glucosamine	A9 adonitol	A10 L-arabinose	A11 D-arabitol	A12 cellobiose
B1 i-erythritol	B2 D-fructose	B3 L-fucose	B4 D-galactose	B5 gentiobiose	B6 α-D-glucose	B7 m-inositol	B8 α-D-lactose	B9 lactulose	B10 maltose	B11 D-mannitol	B12 D-mannose
C1 D-melibiose	C2 β-methyl D-glucoside	C3 D-psicose	C4 D-raffinose	C5 L-rhamnose	C6 D-sorbitol	C7 sucrose	C8 D-trehalose	C9 turanose	C10 xylitol	C11 methyl pyruvate	C12 mono-methyl succinate
D1 acetic acid	D2 cis-aconitic acid	D3 citric acid	D4 formic acid	D5 D-galactonic acid lactone	D6 D-galacturonic acid	D7 D-gluconic acid	D8 D-glusaminic acid	D9 D-glucuronic acid	D10 α-hydroxy butyric acid	D11 β-hydroxy butyric acid	D12 γ-hydroxy butyric acid
E1 p-hydroxy phenylacetic acid	E2 itaconic acid	E3 α-keto butyric acid	E4 α-keto glutaric acid	E5 α-keto valeric acid	E6 D, L-lactic acid	E7 malonic acid	E8 propionic acid	E9 quinic acid	E10 D-saccharic acid	E11 sebacic acid	E12 succinic acid
F1 bromo succinic acid	F2 succinamic acid	F3 glucunoramide	F4 alaninamide	F5 D-alanine	F6 L-alanine	F7 L-alanyl -glycine	F8 L-asparagine	F9 L-aspartic acid	F10 L-glutamic acid	F11 glycyl L-aspartic acid	F12 glycyl L-glutamic acid
G1 L-histidine	G2 hydroxy L-proline	G3 L-leucine	G4 L-ornithine	G5 L-phenylalanine	G6 L-proline	G7 L-pyroglutamic acid	G8 D-serine	G9 L-serine	G10 L-threonine	G11 D, L-carnitine	G12 γ -amino butyric acid
H1 urocanic acid	H2 inosine	H3 uridine	H4 thymidine	H5 phenyl ethylamine	H6 putrescine	H7 2-amino ethanol	H8 2,3-butanediol	H9 glycerol	H10 D, L- α-glycerol phosphate	H11 glucose-1-phosphate	H12 glucose-6-phosphate

Biochemical formatted platforms [Biolog]

- Advantages
  - Is not requiring of expensive equipment
  - System is quick, reproducible and easy to perform
  - Data can be shared between laboratories
  - Can provide a reasonable identification to the genus and species level
- Limitations
  - Requires investment [access] to the library
  - Has limitation in resolving below species level [pathovar separation]
  - Library stronger on human microbials than plant pathogenic bacteria

Fatty acid analysis – the Midi system

Fatty acids

- Gram-negatives
  - Unique hydroxy patterns
  - Some cyclopropanes
  - Few branched acids
- Gram positives
  - Many branched acids
  - Very few hydroxy and cyclopropane acids.

