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The use of applied systematics to identify foodborne pathogens

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15.1 Introduction

Bacterial systematics or classification is of importance to reveal biologically significant, stable groupings. According to the *Bergey's Manual of Systematic Bacteriology*, 'classification' is the orderly arrangement of organisms into taxonomic groups on the basis of similarities or relationships (Staley and Krieg, 1986).The basic taxonomic group in bacterial systematics is the species. Historically, classification of bacteria has been based on similarities in phenotypic characteristics. A bacterial species may be regarded as a collection of strains that share many features in common and differ considerably from other strains (a strain is made up of the descendants of a single isolation in a pure culture). One strain of a species is designated as the type strain and all other strains considered sufficiently similar to this type strain are included in a single species. This concept of species involves subjective judgements. Consequently, some bacterial species have greater phenotypic and genetic diversity than others. A more uniform and rigorous species definition would be desirable (Staley and Krieg, 1986).

In the last decade, it became generally accepted that bacterial classification should reflect as closely as possible the natural relationships between bacteria. Phylogenetic information has increased tremendously mainly through the introduction of molecular methods enabling the determination of genetic relatedness (DNA-DNA hybridization, 16S and 23S rRNA gene sequencing). Nowadays, a species is defined as a group of strains, including the type strain, sharing 70 per cent or greater DNA-DNA relatedness. An alternative is to define the species by percent 16S rRNA similarities (over 97 per cent of 16S rRNA gene sequence homology) (Vandamme *et al*., 1996).

An ‘optimal’ species is one that simultaneously represents a phylogenetic, phenotypic and ecological group. Polyphasic taxonomy aims at the integration of different kinds of data and information (phenotypic, genotypic, phylogenetic) on microorganisms. Variability among organisms is expressed in numerous different molecules (DNA, RNA, proteins, lipids, etc.). Any character which reveals part of this variability is therefore useful and can be considered for classification (Vandamme *et al*., 1996; Stackebrandt, 2002a).

‘Identification’ of unknown organisms is the process of determining whether an organism belongs to one of the established, named taxa (Staley and Krieg, 1986). An identification scheme for a group of organisms can only be elaborated after that group has been classified. It is based on one or more characters, or on a pattern of characters, which all the members of the group have and which other groups do not have. In general the characters chosen for an identification scheme should be easily determinable and few in number. Usually classical phenotypic characteristics such as morphological, physiological and biochemical features are included in identification schemes. However, sometimes the development of simple and restricted identification schemes may not be feasible, especially for particular genera or species that are not susceptible to being characterized by tradional phenotypic tests, e.g. *Campylobacter* sp., *Bacillus* sp., *Aeromonas* sp., pathogenic *Escherichia coli*. In such cases, one may need to apply relatively difficult procedures in order to obtain an accurate identification, e.g. polyacrylamide gel electrophoresis (PAGE) of cellular proteins, cellular fatty acid methyl ester (FAME) composition, PCR detection of specific genes or even 16S rRNA gene sequencing or DNA-DNA hybridization (Krieg, 1986).

In order to choose the most reliable approach for identification of a target organism, i.e. a pathogenic agent or a spoilage organism, it is important to acquire information on its taxonomic position (phenotypic and genotypic diversity, phylogenetic delineation). The present chapter will give an overview of the various types of information and techniques used in classification and their role in identification of bacteria, and will illustrate the use of applying information acquired from systematic studies for accurate identification of foodborne bacterial pathogens.

15.2 Identification based on morphological, physiological and biochemical characteristics

Isolates obtained from a food product can be broadly categorized to a group depending on characteristics such as Gram stain, colony characteristics (pigmentation, mucoid colonies, swarming or pin-point), cell morphology (shape, size, motility and flagellar patterns, endospore formation, inclusion bodies), relation to oxygen (aerobic, facultatively anaerobic, anaerobic, microaerophilic), catalase activity, oxidase activity, ability to dissimilate glucose and the type of metabolism (oxidative or fermentative) (Krieg, 1986; Vandamme *et al*., 1996). Normally, using this approach there is little difficulty in allocating the isolate

into a genus or family. As the category to which the isolate belongs becomes clear, a number of specific diagnostic tests appropriate for the determination of the identity of the isolate in the genus or family should be performed. Which approach and methodology should be used depends on the taxa involved (Krieg, 1986).

Most often, further identification is based on a number of diagnostic characteristics (Vandamme *et al*., 1996):

- physiological features such as growth at different temperatures, pH values, salt concentrations, growth in the presence of various substances such as antimicrobial agents
- biochemical features such as activity of various enzymes, metabolization of carbohydrates.

