FAM INFORMATION

Januar 2001 / 413

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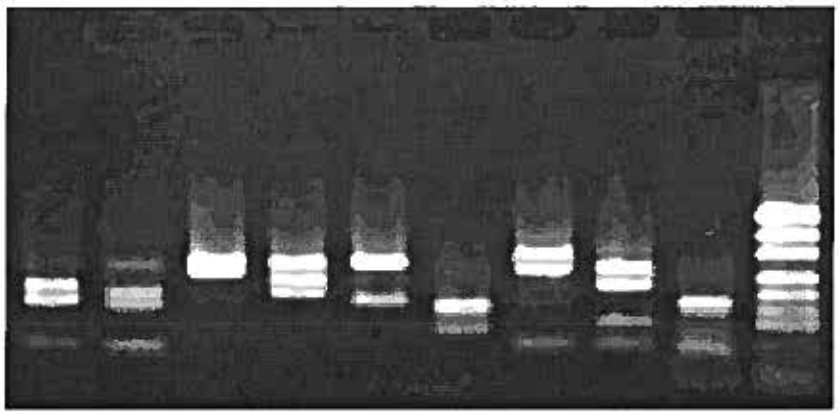
Analytische Möglichkeiten zur Differenzierung von Gärungsorganismen: Eine Situationsanalyse



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1 Einleitung

Die Differenzierung von Gärungsorganismen ist eine wichtige Voraussetzung für deren Selektion. Daher erscheint eine Situationsanalyse über die analytischen Möglichkeiten unerlässlich. Trotzdem wird eine allgemein gültige Aussage, die auf alle Gärungsorganismen-Gruppen wie Propion- oder Milchsäurebakterien, oder Hefen zutreffen wird, kaum möglich sein. Diese Gruppen unterscheiden sich relativ stark voneinander; somit wird sich bei jedem Fall die Frage über das Vorgehen der Differenzierung wieder neu stellen.

Projektziel: Die analytischen Möglichkeiten Gärungsorganismen zu differenzieren sind bekannt und auf ihre Anwendbarkeit für die Selektion hin überprüft.

Jahresziel 2000: Eine Situationsanalyse ist vorgenommen, der Bedarf abgeklärt und zu entwickelnde Methoden sind priorisiert.

Klassische Taxonomie von Gärungsorganismen basiert auf der Morphologie der vegetativen Zellen und auf spezifischen physiologischen und biochemischen Merkmalen. Diese phänotypischen Kriterien werden somit auch zur Differenzierung von Gärungsorganismen herbeigezogen. Solche Methoden sind jedoch arbeits- und zeitintensiv und die Resultate sind nicht immer reproduzierbar.

Im letzten Jahrzehnt wurden verschiedene Anstrengungen unternommen, die Identifizierung und Differenzierung von Gärungsorganismen zu vereinfachen. Kommerziell erhältliche Kits, Software-Programme und chemotaxonomische wie molekularbiologische Methoden kamen zur Anwendung. Letztere Gruppe erlebte den grössten Aufschwung; dazu sind folgende Methoden zu zählen: Elektrophoresenprofile der Zellwand-Proteine sowie gaschromatografische zelluläre Fettsäurespektren, Fourier-Transform Infrarot Spektroskopie (FT-IR), GC-Gehalt der genomischen DNA, DNA-DNA Homologie, Pulsfeldgelelektrophorese, verschiedenste Restriktionsanalysen (genomische, mitochondriale oder genspezifische), RAPD (randomly amplified polymorphic DNA) und viele mehr. Diese Techniken sind vielversprechend, da sie exakte, reproduzierbare, objektive und teilweise auch schnelle Resultate liefern. Einige sind jedoch für den routinemässigen Gebrauch ungeeignet.

Sowohl die klassischen als auch die neueren Methoden liefern wertvolle Angaben zu den Gärungsorganismen. Es ist davon auszugehen, dass zu derer Differenzierung Methoden aus beiden Gruppen herbeigezogen werden müssen.

Im folgenden sollen einige Techniken beschrieben werden, deren Anwendung zur Differenzierung und Identifizierung von Milchsäure- und Propionsäurebakterien an der FAM vielversprechend erscheinen.

2 Klassische Methoden

Im Bereich der klassischen Taxonomie und der Differenzierung von Gärungsorganismen (Milchsäurebakterien und Propionsäurebakterien) aufgrund phänotypischer Eigenschaften hat die FAM eine relativ grosse Erfahrung. Hunderte von Stämmen bzw. Isolate wurden mit verschiedenen Methoden charakterisiert; die wichtigsten davon werden hier erwähnt.

2.1 Biochemische Tests („Biochemische Reihe")

Eine klassische Methode zur Identifizierung von Bakterien ist die Bestimmung ihres Fermentations-Profils. Auf dem Markt werden seit Jahren verschiedene Schnellmethoden angeboten, welche auf diesem Prinzip basieren. Diese wurden vor allem für medizinisch relevante Bakteriengruppen wie z.B. *Enterobacteriaceae* entwickelt; sie werden aber auch immer mehr auf dem Gebiete der Lebensmittelmikrobiologie eingesetzt. Das Kit API 50 CHL (API bioMérieux) wird für die Identifikation von Milchsäurebakterien (Laktobazillen, Laktokokken, Streptokokken und Pediokokken) angeboten. Zur Auswertung der Resultate bietet die Lieferfirma seit einigen Jahren ein EDV-Programm (APILAB) an.

Die relativ grosse Erfahrung in der FAM (ca. 1000 Isolate) hat gezeigt, dass diese Methode, wenn sie alleine eingesetzt wird, oft zu unsicherer bzw. zu falscher Diagnose führt. Die Kombination dieser Methode mit der Bestimmung der Konfiguration der gebildeten Milchsäure-Isomere führt hingegen zu guten Resultaten; zudem können noch einige Zusatzkriterien wie z.B. Gasbildung aus Glukose und Glukonat, u.a. miteinbezogen werden (Isolini et al., 1990).

Dasselbe Prinzip kann auch für die Identifizierung von Propionsäurebakterien verwendet werden. In der FAM wurde eine grosse Reihe von Propionsäurebakterien auf dieser Weise identifiziert (Zehntner, 2000), allerdings nicht mit käuflichen Kits, sondern mittels Mikrotiterplatten (die Medien werden in die Platten abgefüllt, eingefroren und nach Bedarf aufgetaut). Die Fähigkeit Nitrat zu reduzieren kann genützt werden, um *Propionibacterium freudenreichii* subsp. *shermanii* von *P. freudenreichii* subsp. *globosum* zu unterscheiden. Diese Methode, mit einer geeigneten molekularbiologischen Technik kombiniert, erlaubt die Differenzierung von einzelnen Stämmen dieser beiden Subspezies in der Kultur PROP01 (Sollberger et al., 2000).

Über 70 Clostridien- und 40 Enterokokken-Stämme unserer Sammlung konnten ebenfalls mit den entsprechenden käuflichen Kits neu identifiziert werden (Isolini et al., 1987; Isolini et al. 1990).

2.2 Gaschromatographische Trennung zellulärer Fettsäuren

Die Lipide werden verseift und die basisch freigesetzten Fettsäuren werden mit einem Gemisch aus Salzsäure und Methanol in leichter flüchtige Methylester überführt. Danach werden die Reaktionsprodukte extrahiert und von Beiprodukten gereinigt. Anschliessend werden die Proben gaschromatographisch getrennt. Die Auswertung der Resultate erfolgt durch den statistichen Vergleich der erhaltenen Daten mit denjenigen einer Datenbank.

Mit dieser Methode (Jakob, 1995) sind in der FAM milchwirtschaftlich interessante Propionsäurebakterien Stämme untersucht und taxonomisch bis auf Stufe Spezies (*P. acidipropionici, P. freudenreichii, P. jensenii* und *P. thönii*) eingeteilt worden. Für die Differenzierung der Subspezies von *P. freudenreichii*, welche nur aufgrund einiger wenigen biochemischen Unterschiede auseinandergehalten werden können, ist die Fettsäureanalyse nicht geeignet. Eine weitergehende Unterscheidung von Isolaten im Sinne eines Fingerprints ist nicht möglich.

2.3 Polyacrylamidgel-Elektrophorese der Zellproteine

Die Trennung eines Gemisches von Zellproteinen durch Polyacrylamidgel-Elektrophorese (PAGE) ergibt ein komplexes Elektropherogramm, welches als „fingerprint" eines Stammes betrachtet werden kann. Aus den gewaschenen Zellen werden meistens durch Ultraschall zellfreie Extrakte vorbereitet; die Proteine werden durch SDS (sodium dodecyl sulfate), bei 95° bis 100°C, denaturiert und mit Mercaptoethanol oder Dithiothreitol werden mögliche Disulfidbrücken reduziert. Die Komplexe aus SDS und denaturiertem Protein werden dann der Polyacrylamid-Elektrophorese unterworfen; in Anwesenheit von SDS werden die Zellproteine aufgrund ihrer Molekularmasse getrennt. Im Gel können die Proteine schliesslich durch Silberfärbung oder mit einem Farbstoff wie Coomassie-Blue sichtbar gemacht werden. Die Proteinprofile können visuell verglichen werden; sie können auch photographiert und digitalisiert werden. Durch Standardisierung mittels Referenzstämmen oder Markern mit bekannter Molekularmasse kann eine EDV gesteuerte Auswertung stattfinden.

Die Elektrophorese-Profile von Bakterienstämmen sind gut reproduzierbar, wenn das Züchtungsverfahren und die Methode gut standardisiert sind. Bakterienstämme, welche ein DNS-DNS-Hybridisierungs-Niveau von 90% bis 100% aufweisen, zeigen nur minime Unterschiede im Proteinprofil; bei einer DNS-Homologie von mindestens 70% sind die Elektrophorese-Profile noch ziemlich ähnlich.