The Midi system

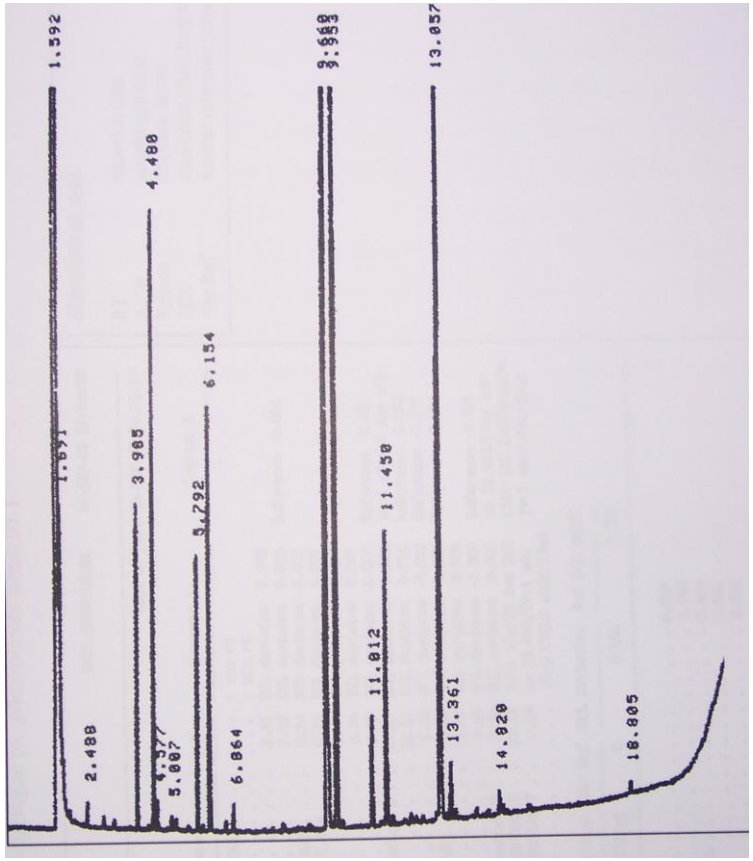
- Commercial and / or lab produced – [http://www.midi-inc.com/]
- Based on comparisons of types and amounts of acids
- Interrogates library for identification
- Typical “return”:

NCPPB Rev 3.0	Agrobacterium biovar 1 …….	0.814
	Agrobacterium biovar 2 …….	0.567
	Agrobacterium biovar 3 [vitis] …	0.316

Fatty acid extraction process

- Culture Cells [i.e. 24hr on TSBA]
- Harvest Cells
- Saponify Lipids
- Methylate Fatty Acids [FAMEs]
- Extract and Purify
- GC analysis
- Comparison to library

MIDI system print outs



Sherlock Version: 3.10 DATA:E00915545A 16-SEP-00 09:06:00

ID: 2032 NM-P. PHAS. 95.1 Date of run: 16-SEP-00 08:35:27
Bottle: 41 SAMPLE [TSBA40]

RT	Area	Ar/Ht	Respon	ECL	Name	%	Comment 1	Comment 2
1.604	309664512	0.031	. . .	7.021	SOLVENT PEAK	. . .	< min rt	
2.497	656	0.026	. . .	8.942	. . . . .	. . .	< min rt	
3.995	10872	0.032	1.053	11.424	10:0 3OH	2.41	ECL deviates 0.002	
4.490	23184	0.035	1.031	12.000	12:0	5.03	ECL deviates 0.000	Reference 0.001
4.587	1112	0.034	1.028	12.090	11:0 ISO 3OH	0.24	ECL deviates 0.001	
5.015	600	0.037	1.015	12.485	unknown 12.484 . . .	0.13	ECL deviates 0.001	
5.803	11480	0.038	0.996	13.177	12:0 2OH	2.40	ECL deviates 0.000	
6.164	18088	0.039	0.989	13.455	12:0 3OH	3.76	ECL deviates 0.001	
6.872	1216	0.038	0.978	14.000	14:0	0.25	ECL deviates -0.000	Reference 0.001
9.670	165296	0.046	0.954	15.819	Sum In Feature 3 . .	33.17	ECL deviates -0.003	16:1 w7c/15 iso 2OH
9.963	141496	0.046	0.953	16.001	16:0	28.35	ECL deviates 0.001	Reference 0.002
11.021	6136	0.049	0.949	16.628	17:0 ISO	1.22	ECL deviates -0.002	Reference -0.001
11.460	16376	0.049	0.948	16.889	17:0 CYCLO	3.26	ECL deviates 0.001	Reference 0.001
13.068	94856	0.050	0.946	17.824	18:1 w7c	18.87	ECL deviates 0.001	
13.372	3184	0.050	0.946	18.000	18:0	0.63	ECL deviates -0.000	Reference -0.001
14.830	1384	0.051	0.945	18.848	Sum In Feature 7 . .	0.28	ECL deviates 0.002	un 18.846/19:1 w6c
*******	165296	. . .	. . .	. . .	SUMMED FEATURE 3 . .	33.17	16:1 w7c/15 iso 2OH	15:0 ISO 2OH/16:1w7c
*******	1384	. . .	. . .	. . .	SUMMED FEATURE 7 . .	0.28	un 18.846/19:1 w6c	19:1 w6c/.846/19cy
*******	. . .	. . .	. . .	. . .	. . . . .	. . .	19:0 CYCLO w10c/19w6	