Die Erfahrung mit dieser Methode in der FAM ist ziemlich gross; hierunter sind einige Beispiele erwähnt: Über 150 Isolate (*Lactobacillus delbrueckii* subsp. *lactis*) aus Rohmischkulturen und aus Käse wurden gruppiert und mit Stämmen anderer Spezies (*L. helveticus*, *L. fermentum* und *L. acidophilus*) verglichen (Baer et al., 1985). Im Rahmen einer Dissertation (Fessler, 1997) sind über 400 Stämme von Propionsäurebakterien bis zur Spezies identifiziert worden; zum Teil wurden die Ergebnisse durch die Resultate der Restriktionsanalyse vom 23S rRNS-Gen bestätigt. Eine andere Gruppe (Weinrichter et al., 2000) hat mehrere Stämme von fakultativ heterofermentativen Laktobazillen durch SDS-PAGE bis zur Spezies identifiziert.

Im Rahmen einer weiteren Arbeit (Meyer et al., 1986) wurden einige Enzyme von über 250 Laktobazillen und von ca. 200 Streptokokken aus 10 verschiedenen RMK verglichen. Die Gels werden nach der Elektrophorese mit den entsprechenden Substraten inkubiert; die Anwesenheit der Enzyme können direkt (Verfärbung) oder unter UV-Licht (Fluoreszenz) abgelesen werden. Mit der gleichen Methode sind auch 28 verschiedene Milchsäurebakterien-Referenz-Stämme (Laktobazillen, Streptokokken und Laktokokken) untersucht worden (Meyer et al. 1992).

Die Differenzierung verschiedener Laktobazillen-Spezies aufgrund ihrer Autolysine ist ebenfalls mit Polyacrylamidgel-Elektorphorese möglich. Dem Gel kann als Substrat für die Autolysine ein Standardpräparat von *M. luteus*-Zellwand oder autoklavierte

Laktobazillus-Zellen inkorporiert werden. Die Visualisierung der Hydrolasen-Bande ist vom verwendeten Substrat abhängig; jedoch sind Unterschiede innerhalb der verschiedenen Spezies sichtbar (Jimeno, 1999).

2.4 Autolyse

Verschiedene Enzyme, welche in der Proteolyse involviert sind, sind auch bei den Milchsäurebakterien im Zellinneren lokalisiert. Nach der Vermehrung der Keime im Käse sind die Enzyme inaktiv, da Membran und Zellwand sie vom Substrat trennen; sie können aber durch die Autolyse freigesetzt werden. Nicht nur die Aktivität dieser Enzyme ist für die Käsereifung wichtig, sondern auch der Zeitpunkt ihrer Freisetzung. Es ist somit interessant, die Stämme aufgrund ihrer Autolyse-Geschwindigkeit zu differenzieren.

Nach Zentrifugation der Bakteriensuspension wird die Enzymaktivität im Sediment und im Überstand gemessen. Die enzymatische Aktivität im Überstand ist auf die lysierten Zellen und diejenige im Sediment auf die Zellen mit intakter Membran zurückzuführen. Die Summe der beiden gemessen Aktivitäten ergibt die Gesamtaktivität; somit lässt sich der prozentuale Anteil lysierter Zellen messen.

Die Autolyse-Geschwindigkeit von ca. 150 Stämmen von *L. delbrueckii subsp. lactis* aus 8 verschiedenen Rohmischkulturen wurde gemessen; als Marker-Enzym ist X-Prolin-Dipeptidylpeptidase verwendet worden. Die untersuchten Stämme konnten in vier verschiedene Gruppen eingeteilt werden (Meyer et al., 1992). Die etwa 100 untersuchten Stämme von *Streptococcus salivarius* subsp. *thermophilus* konnten in zwei Gruppen eingeteilt werden: beim grössten Teil waren die Zellen nach einem Tag vollständig lysiert; bei den restlichen waren die Enzyme nach einem Monat noch nicht freigesetzt (Meyer et al., 1992).

Durch die Verfolgung der Trübung im flüssigen Medium ist die Autolyse-Geschwindigkeit von mehreren Stämmen von fakultativ heterofermentativen Laktobazillen bestimmt worden; dabei konnte man eine Korrelation zwischen Abnahme der Trübung und Freisetzung von Enzymen wie z.B. β-Galactosidase feststellen (Weinrichter et al., 2000).

2.5 Aktivität von Enzymen

Die quantitative Bestimmung der spezifischen enzymatischen Aktivitäten mittels geeigneten Substraten kann zur Differenzierung von Gärungsorganismen verwendet werden.

Die Aktivität verschiedener Enzyme (Glycolyse, Lipolyse und Proteolyse) von über 400 Isolaten aus Rohmischkulturen (Laktobazillen und Streptokokken) wurde untersucht. Unterschiede, sowohl auf Spezies- als auch auf Stammebene, konnten festgestellt werden (Meyer et al., 1986). In einer weiteren Arbeit wurden 28 Referenz-Stämme verschiedener Spezies (Laktokokken, Streptokokken und Laktobazillen) nach den gleichen Methoden charakterisiert (Meyer et al. 1992).

Die Aspartase Aktivität der Propionsäurebakterien spielt während der Käsereifung eine wichtige Rolle (Wyder et al., 2000); die Aktivität ist stammspezifisch und somit ein

wichtiges Differenzierungsmerkmal. Zu diesem Zweck wurde in der FAM eine Mikrotiterplatte-Methode zur Bestimmung der Aspartase-Aktivität entwickelt. Massgebend ist die Menge des gebildeten Ammoniaks, welches neben Fumarat beim Abbau vom Aspartat entsteht. Etwa 30 Stämme sind bereits untersucht und aufgrund dieser Eigenschaft gruppiert worden (Meyer et al., 1999).

Zur Messung von der Oligopeptidase pepO, Dipeptidylpeptidase sowie für die Proteinase (markiertes Casein) mittels Mikrotiterplatten sind in der FAM Methoden entwickelt worden. Über 300 Isolate und Stämme von Laktobazillen und Streptokokken wurden untersucht (Meyer, 1998). In einer weiteren Arbeit (Chavagnatet al., 2000) ist die Aminopeptidase-Aktivität pepN von über 80 Stämmen von *S. salivarius subsp. thermophilus* bestimmt worden.

2.6 Flüchtige Aromakomponente

Die Bildung von flüchtigen Aromakomponenten aus Aminosäuren kann ein Kriterium zur gezielten Differenzierung von Gärungsorganismen sein. Dazu wird zur Zeit in der FAM eine Methode entwickelt, welche auf der Bebrütung der Zellen mit einem Aminosäuregemisch und der anschliessenden Analyse der flüchitgen Komponenten basiert (Bosset et al., 1999).

2.7 Verwendung verschiedener Energiequellen

Die Eigenschaft von Gärungsorganismen verschiedene Substrate als Energiequelle zu verwenden, ist in Bezug auf die Käsereifung von Bedeutung. Die Methode ist einfach und erlaubt eine Differenzierung innerhalb verschiedener Arten, Spezies und Stämme (Jimeno et al., 1998; Jimeno, 1999; Jimeno, 2000).

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3 FT-IR spektroskopische Identifikation von Mikroorganismen

3.1 Prinzip der Methode

Die Fourier Transform Infrarot (FT-IR) Spektroskopie beruht auf der Absorption von Strahlungsenergie durch molekulare Schwingungen. Die Hauptbestandteile der Mikroorganismen (Proteine, Lipide, Polysaccharide) erzeugen ein charakteristisches Absorptionsmuster (IR-Spektrum), das durch die chemische Beschaffenheit, aber auch durch strukturelle Feinheiten des Aufbaus (z.B. durch die Sekundärstruktur von Proteinen) bestimmt ist. Wenn die chemisch-physikalischen Unterschiede zwischen zwei verschiedenen Keimen Anlass zu systematisch verschiedenen IR-Spektren geben, so kann die FT-IR Spektroskopie – zusammen mit geeigneten Computerprogrammen – zur Identifikation eingesetzt werden.

Voraussetzung ist in jedem Fall eine Spektrenbibliothek, worin jedes einzelne Spektrum eindeutig zu einem Genus und einer Spezies (evtl. Subspezies) zugeordnet werden kann. Der Aufbau solcher Spektrensammlungen, insbesondere aber die mit der Taxonomie verbundene Arbeit, ist recht aufwendig. Die Identifikation auf der Basis bestehender Bibliotheken ist dann allerdings als Schnellmethode zu bezeichnen.

Eine gute Einführung in die Grundlagen der Methode gibt Naumann (Naumann D. et al., 1991).

3.2 Stand der Entwicklungen

Die Literatur zur IR-spektroskopischen Identifizierung von Mikroorganismen ist bereits sehr umfangreich. Hier beschränken wir uns auf die Erwähnung der für das Projekt 33-2-1 wichtigsten Publikationen und eigenen Arbeiten.

3.2.1 Milchsäurebakterien

Das Potential der FT-IR Spektroskopie zur Identifikation verschiedener Milchsäurebakterien (Genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* und *Weissella*) auf Stufe Spezies und Subspezies wurde kürzlich erneut bestätigt (Amiel et al., 2000). Für Referenzstämme wurde bezüglich Genus und Spezies eine Treffsicherheit von 100 % erreicht, auf Stufe Subspezies waren 86 % korrekt identifiziert. Frühere Arbeiten dieser Forschungsgruppe sowie andere wichtige Arbeiten findet man in der Literaturliste der erwähnten Publikation.

Ebenfalls in einer neueren Arbeit wurde gezeigt, dass bei idealen Bedingungen sogar verschiedene Stämme der Subspezies *Lactococcus lactis lactis* bzw. *Lactococcus lactis cremoris* IR-spektroskopisch differenzierbar sind (Lefier et al., 2000).

In der FAM ist im Zusammenhang mit der Dissertation von B. Weinrichter eine sehr umfangreiche Sammlung taxonomisch gesicherter Spektren erstellt worden. Eine Publikation der Ergebnisse ist in Vorbereitung.

Im laufenden Jahr wurde mit der Erstellung von Spektrenbibliotheken der *Lactobacillus acidophilus* Gruppe (*L. acidophilus, L. amylovorus, L. crispatus, L. gallinarium, L. gasseri, L. johnsonii, L. helveticus*) begonnen (Prj. 34-1-1). Jene Keime, deren

Taxonomie gesichert ist, zeigen in der Diskriminanzanalyse der IR-Spektren eine eindeutige Differenzierung.

Wir haben zudem Spektrensammlungen von Streptokokken und Lactokokken, die noch bearbeitet werden müssen.

3.2.2 Propionsäurebakterien

In der Literatur gibt es keine Hinweise, dass Propionsäurebakterien bereits IR-spektroskopisch untersucht wurden. Unsere Arbeiten haben gezeigt, dass die vier Spezies (*Propionibacterium acidipropionici, P. freudenreichii, P. jensenii, P. thönii*) IR-spektroskopisch identifiziert werden können. Die Differenzierung der Subspezies *P. freudenreichii freudenreichii* und *P. freudenreichii shermanii* ist in der Diskriminanzanalyse nachgewiesen. Eine Publikation dieser Ergebnisse ist in Vorbereitung.

3.3 Ausblick

Die weitere Entwicklung der FT-IR spektroskopischen Methode zur Identifizierung von Mikroorganismen wird vornehmlich im Projekt Biotechnologie (34-1-1) erfolgen. Voraussetzung ist eine enge Zusammenarbeit zwischen den Fachbereichen Mikrobiologie, sowie Physik und Chemie. Methodisch ist eine Erweiterung durch die IR-Mikrospektroskopie zu prüfen.

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4 Differentiation of bacteria by molecular biological techniques

In modern bacteriology, the major challenges include purification, enumeration, identification, and differentiation of bacteria. Protocols available today tend to concentrate on pathogenic organisms, an emphasis that merely reflects the way in which these techniques are frequently applied and need not be a barrier to their more wide-spread use. Indeed, some of the techniques presented here have found use in both the study of microbes and of higher Eukaryotes. Methods are available for the identification of bacteria at the species, sub-species, and strain level. Sometimes a slight variation of a method may permit its application at different levels.

The techniques of molecular biology are entirely concerned with nucleic acids. The following table 1 gives the size of the genomes of various bacterial species involved in the dairy industry. For comparison some other well known bacteria have been included.

Table 1: Size of genomes of bacteria involved in the dairy industry

Species	Number of base pairs
Bacillus subtilis	4'200'000
Escherichia coli	4'600'000
Helicobacter pylori	1'700'000
Lactobacillus acidophilus	2'000'000
Lactobacillus delbrueckii ssp. bulgaricus	2'300'000
Lactobacillus delbrueckii ssp. lactis	2'300'000
Lactobacillus helveticus	2'000'000
Lactococcus lactis	2'500'000
Propionibacterium freudenreichii	2'500'000
Streptococcus thermophilus	1'800'000

As can be seen the genomes of the dairy bacteria are relatively small, however, the sequencing of the entire genome for the identification of a bacterium is neither practical nor financially feasible. Since sequencing of the complete genome is not practical various strategies have been developed to partially analyse it. These techniques may be divided into four main groups: electrophoresis, hybridisation, DNA sequencing, and amplification. Sometimes a combination of two or more of these methods is used.

4.1 Preparation of Bacterial Genomic DNA

In a preliminary step, before any of the above mentioned techniques may be applied, relatively pure nucleic acids must be isolated from the product of interest e.g., milk, starter, cheese, etc. If a technique involving nucleic acid amplification is used the preparation must be very pure since it is known that foodstuffs contain inhibitors of the amplification processes (Rossen et al.). Today, many commercial kits are available for the preparation of nucleic acids. The following table lists the major companies that

supply nucleic acid purification kits. Kits tested by us are marked with an asterisk (*). Most of these kits have been developed for the extraction of DNA from *E. coli*. The majority of microorganisms used in the dairy industry are Gram-positive bacteria. These have a cell wall which is more resistant to lysis and so special protocols involving incubation with lytic enzymes have been developed e.g. lysozyme, mutanolysin. The use of strong detergents such as SDS may also be necessary. The use of detergents is not always compatible with commercial kits and so care should be taken when selecting a kit. In our hands we have found that the kit purchased from Roche Molecular Biochemicals gave the best results.

5' Prime – 3' Prime, Inc. *	ABgene
Advanced Biotechnologies Ltd.	Alltech Bioscience
Althea Technologies Inc.	Ambion, Inc. *
American International Chem.	Amersham Pharmacia Biotech *
ard-Schlesinger Industries	Autogen, Inc.
Barnstead/ Thermolyne	Beckman Coulter Corp.
BIO 101*	Bionexus
Bioprobe Systems	Bio-Rad Laboratories, Life Science Group *
BIOS Corp.	Biosearch Technologies, Inc.
Biotecx Labs	Bioventures
Calbiochem *	Clontech Laboratories, Inc. *
Cpg, Inc.	Cruachem*
Dynal *	Epicentre *
Eppendorf	Geno Technology, Inc.
Gentra Systems, Inc.	ICN Pharmaceuticals, Inc.
Intermountain Scientific Corporation	Invitrogen
Kirkegaard & Perry Labs	Life Technologies, Inc. *
MacConnell Research	Machery-Nagel*
Mallinckrodt Specialty Chemicals Co.	MBI Fermentas
Merck	Midland Certified Reagent

Millipore S.A.	Oncor Appligene
Panvera	PE Applied Biosystems
Perseptive Biosystems	Phenix Research Products
Pierce Chemical	Polysciences, Inc.
Promega*	Qiagen *
Roche Molecular Biochemicals*	Sigma BioSciences
Stratagene GmbH *	Supelco
Tetra Link International	Topview Analysis International
Transgenomic	Worthington Biochemical Corp.

4.2 Electrophoretic Techniques

4.2.1 Total DNA Restriction Pattern Analysis

Total DNA restriction pattern analysis is one of the techniques commonly used to identify and group bacteria. This technique can be used to distinguish separate species or to distinguish similar bacterial strains within the same species or sub species. Restriction pattern analysis is based on the well-known feature of bacterial restriction endonucleases, namely, that they cut the double strand of DNA when they recognise short specific sequences. Fragments obtained by the digestion of genomic DNA with a restriction endonuclease can be separated by electrophoresis generating a band pattern that constitutes the stable "fingerprint" of a single bacterial strain, since the number and location of the restriction sites are specific to each genome.

Electrophoresis of restriction digests can be performed either on agarose or polyacrylamide gels. The former method is usually used to identify species and subspecies. The latter method, which is more time consuming, has a very high resolution power and can separate DNA molecules that differ by as little as 0.2% in length. It is more appropriate for differentiating between strains of the same species. Now, with the appearance on the market of the Lab-on-a-Chip from Agilent Technologies for the rapid separation of nucleic acids, the resolution power may be higher than ever. Nevertheless this remains to be confirmed.

The electrophoretic pattern of restriction fragments, reflecting the structure of the genome, can be used to evaluate the similarity among microorganisms. With this approach, strains are assigned to groups based on their electrophoretic pattern. This type of analysis has an established application in epidemological and taxonomic studies.

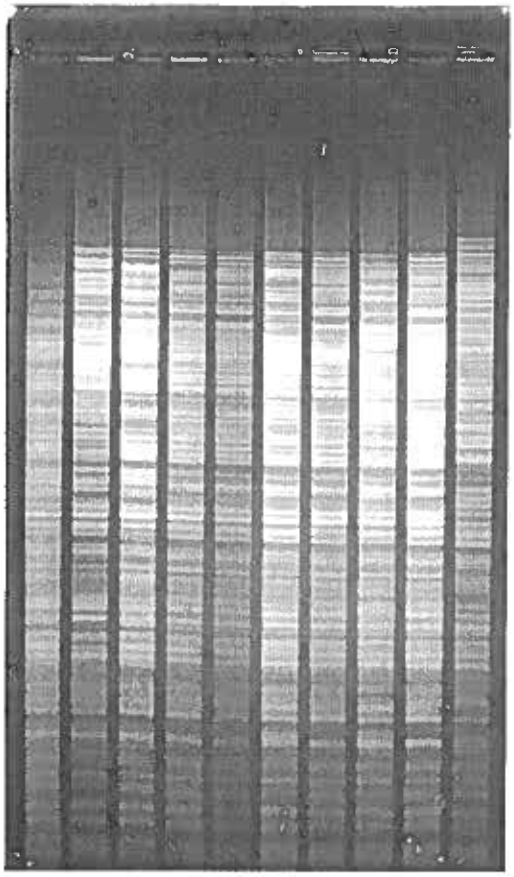


Figure 1. Total DNA Restriction Pattern Analysis of various strains of *Streptococcus thermophilus*.

A preliminary experiment with a number of nucleases is necessary to find the most suitable for a given group of bacteria. This method has been successfully used to differentiate between lactic acid bacteria in Liebefeld (see Jimeno et al., 1989a) Figure 1 shows a typical example of Total DNA Restriction Pattern Analysis separated on an agarose gel after digestion with the nuclease *EcoR* I. As can be seen the different strains are similar but not always identical.

4.2.2 Pulse Field Gel Electrophoresis

The technique of pulse field gel electrophoresis (PFGE) enables researchers to separate linear DNA fragments of up to 10'000'000 base pairs. The technique differs from conventional gel electrophoresis by requiring the DNA molecules periodically to change their direction of migration. Several types of PFGE have been described; all of which utilise the basic principle of applying an electric field alternatively in two different directions. The difference lies in the manner in which the alternating electrical fields are generated.

A number of factors are known to affect the resolution of linear DNA fragments by PFGE. These include the topology of the DNA, the time interval between reorientation of the electrical field, the angle at which the alternating electric fields are applied, the

field strength, the gel concentration, and the temperature at which the electrophoresis is run.

PFGE has provided a means of analysing the genome of bacteria that could not be studied by classical genetic techniques. The pattern of restriction fragments obtained by digestion of bacterial genomes with restriction enzymes, which cut the genome infrequently, has been successfully applied to the identification of bacterial strains.