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	ECL Deviation	Ref ECL Shift
309664512	495280	495280	100.00	475532	6	0.001	0.001

IMIBAC (Rev 1.0) * NO MATCH *
TSBA40 (Rev 4.10) Pseudomonas 0.899
P. syringae 0.899
P. s. phaseolicola 0.899
P. s. syringae 0.841
P. s. glycinea 0.820

- GC trace [left]
- Library analysis [above]

Key acids from 4 genera

Acid	*Acidovorax*	*Ralstonia*	*Pseudomonas*	*Burkholderia*
10:0 3OH	+		+	+
12:0 2OH			+	
12:0 3OH			+	
14:0 3OH		+	+	+
16:0 2OH		+		+
16:0 3OH			+	+
16:1 2OH		+		
18:1 2OH		+		+

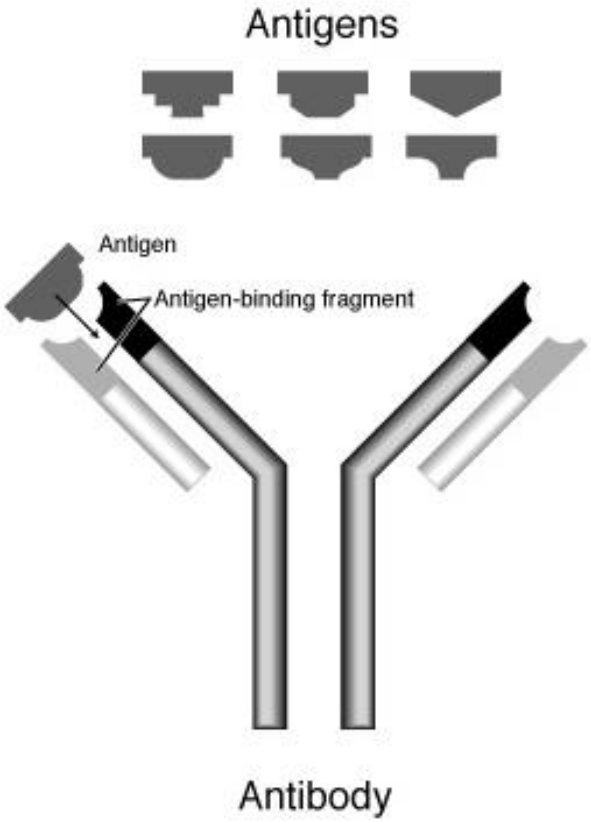
Fatty acid analysis

- Advantages
  - System is quick, reproducible and easy to perform
  - Data can be shared between laboratories
  - Can provide a reasonable identification to the genus and species level
- Limitations
  - Requires investment in GC equipment and MIDI library
  - Has limitation in resolving below species level [pathovar separation]
  - Library stronger on human microbials than plant pathogenic bacteria

Serological approaches

Serological approaches

- Rapid
- Sensitive
- Specific
- Diagnose diseases



Immunoassays are based on antibodies….

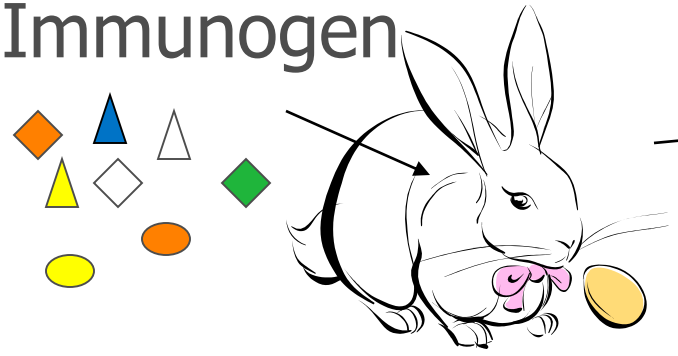
- Mammals produce antibodies that specifically recognize binding sites (epitopes) on proteins, glycoproteins, lipopolysaccharides, carbohydrates (antigens)
  - Polyclonal antibodies
  - Monoclonal antibodies
- Antibodies specifically bind antigens
- Bound antibodies are detected with various markers