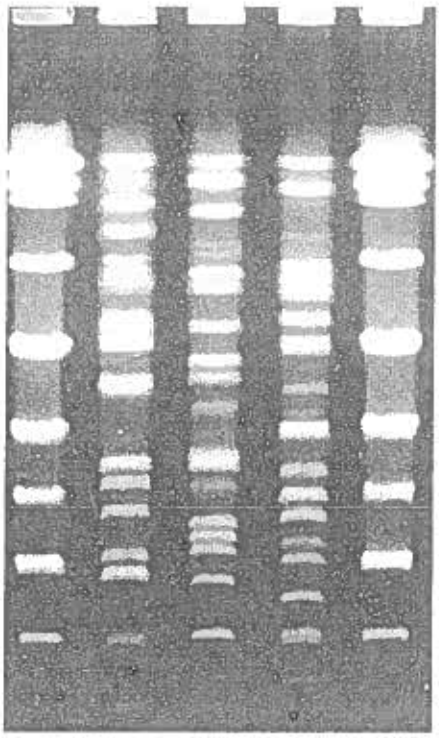


Figure 2. PFGE patterns of *Sgr*A1 digests of genomic DNA from *L. helveticus*. Lanes 1 and 5, molecular size markers. Lanes 2, 3, 4, three different strains of *L. helveticus*. (from Lortal et al.)

The main drawback of this method is that it is very time consuming. The whole procedure takes 3-4 days. It should also be pointed out that the electrophoresis apparatus is relatively expensive and that only about 24 samples can be processed at the same time.

In order to avoid shearing of the genome before electrophoresis takes place the genomic DNA has to be prepared in agarose plugs. Bacterial cells are mixed with liquid agarose at 42°C. The mixture is dispensed into wells and allowed to solidify. The plugs are then incubated for up to 24 h with lysing buffer in order to lyse the cells. After washing for up to 24 h the DNA in the plugs is then treated with restriction endonucleases for a minimum of 3 h. The plugs are again washed and PFGE is then performed at 15°C for up to 24 h.

Figure 2 shows an example of PFGE of three different strains of *L. helveticus*. We have used this method with success to determine the molecular size of various bacteriophages (see Jenni et al.). Nevertheless, because of the large amount of time and expense involved we would not recommend the use of this method for routine screening of bacterial strains.

4.2.3 Plasmid Profile Typing

Plasmids are extra-chromosomal molecules of DNA capable of autonomous replication. Such molecules have been identified in many bacterial genera and usually exist as covalently closed circular molecules. Plasmids range in size from less than one thousand to several hundred thousand base pairs. Many plasmids code for properties such as resistance to antimicrobial drugs or other properties which enhance the virulence of their host strains for humans or animals; other plasmids have no obvious phenotypic properties and are regarded as "cryptic". The identification of plasmids in bacterial strains by agarose gel electrophoresis in terms of their numbers and molecular weight has provided invaluable information that has been used on numerous occasions to supplement the traditional forms of bacterial typing such as serotyping and phage typing.

The protocol usually used is based on the alkaline lysis method. This method is based on the resistance of plasmid DNA to high pH, whereas chromosomal DNA is sheared into linear fragments during the extraction process. Following denaturation at high pH and rapid neutralisation with sodium or potassium acetate, chromosomal DNA is precipitated leaving the plasmid DNA in solution. Plasmid DNA can then be precipitated by the addition of alcohol. The plasmid extract is then dissolved in buffer and electrophoresis is usually performed in 0.6% agarose. This method has been successfully used in the FAM (see Jimeno et al. 1989b) Figure 3 shows an example of plasmid profile typing of various strains of *Lactobacillus delbrueckii* ssp. *lactis.*

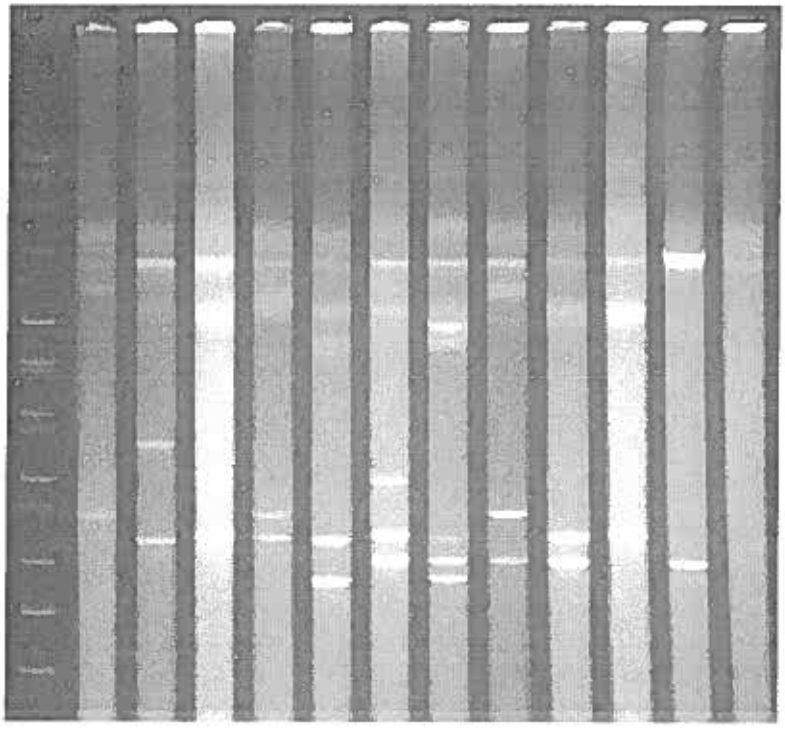


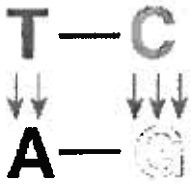
Figure 3. Plasmid profile typing of various strains of *Lactobacillus delbrueckii* ssp. *lactis*. Lane 1, molecular size markers. Lanes 2 –12 various strains of *L. delbrueckii lactis* isolated from RMKs from Liebefeld.

Unfortunately not all bacteria contain plasmids. Also although plasmids may be of the same or similar molecular weight, this does not necessarily mean that they are identical,

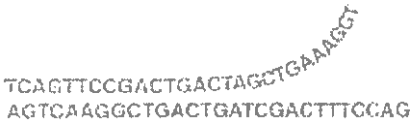
or even related. Thus this method may possibly give an indication that two strains are either identical or different but there is no guarantee that this is always possible.

4.3 Hybridisation Techniques

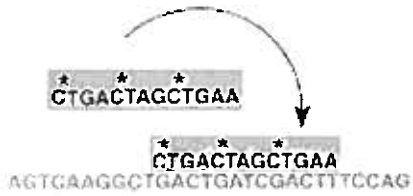
Hybridisation is the coming together, or binding, of two genetic sequences. The binding occurs because of the hydrogen bonds between base pairs. Between a A base and a T base, there are two hydrogen bonds; between a C base and a G base, there are three hydrogen bonds.



When making use of hybridisation in the laboratory, DNA must first be denatured, usually by using heat or chemicals. Denaturing is a process by which the hydrogen bonds of the original double-stranded DNA are broken, leaving a single strand of DNA whose bases are available for hydrogen bonding.



Once the DNA has been denatured, a single-stranded probe can be used to see if the denatured DNA contains a sequence similar to that on the probe. The denatured DNA is put into a plastic bag along with the probe and some saline liquid; the bag is then shaken to allow mixing. If the probe finds a fit, it will bind to the DNA.



The fit of the probe to the DNA does not have to be exact. Sequences of varying homology can stick to the DNA even if the fit is poor; the poorer the fit, the fewer the hydrogen bonds between the probe and the denatured DNA. The ability of low-homology probes to still bind to DNA can be manipulated through varying the temperature of the hybridisation reaction environment, or by varying the amount of salt in the buffer.

4.3.1 Dot Blot Hybridization

For dot blot hybridisation the genomic DNA from a bacteria is first denatured with alkali and heat for 5 min followed by rapid neutralisation and cooling. The denatured DNA is then applied to a membrane made of nitro-cellulose or nylon. It is then fixed to the membrane by heating at 80°C for 2 hours or by treatment with UV light. This is followed by a pre-hybridization step for between 2 – 6 hours. Pre-hybridization is necessary in

order to block binding sites on the membrane which may bind the probe non-specifically. The membrane is then mixed with a buffer containing the probe and incubated for 15 – 24 hours. Finally the membrane is washed at a specific temperature and salt concentration in order to remove any unbound probe. The higher the temperature and the lower the salt concentration the greater the homology between the probe and the membrane bound DNA.

Originally probes were radioactively labelled; however, today several different techniques are available for labelling probes. Detection of radioactively labelled probes bound to the membrane involves the least amount of work. Usually the membrane is placed in contact with an X-ray film in the dark and exposed for maximum signal (typically 24 h). The film is developed and blackening identifies the spots containing the desired DNA. Probes that are labelled fluorescently, with biotin or with digoxigenin can also be detected on the membrane but this involves far more work since it is usually necessary to detect the probes enzymatically.

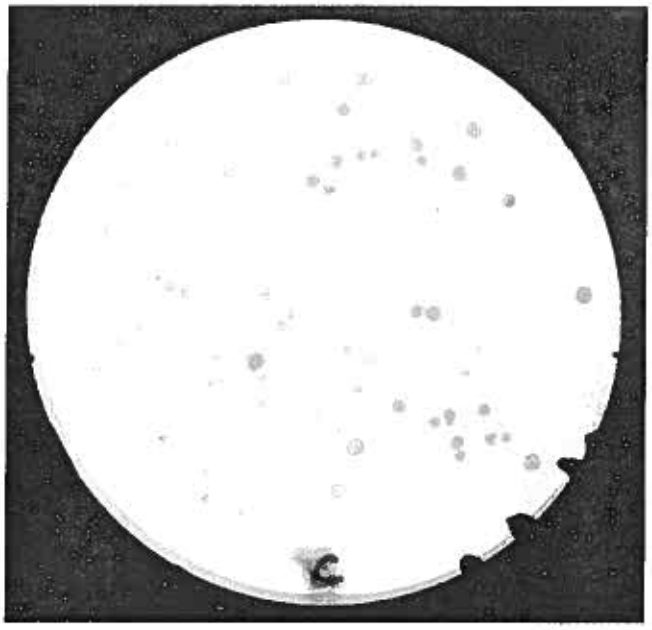


Figure 4. Bacterial colonies hybridised with a probe specific for *Clostridium tyrobutyricum*.

Instead of denaturing the DNA in solution it is also possible to lyse colonies of bacteria directly on the membrane and followed by denaturing, binding and hybridisation. Probes may be of any size from a specific DNA sequence of as little as 20 base pairs up to the complete genome. This method has been successfully used in the FAM (see Casey et al. 1995) Figure 4 shows bacterial colonies on a membrane after hybridisation with an oligonucleotide probe labelled with digoxigenin and specific for *Clostridium tyrobutyricum*.

Dot blot hybridisation is an excellent technique for detecting and identifying bacteria when a specific probe is available. Specific probes can easily be developed to distinguish between different species; however, specific probes to distinguish between strains can only be developed when the specific sequence is known. The main

drawback of the method is that it is very slow and time consuming and so is not suitable for routine detection and identification.

4.3.2 Ribotyping: A Special Case of Southern Blotting

Small differences between related DNA molecules can be readily detected because their restriction fragments can be separated and displayed by gel electrophoresis (see above, Total DNA Restriction Pattern Analysis). However, total restriction patterns of bacterial genomic DNA are often too complicated to interpret. A powerful technique known as Southern blotting was devised by E. M. Southern to overcome this problem. A mixture of restriction fragments is separated by electrophoresis through an agarose gel, denatured to form single-stranded DNA and transferred to a nitro-cellulose or nylon sheet. The position of the DNA fragments in the gel are preserved on the membrane sheet where they can be hybridised with a labelled single-stranded DNA probe as described above. Suitable probes for this procedure can be random DNA sequences or parts of known genes. An animated description of Southern blotting is available at **http://vector.cshl.org/Shockwave/southan.html**.

Ribotyping: Most often, the application of a gene probe is limited to the bacterial species from which the probe derives. However, some common repeated sequences have been found in phylogenetically related species. Our present knowledge is that no gene is more universal than rRNA genes. rRNA sequences contain some extremely conserved regions that can hybridise to rRNA genes of bacteria irrespective of their phylogenetic position. It is then possible to characterise, identify, and type bacteria by studying their rRNA gene restriction patterns. The determination of rRNA gene restriction patterns has also been termed ribotyping.

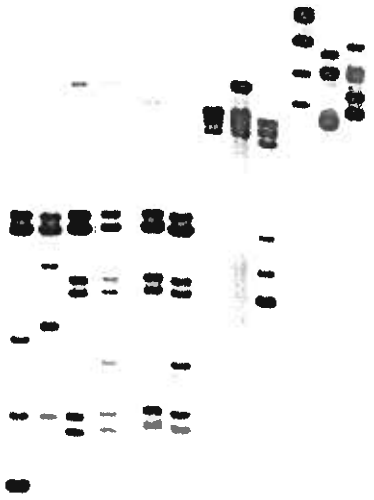


Figure 5. Ribotyping of different isolates of *Lactobacilli* (from Zhong et al.)

Normally ribotyping has been used to identify species of bacteria but the method has also been applied for differentiating between strains. Fig. 5 shows the results of ribotyping of different isolates of *Lactobacilli.* In our laboratory we attempted to use the

method to differentiate between Propionic acid bacteria. The method permitted differentiation between species but not between strains (see Fessler 1997).

The method is very elegant but labour intensive. It involves labelling of probes, purification of genomic DNA, cleavage with restriction enzymes, agarose gel electrophoresis, transfer of DNA fragments to a membrane, hybridisation with labelled probes, and finally detection of labelled probes on the membrane. The method involves 2 – 3 days work before results are available and usually not more than 20 samples can be processed at the same time. This method should not be considered for routine laboratory work. Nevertheless, an automated microbial characterisation system is available on the market which claims that "an automated genetic fingerprint of any bacterium...in less than eight hours...with no special operator skills" is possible (see **http://www.qualiconweb.com/rp.html**).

4.3.3 Subtraction Hybridization

Subtraction hybridisation offers an approach for the generation of strain specific probes or PCR primers (see below chapter on PCR). Genomic DNA of the target strain and the subtracter strains are digested by restriction enzymes. Subtracter DNA fragments are labelled with biotin and hybridised in excess to the probe DNA fragments in solution. Labelled hybrids are immobilised using streptavidin coated magnetic beads, which bind biotin labelled DNA. The magnetic beads are separated from the unlabelled strain specific DNA fragments. After cloning and sequence analysis of these fragments, probes or primers for the specific strain can be designed. In Fig. 6 the basic steps are schematically shown.

The method for the preparation of the strain specific DNA takes approximately 3 days but very little "hands-on" work is necessary. The method allows one to distinguish the selected strain from the subtracter strains very specifically but it is not a real indication of a specific strain since other strains not included in the subtracter group may also contain the selected DNA.

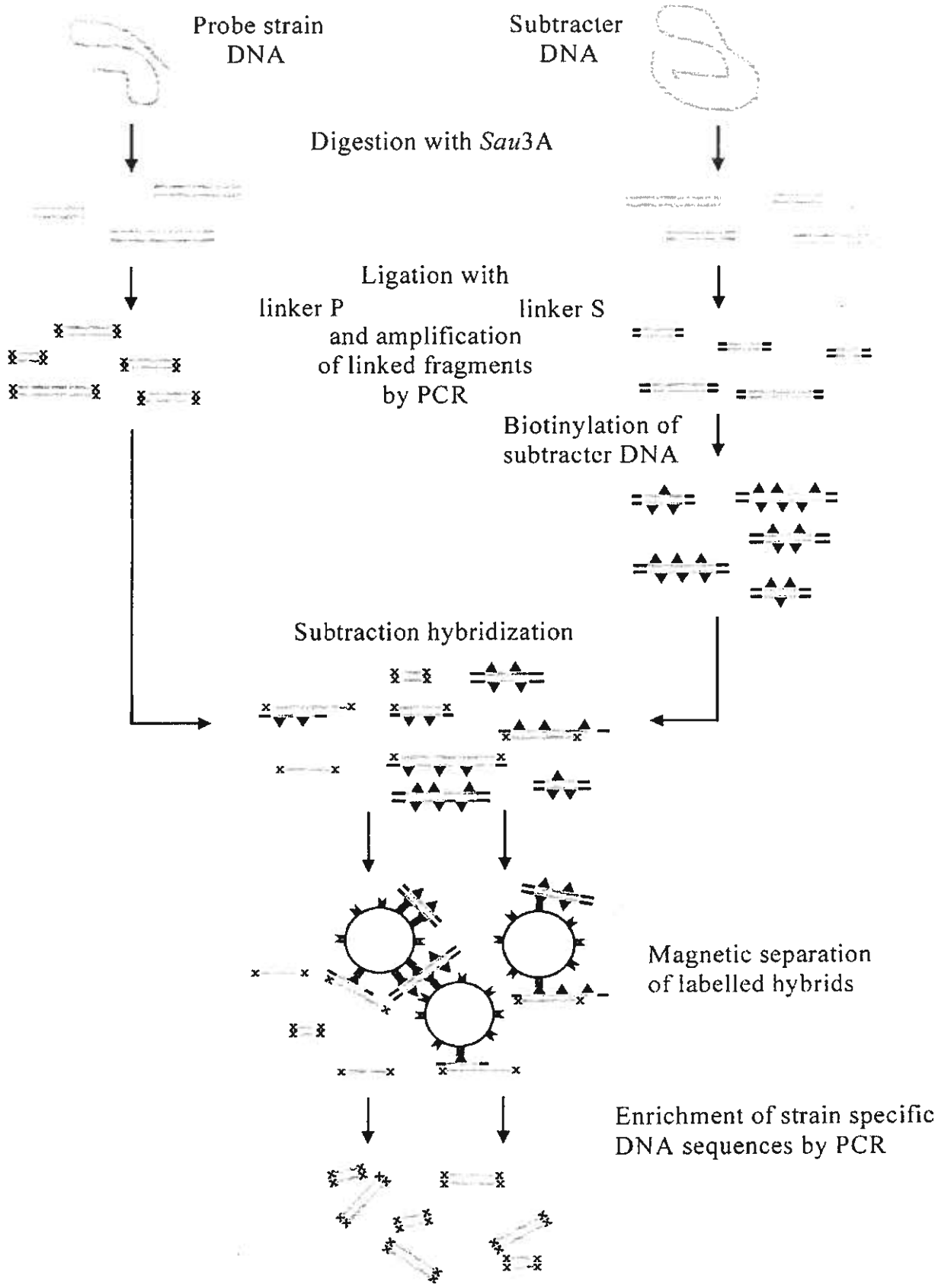


Figure 6. Schematic illustration of subtractive hybridisation after Wassill et al.

4.4 Amplification techniques

Ever since the advent of the Polymerase Chain Reaction (PCR) many different approaches using this technique have been developed for the identification and detection of microorganisms. An animated description of the PCR technique is available at **http://vector.cshl.org/Shockwave/pcranwhole.html**. In order to amplify a specific sequence of DNA both ends of the sequence must be known in order to construct specific primers. Different genes have been chosen to amplify, either partially or fully, DNA segments for specific identification of a bacterial species. A second related strategy involves the amplification of intergenic regions. Because these spacer regions have no known structural functions they are under less selective pressure and ought to be more variable. These regions are usually used to select primers in order to differentiate between strains of the same species.

4.4.1 Ribosomal RNA Amplification

As mentioned previously no genes are more universal than rRNA genes. In bacteria, the DNA encoding rRNA is arranged in an operon consisting of three genes, which represent the 16S, 23S, and 5S RNAs (see Fig. 7).