Polyclonal Antibodies

- Immunogens (preparations containing antigens that are used to immunize an animal)
  - Various degrees of purification of immunogens
    - Whole cells
    - Cell (surface) washings
    - Virus particles
    - Broken cells
    - Purified cell components
- Immunogens injected into animals for antibody production

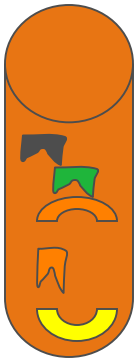
Polyclonal antibody production

Immunogen



Collect blood; separate serum

Purify antiserum



Immunoassay

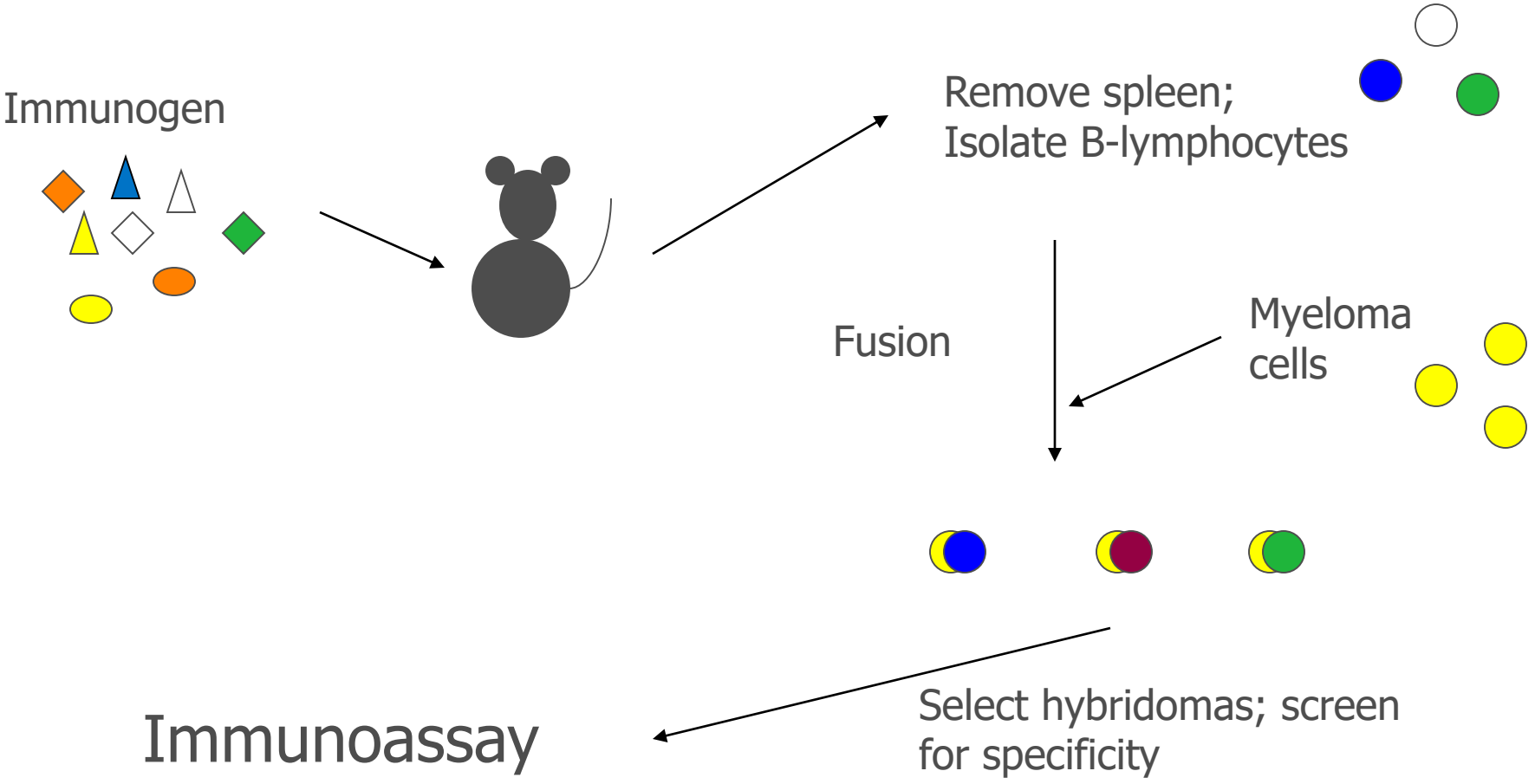
Characteristics of polyclonal antisera

- High sensitivity
- Varying specificity - depending on purity of immunogen/number of epitopes
- May vary from batch to batch