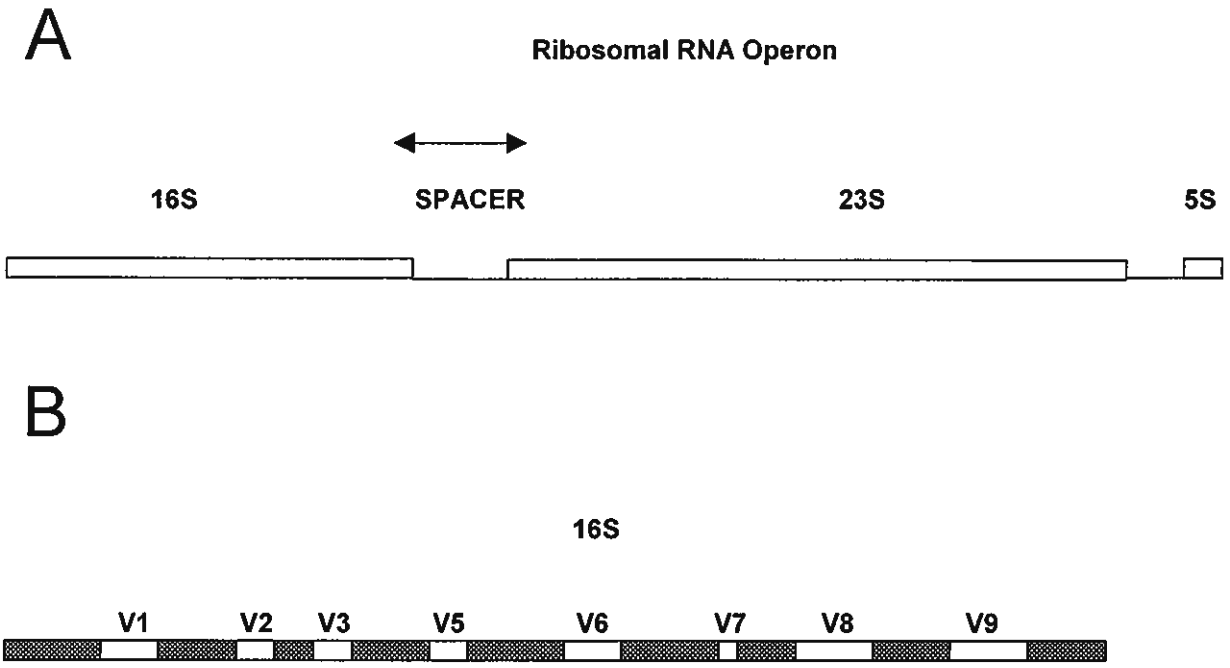


Figure 7. Schematic representation of a prokaryotic ribosomal operon. (A) Each rRNA operon consists of three genes, 16S, 23S, and the 5S genes. The position of the intergenic or spacer region is indicated. (B) The 16S gene showing the constant ( ) and variable ( ) regions.

Operons encoding rRNA are present in up to 10 copies in bacteria. They are essential constituents of prokaryotic and eukaryotic ribosomes and, because of their pivotal role,

they are highly conserved in structure in all organisms. However, within the rRNA genes there are regions of conserved sequence among species interspersed with regions of sequence variation. The constant and variable regions of microorganisms can be compared to each other for homology and can be used to establish evolutionary relationships and draw phylogenetic trees. Variable regions of the

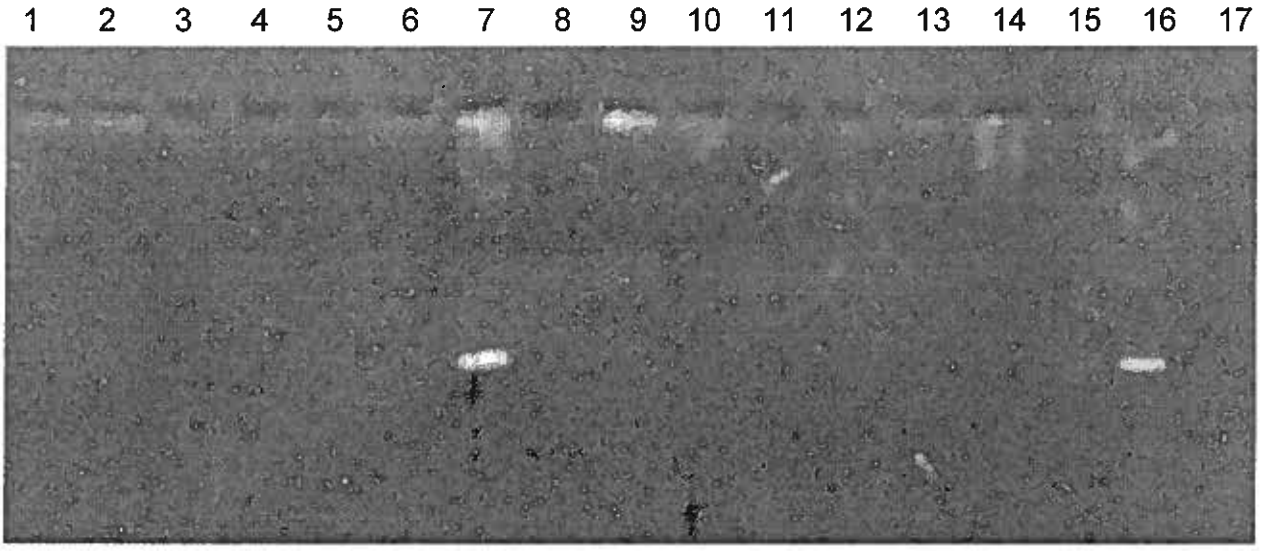


Figure 8. Analysis of PCR amplification specificity using primers specific for Clostridium tyrobutyricum. 1, C. bifermentans, 2, C. butyricum, 3, C. clostridiforme, 4, C. difficile, 5, C. tertium, 6, C. sporogenes, 7, C. tyrobutyricum, 8, M. lacticum, 9, C. glutamicum, 10, L. mesenteroides, 11, Lcctococcus lactis, 12, Bacillus subtilis, 13 –15, no sample, 16, C. tyrobutyricum, 17, negative control. (From Colleran 1994)

16S rRNA gene can be amplified from any bacterium using universal primers from the neighbouring constant regions. The DNA sequence of the variable regions can then be determined by direct sequence analysis of the PCR product or, in the case of multiple products, by subcloning PCR products prior to sequencing. By sequence alignment of the variable regions, targets for the design of species-specific DNA primers can be chosen. Homology alignments to rRNA sequences in the Genbank and European Molecular Biology Laboratory (EMBL) databases can also be carried out using available sequence analysis software packages.

Figure 8 shows an example of a PCR assay for *Clostridium tyrobutyricum*. Only DNA from this species gave positive results. This method has been successfully used at Liebefeld (see Casey et al. 1995)

This PCR method based on sequences from rRNA is highly specific and since very many sequences from other species are available from the Genbank and European Molecular Biology Laboratory (EMBL) databases it is possible to confirm the uniqueness of the chosen primer sequences. This is probably the method of choice for detecting the presence of a microorganism in a given sample.

4.4.2 Amplification of Other Conserved Genes

A variety of conserved genes, including the heat shock protein 60 (HSP60 or CPN60) gene, the major cold shock protein gene, the *sod* gene and the *tuf* gene, have been exploited for the detection of bacteria.

A set of universal degenerate primers which amplified, by PCR, a 600-bp oligomer encoding a portion of the 60-kDa heat shock protein (HSP60) of both Staphylococcus aureus and Staphylococcus epidermidis were developed. Degenerate primers were developed which were shown to amplify an anticipated 600-bp PCR product from all 29 Staphylococcus species and from all but 2 of 30 other microbial species, including various gram- positive and gram-negative bacteria, mycobacteria, and fungi. These conserved genes could also be exploited for the specific detection of microorganisms.

Using two universal PCR primer oligomers from conserved regions of the major cold-shock protein gene, a 200 base-pair DNA sequence from more than 30 diverse Gram-positive and Gram-negative bacteria, including representatives from the genera Aeromonas, Bacillus, Citrobacter, Enterobacter, Enterococcus, Escherichia, Klebsiella, Lactobacillus, Lactococcus, Listeria, Pediococcus, Photobacterium, Proteus, Salmonella, Shigella, Staphylococcus, Streptococcus, and Yersinia were amplified. Sequence analysis of the amplified products confirmed a high level of DNA homology. Significantly, however, there are sufficient nucleotide variations to allow the unique allocation of each amplified sequence to its parental bacterium.

The superoxide dismutase (*sod*) gene has been identified as a target in screening for the presence of mycobacteria on the genus level and differentiating relevant mycobacterial species from one another by PCR. Consensus primers deduced from known superoxide dismutase gene sequences allowed the amplification of DNAs from a variety of bacteria, fungi, and protozoa. Selected amplicons from Actinomyces viscosus, Corynebacterium diphtheriae, Corynebacterium pseudodiphtheriticum, Mycobacterium avium, M. fortuitum, M. gordonae, M. intracellulare, M. kansasii, M. scrofulaceum, M. simiae, M. tuberculosis, M. xenopi, and Nocardia asteroides were subsequently cloned and sequenced. The alignment of those sequences facilitated the selection of primers targeting conserved regions present in myobacterial species but absent in non mycobacterial species and thus allowed the genus-specific amplification of all 28 different mycobacterial species tested.

The *tuf* gene, encoding elongation factor EF-Tu, is involved in peptide chain formation and is an essential constituent of the bacterial genome. These characteristics make it a target of choice for diagnostic purposes. PCR-based assays in which the tuf gene serves as the target sequence have been developed for *Mycoplasma fermentans*, *M. pneumoniae* and enterococci. Here in Liebefeld we have used the *tuf* gene to develop specific PCR primers for *Lactobacillus lactis*.

These methods are useful for detecting the presence of a microorganism in a given sample and permit one to distinguish between species and possibly subspecies.

4.5 Differentiation Between Strains

In order to distinguish between strains from the same species a different approach is necessary. Again since it is neither practical nor financially feasible to sequence the entire genome intergenic or spacer sequences have been chosen since this region is

not subject to the same selective pressure as the structural genes. It has been shown that for the rRNA 16S-23S intergenic spacer there is considerable variability in sequence and also size between microorganisms (Leblond-Bourget et al.). Unfortunately other authors (Nour et al.) have obtained different results. It was found that a series of *S. thermophilus* strains all exhibited the same sequence, which is an indication for a strong intraspecific conservation of the 16S-23S intergenic spacer sequence. Because of these contradictory results we have not applied this approach for differentiating between strains.

4.5.1 Random Amplified Polymorphic DNA (RAPD)

RAPD is a PCR based system that can be used to provide DNA fingerprints to identify differences between strains and species. The process uses single primers of arbitrary sequence, usually 10 bases long, and these can be used on any DNA target irrespective of any knowledge of its DNA sequence. This offers a substantial time saving advantage as the DNA region need not be identified, cloned, and its sequence determined before it can be used as a DNA marker.

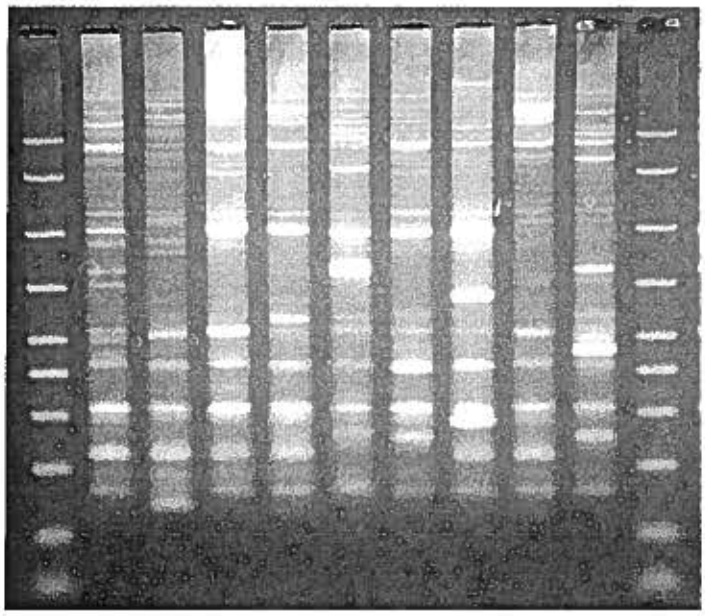


Figure 9. RAPD profiles of Propionic acid bacteria. Lanes 1, 11: molecular size standards. Lanes 2-10 various wild strains isolated from raw milk.

DNA is made up of an ordered sequence of four constituent bases, thus the sequence of a random primer 10 bases long should occur once every 4^{10} or approximately 1'000'000 bp on any target DNA template. The genomes of lactic acid bacteria are about 2'000'000 bp and so would be expected to carry only two copies of any random 10-base sequence. To form a PCR product, two such binding sites should be present on opposite strands of a target DNA template so that PCR will copy the region between the priming sites. Thus one would expect to obtain only one PCR product per genome of lactic acid bacteria. However, in practice this is not the case and usually several PCR products of differing sizes are obtained. This is probably because mismatch priming

occurs. Because of this dependence on mismatch priming the method has been severely criticised by many authors.

The pattern of PCR products seen after agarose gel electrophoresis of an RAPD analysis is termed an RAPD profile and differences in the RAPD profiles of even closely related individuals can be obtained using appropriate primers. This forms the basis of a species/strain identification system and RAPD fingerprinting has now been successfully applied to a variety of organisms, including bacteria, fungi, plants, and animals.

In practice when using genomic DNA from lactic acid bacteria, some random primers give no PCR products whereas others give multiple products. Since it is possible that the production of some products is due to mismatch priming it is essential to ascertain that DNA samples from different isolates of a strain or species should all have identical and unique RAPD profiles.

In practice, when developing an RAPD technique to differentiate between strains of lactic acid bacteria, sufficient effort should be made to choose the appropriate primer. The sample DNA should be as pure as possible in order to avoid effects of PCR inhibition. The same amounts of sample DNA should be used each time in order to avoid variations in the intensity of the profile. Even if a DNA sample always gives the same RAPD profile different DNA samples from the same strain should be tested in order to be certain that the method of extraction does not influence the result. The choice of primer can only be decided upon when one is certain of the reproducibility of the method. We have successively used RAPD to differentiate between strains of Propioni bacteria in our laboratory. (see Fessler et al. 1999) Fig. 9 shows the RAPD profiles of different wild strains of Propionic acid bacteria.

In summary this method is good for differentiating between strains provided enough initial effort is put into the selection of the proper primer and conditions of amplification.

4.5.2 Repetitive DNA Sequence PCR (rep-PCR)

Because of the severe criticism of RAPD by many authors on its poor reproducibility, several workers have explored different ways to approach to the problem. Among others is the use of naturally occurring interspersed repetitive sequences in prokaryotic genomes. Both prokaryotic and eukaryotic genomes contain dispersed repetitive sequences separating longer single-copy DNA sequences. Various classes of repeated DNA sequences are present in diverse prokaryotic genomes. Coding sequences, such as ribosomal RNA genes and insertion sequences, represent larger repeated elements that are present in relatively low copy number.

In contrast to these larger repeated elements, relatively short polynucleotide sequence patterns may represent highly repetitive sequences in bacterial genomes. The oligonucleotide sequences GTGGTGGTGGTGGTG and GCCGCCGCCGCCGCC have been found to be highly repetitive in the genomes of *E. coli* and *Salmonella typhimurium.* Interspersed repetitive DNA sequences typically are between 15 and several hundred base pairs in length and are widely dispersed in bacterial chromosomes. These elements are noncoding and present in high copy numbers relative to the larger repeated elements that contain coding sequences.

Multiple examples of interspersed repetitive DNA sequences have been described in bacteria and such elements have been isolated from Gram-positive and Gram-negative bacteria. Although these elements were originally described in a single bacterial species

or genus, several of these elements are conserved and dispersed in the genomes of diverse bacteria. The most well known examples of evolutionary conserved repetitive DNA sequences are BOX (Martin et al.), ERIC and REP (Versalovic et al.).

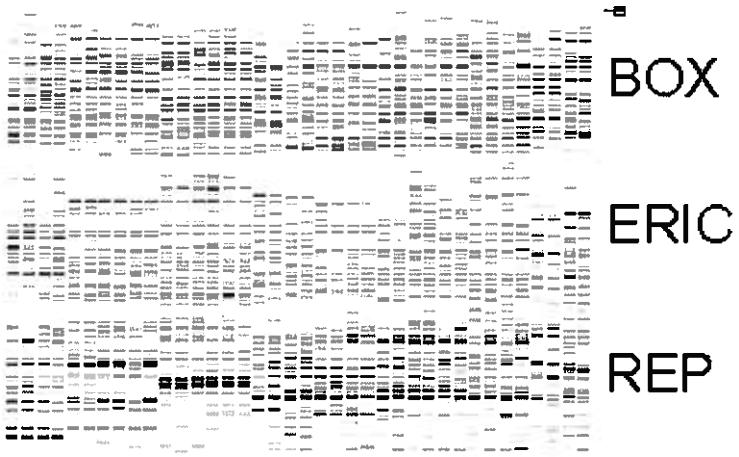


Figure 10. Cluster analysis of 19 Xanthomonas strains belonging to 6 DNA homology groups using combined REP, BOX and ERIC fingerprints (from de Bruijn et al.).

Genomic fingerprinting with interspersed repetitive sequence-based primers distinguishes unrelated organisms because individual bacterial strains vary with respect to the distances between the repetitive sequences. The rep-PCR methods have the advantage that, since primers are designed to be complementary to endogenous interspersed repetitive sequences, they enable a rational approach to primer design and a limited set of primers can be used with virtually any bacteria. The primers are longer in length (18- 22 mers) and therefore, higher annealing temperatures that enable greater stringency and less artificial variation of the rep-PCR can be used. Fig 10 shows the cluster analysis of 19 *Xanthomonas* strains belonging to 6 DNA homology groups using combined REP, BOX and ERIC fingerprints.

In Liebefeld we have successively used Rep-PCR to distinguish between different strains of Propionic acid bacteria. Besides the well-known BOX, ERIC and REP sequences many others have been found. In practice different primers corresponding to the various interspersed repetitive sequences should be tested before choosing the most appropriate for distinguishing between strains of a given species.

4.5.3 Insertion Sequence PCR (IS-PCR)

Insertion sequences are segments of DNA, found in virtually all cells that move or "hop" from one place on a chromosome (the donor site) to another on the same or a different chromosome (the target site). No homology is usually required for the movement, called transposition, to occur. The new location is chosen more or less randomly. Because insertion of the sequence in an essential gene could kill the cell, the events are tightly regulated and occur very infrequently (perhaps once in a million cell divisions). Insertion sequences contain only the sequences required for their transposition and the genes for proteins (transposases) that promote the processes. Insertion sequences that contain

one or more genes besides those needed for transposition are termed complex transposons. These additional genes often confer resistance to antibiotics. Bacterial insertion sequences vary in structure, but most have short repeats at the two ends of the element that serve as binding sites for the transposase.

Here in Liebefeld we have developed a method for distinguishing between bacterial strains based on the short repeats at the two ends of the insertion sequence. The sequences chosen as primers face outwardly from the insertion sequence so that after PCR it is the segments between the insertion sequences that are amplified. Since individual bacterial strains vary with respect to the distances between the insertion sequences they should yield different IS-PCR profiles. The DNA sequences of insertion sequences from several different lactic acid bacteria have been published and are available for the construction of appropriate primers.