Monoclonal antibodies

- Single type of antibody
- Highly specific
  - Recognize single epitope
- Sensitivity varies
- Produced by hybridoma cell lines that are theoretically immortal

Monoclonal antibody production



Immunoassay formats

- Enzyme-linked immunosorbent assay (ELISA)
  - Enzyme conjugated to antibody = marker
    - Alkaline phosphatase
    - Peroxidase
- Lateral flow immunoassay
  - Ab-Ag binding occurs as mixture flows through solid phase in liquid
- Immunofluorescence
  - Fluorescent molecule marks Ab-Ag reaction

ELISA

- Positive reaction indicated by enzymatic reaction with chromogenic substrate = color change
  - Antigen capture/plate-trapped antigen
    - Antigen bound to solid phase
  - Indirect vs. direct
    - Direct = detecting antibody conjugated with enzyme
    - Indirect = enzyme conjugated to secondary antibody
  - Sandwich ELISA (double antibody, triple antibody)

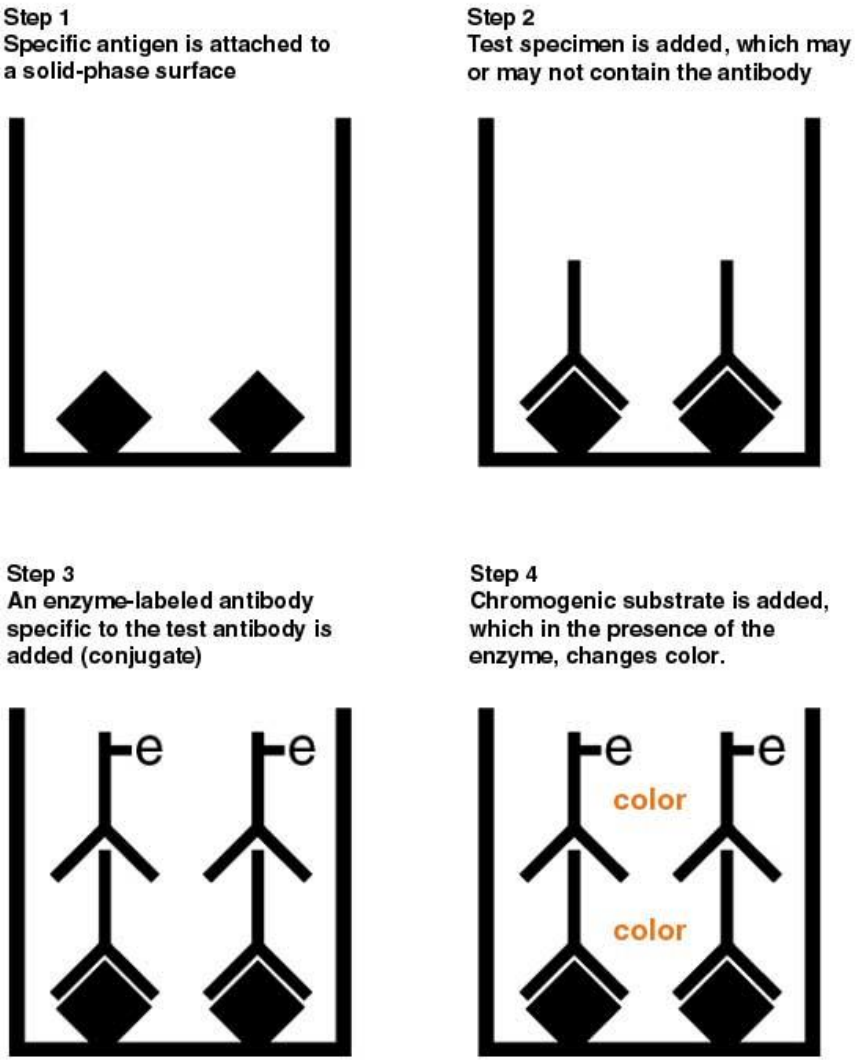
Direct, Double Ab Sandwich ELISA

DAS-ELISA

Indirect, Plate-trapped Antigen ELISA

Primary Ab specific to Antigen

Secondary Ab produced in a different species, e.g. goat - specific to primary Ab



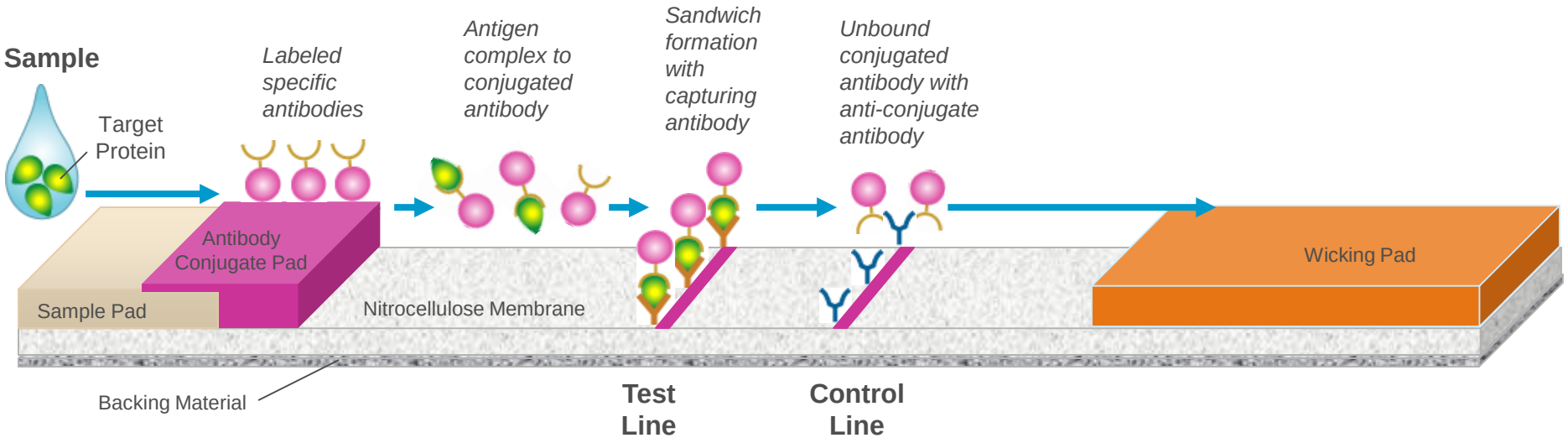
Multiwell Immunoasay

- Many commercially available
- Most detect various viruses
- Also for bacteria, fungi