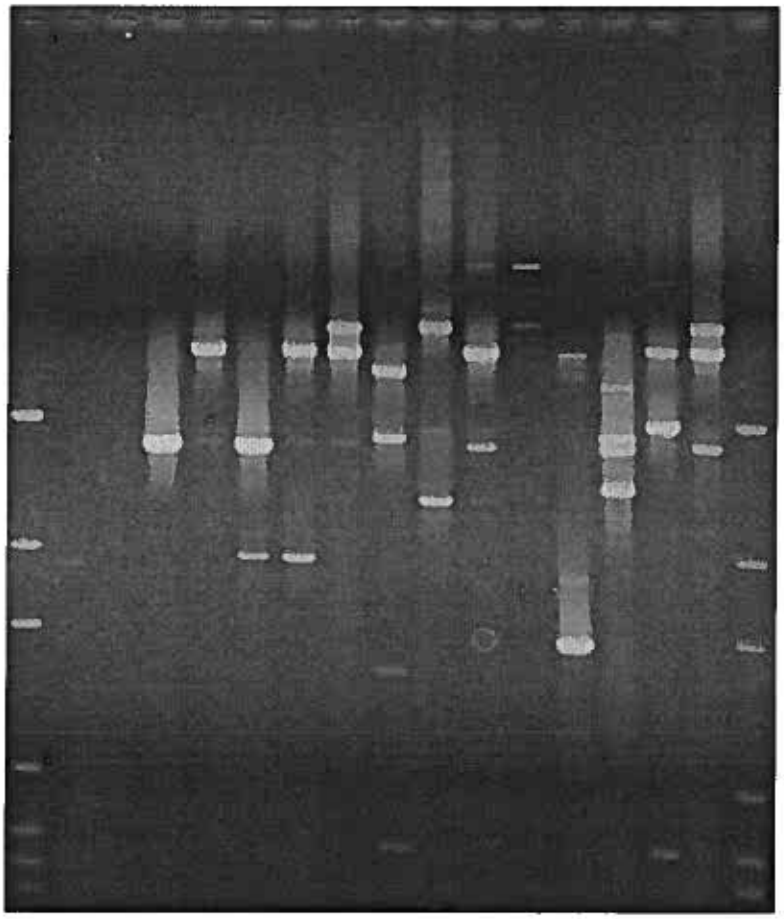


Figure 11. IS-PCR profile of different wild strains of *S. thermophilus* isolated from raw milk.

It has been demonstrated that there is horizontal transfer of insertion sequences between *S. thermophilus* and *Lactococcus lactis* (Bourgoin et al.). We have chosen a battery of primers based on the short repeats of insertion sequences found in the lactic acid bacteria of lactobacilli, streptococci, and lactococci. Fig 11 shows how it was possible to differentiate between various strains of *S. thermophilus* using this technique.

In practice sufficient effort should be invested into the choice of primers. If possible a battery of primers with similar melting temperatures should be chosen in order to increase the number of amplicons. This will yield a more complex profile.

4.5.4 Infrequent-Restriction-Site PCR (IRS-PCR)

The main disadvantage with the PCR methods described so far is, that before primers can be chosen, it is necessary to screen several strains of a species in order to see if they are suitable. Mazurek et al. have developed a method based on the presence of infrequent restriction enzyme sites. Normally after treatment with restriction enzymes the large restriction fragments produced are separated by pulsed-field gel electrophoresis as described above. The resulting electrophoretic patterns are highly discriminatory for a wide range of organisms. However, large amounts of high molecular weight DNA are needed. Purification of high molecular weight DNA in agarose and the electrophoretic separation of the large restriction fragments are time-consuming and tedious. The method described by Mazurek consists of amplifying DNA sequences flanking infrequent restriction sites to produce strain-specific electrophoretic patterns from crude bacterial lysates. The method is referred to as infrequent-restriction- site PCR (IRS-PCR) fingerprinting. Identical enzymes, adapters, primers, and PCR conditions were used to characterise 32 *Mycobacterium avium-M. intracellulare* isolates, 4 Pseudomonas aeruginosa isolates, and 4 Staphylococcus aureus isolates.

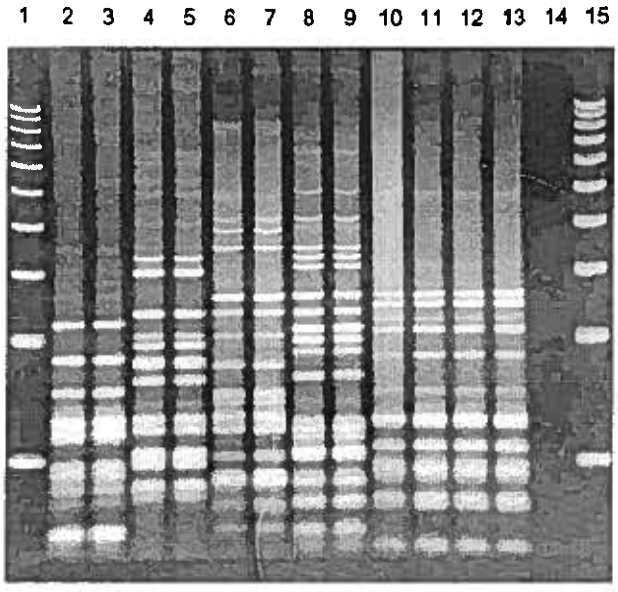


Figure 12. IRS-PCR profiles of *Mycobacterium avium-M. intracellulare* isolates from multiple clinical specimens from five patients. Lanes 1 and 15 100-bp ladder. Lanes 2 and 3 patient 1. Lanes 4 and 5 patient 2. Lanes 6 and 7 patient 3. Lanes 8 and 9 patient 4. Lanes 10–13 patient 5. From Mazurek et al.

The strategy used will not be described in detail here. Suffice it to say that by using two restriction enzymes, one that cuts frequently e.g. *Hha*I and a second that cuts infrequently e.g. *Xba*I, followed by ligation with appropriate primers and PCR only the

DNA between the binding sites for *Xba*I and *Hha*I is amplified. The patterns generated by IRS-PCR were shown to be reproducible. Patterns generated with multiple bacterial isolates from the same patient were essentially identical but different from those generated with isolates recovered from different patients (see fig. 12).

We have not yet investigated the use of this method here in Liebefeld but hope to do so in the near future.

4.6 Conclusion

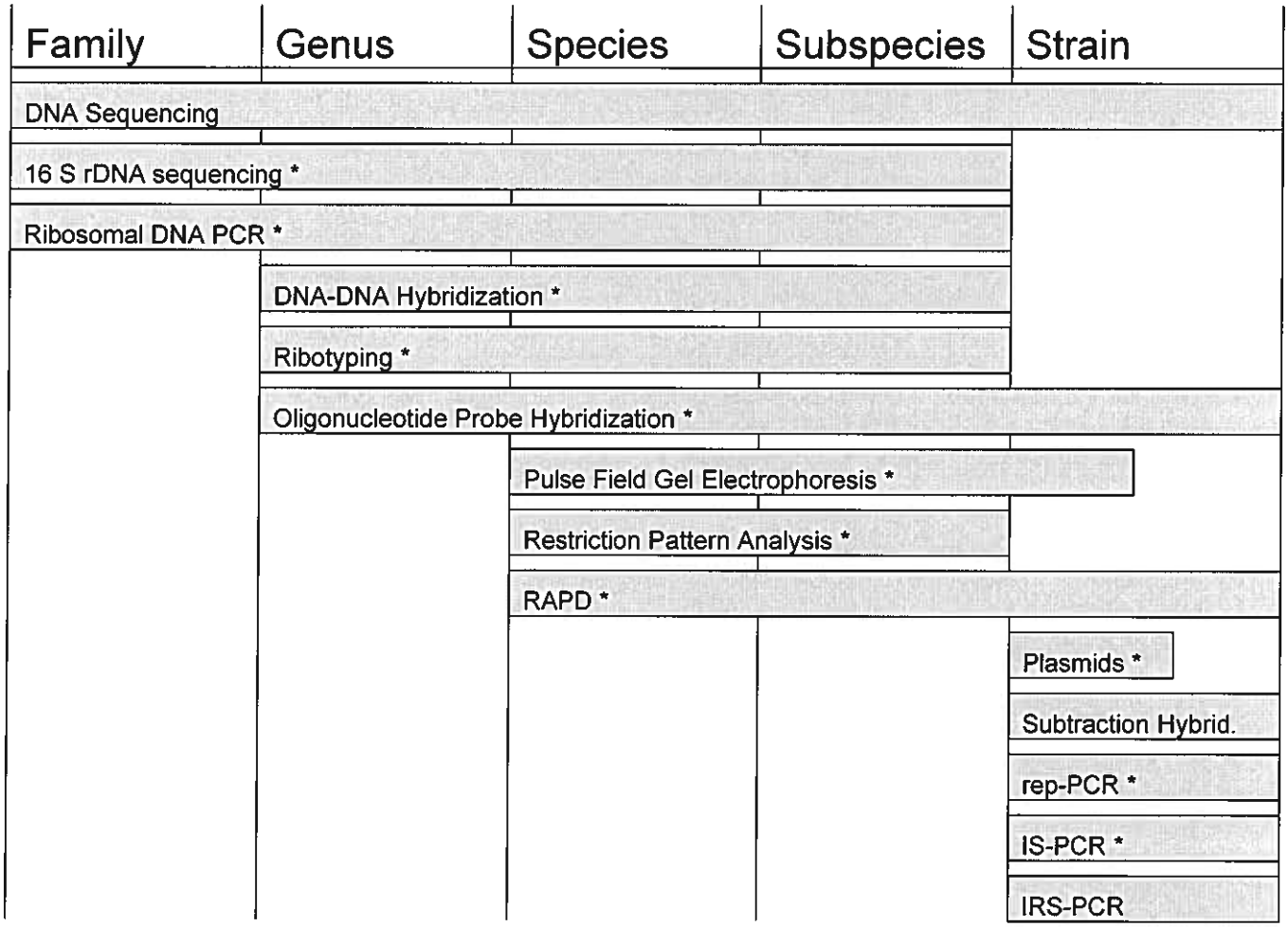


Figure 13. Relative resolution of various fingerprinting and DNA techniques.
(* Methods successfully used in Liebefeld)

Modern molecular biology techniques are extremely useful for differentiating between bacteria. Nevertheless it should be rembered that these techniques are far more complex than classical methods. In general they are more time consuming, more expensive, involve specially trained laboratory staff and equipment. Time should be invested on deciding which is the appropriate technique. Figure 13 shows the relative resolution of various fingerprinting and DNA techniques. Today it is possible to differentiate microorganisms anywhere between the family and the strain level. New

methods are continually being described in the literature. Hopefully in the future ways will be found to reduce both the cost and "on hands" time involved.

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