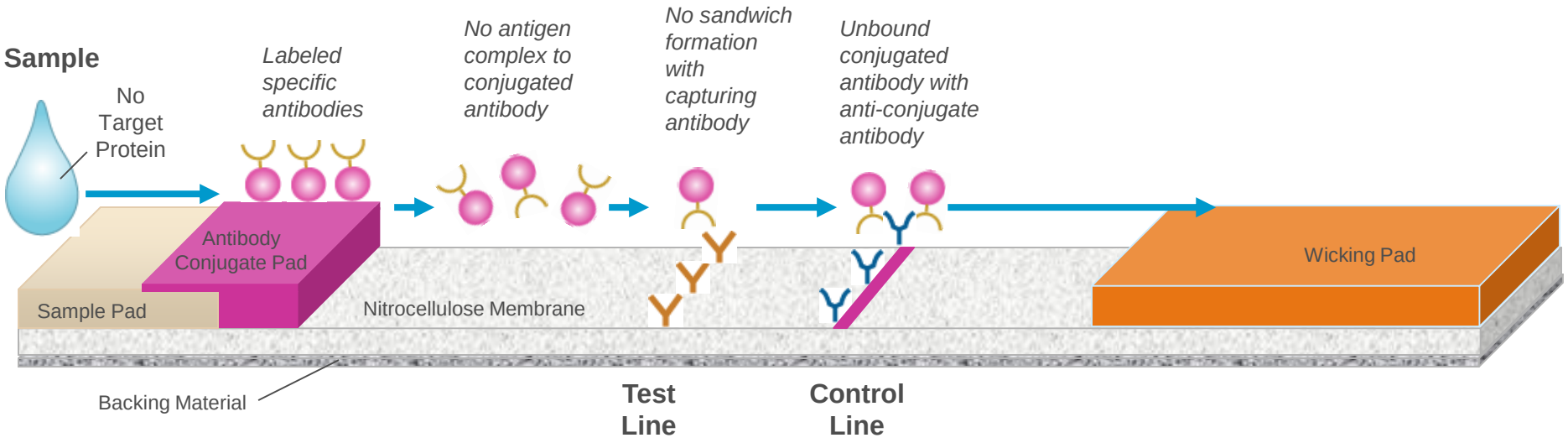
Immunostrip (Lateral Flow) Assays

- Very fast - 3-5 minutes
- Sensitive
- Some are available commercially
- Extracts diffuse through paper strips
- Marker may be gold microparticles

LFD Cross-Sectional View
Positive Result



LFD Cross-Sectional View
Negative Result



Environlogix, Inc.

DNA approaches

DNA sequencing

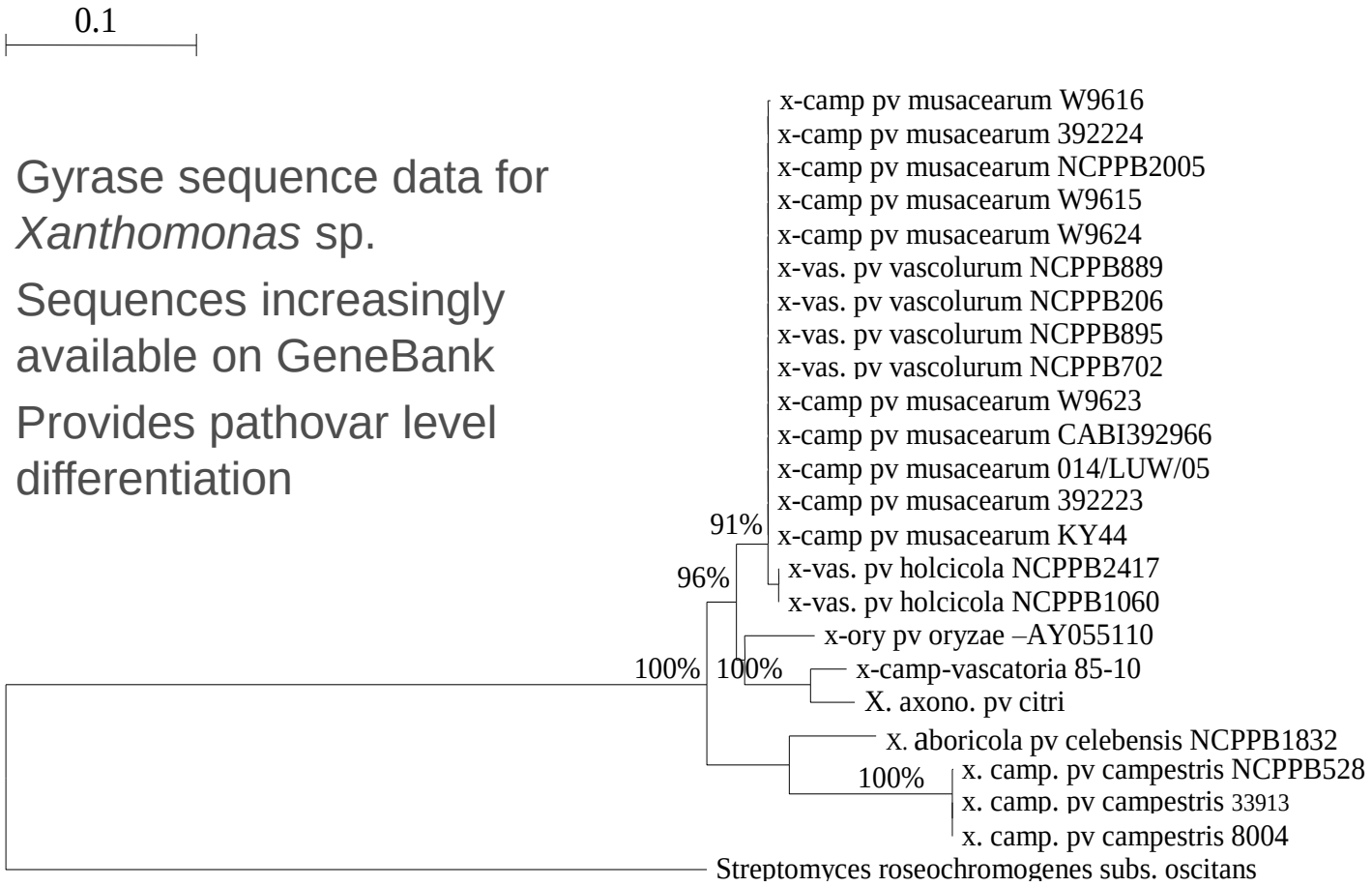
- 16s rDNA sequencing
  - One example: 27F and 1492R primers amplification followed by 518F and 800R primers
  - Stringent annealing conditions
  - BLAST search for nearest relatives
  - Assemblage of closest relatives

Sequencing of other genes

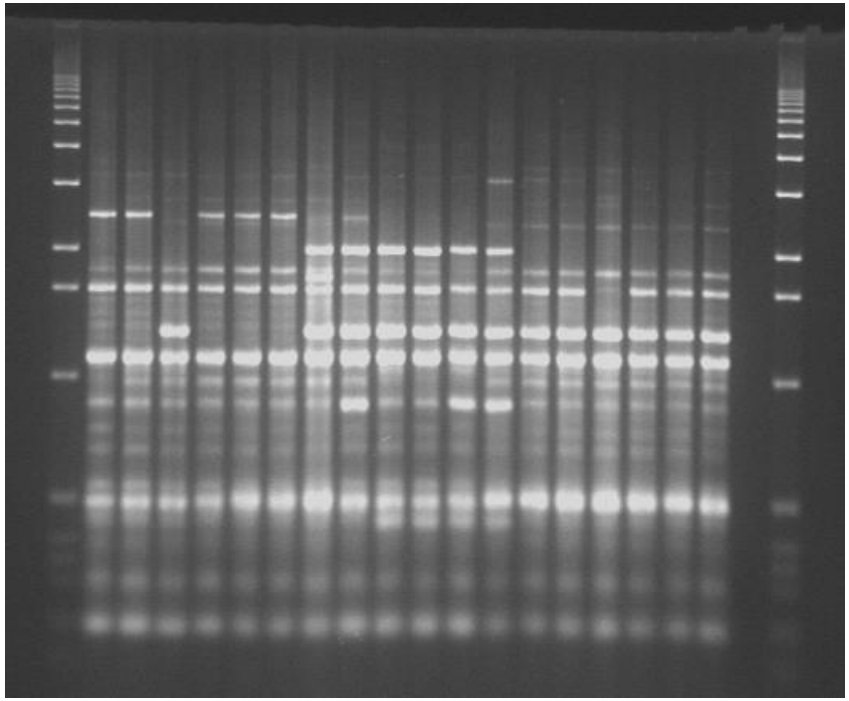
- Whilst 16S rDNA is the normal target for sequencing, for some bacteria insufficient variation may be present to allow differentiation below the species level [pathovar level]
- For these bacteria different target sequences can be used which present more variation
  - Examples include:
    - Hrp genes
    - Gyrase gene
    - 16-23S rDNA interspacer region

Sequence alignment

- Gyrase sequence data for *Xanthomonas* sp.
- Sequences increasingly available on GeneBank
- Provides pathovar level differentiation



DNA fingerprinting



- By comparing DNA fingerprint of unknown to known strains an identification can be achieved
- Is particularly appropriate for pathovar level identifications
- Require access to known strains [genetic resource collection]

DNA approaches to identification

- Advantages
  - Commercial services available for sequencing
  - Data can be shared between laboratories
  - By a combination of approaches identification to the genus, species and pathovar level can be achieved
- Limitations
  - Requires investment in PCR and gel equipment
  - Cost of molecular consumables is high
  - Technically demanding; PCR is notorious for ‘random’ problems