Rigorous standardization of phenotypic methods is of the utmost importance to obtain reproducible results. The results of physiological and biochemical tests may vary depending on the size of the inoculum, the incubation temperature, length of the incubation period, composition of the medium and the criteria used to define a 'positive' or 'negative' reaction. Therefore it is advisable to include type or reference strains, available from international and national culture collections to use as comparators when using a phenotypic identification scheme in order to check the performance of the test under the conditions employed in one's own laboratory (Krieg, 1986).

Also morphology can depend on the age of the culture, the medium used and other growth conditions, e.g. motility of *Listeria monocytogenes* only occurs after incubation at 30ºC while they are non-motile at 37ºC, spiral bacteria such as *Campylobacter* generally develop into coccoid forms after extended incubation whereas spore formation of *Bacillus* species is only noticed after prolonged incubation. In addition, it is important to start from a pure culture for identification of an organism. The single selection of a colony from a plate, especially if selective media are used, does not assure purity. Purification on non-selective media is preferred for final isolation (Krieg, 1986).

A number of convenient miniaturized systems are commercially available for identification of various taxa, particularly those pathogens and spoilage organisms which are well characterized and commonly found in medical, food and industrial microbiology. These systems are generally composed of a battery of tests using dehydrated reagents and addition of a standardized inoculum initiates the reaction (growth, production of enzymatic activity, acid production from sugars) (Vandamme *et al*., 1996). The results are interpreted as recommended by the manufacturer and are readily available by comparison of the outcome of the test with diagnostic tables or a database furnished by the manufacturer. The use of commercial galleries of biochemical tests such as API galleries (bioMérieux, Marcy-l'Etoile, France), Biolog microplates (Biolog Inc., Hayward, USA), the BBL Crystal ID system (Becton Dickinson, Meylan Cedex, France) etc. can aid in reducing the workload and increases the standardization of the tests among the various laboratories because of the use of quality-

controlled media and reagents. Although some of these commercial galleries may include the same substrates, the results might differ, e.g. the API galleries test the production of acid from carbohydrates whereas the BIOLOG system tests the ability of an organism to utilize a particular carbohydrate, and this may cause discrepancies between the two test kits (Arnaut-Rollier *et al*., 1999).

The approach for identification of unknown organisms using diagnostic tables or computer-assisted comparison with a database in these commercial test systems is designated as numerical identification. In the late 1950s numerical taxonomy developed in parallel with computers and allowed comparison of large numbers of phenotypic characteristics (n) for a large number of strains (t) (Sneath and Sokal, 1973). The $n \times t$ table can then be analyzed to yield the similarities between each pair of strains and a similarity matrix can be constructed. This table of similarities can than be subjected to cluster analysis. In cluster analysis, the principle is to search the table of similarities for high values that indicate the most similar pairs of strains. These form the nuclei of the clusters and the computer searches for the next highest similarity levels and adds the corresponding strains onto these cluster nuclei, etc. The result of the former is a tree-like diagram or dendogram (more precisely a phenogram because it expresses phenotypic relationships). The phenogram reveals a general picture of the phenotypic consistency of a particular group of strains. Although there are no objective criteria to automatically equate a group with highly consistent phenotypic traits with a taxon, it is however commonly found that these phenotypic groups formed at about 80 per cent similarity are equivalent to bacterial species. Description of these groups (species) can now be made by referring back to the original table of phenotypic characteristics for all strains included in the study. Identification tables can be established by calculating the percentage frequencies of occurrence of characteristics in strains of significant clusters using a software program. The better diagnostic characteristics can be chosen, being those which are very constant within groups but vary between groups. These make up the simple but restricted number of morphological, physiological or biochemical tests which should be performed for identification of an unknown organism which is already located to a family or genus on the basis of some superficial initial test as described above. It is these diagnostic characteristics that are described as confirmation tests in official microbiological analytical procedures for foodborne microorganisms (Sneath, 1986a). Recently, a numerical taxonomy study has been described for *Pseudomonas* (Arnaut-Rollier *et al*., 1999) and *Aeromonas* (Valera and Esteve, 2002).

With many genera and species, for example the members of the family of *Enterobacteriaceae*, lactic acid bacteria, or bacilli, identification may not be based on only a few tests, but rather on a the pattern given by applying a whole range of tests. To avoid inoculating large numbers of media, commercially-available miniaturized test systems are used for these purposes. Numerical identification is then used by the software furnished by the manufacturer to correlate the results of the numerous tests into an approximate identification. The operation of numerical identification is to compare an unknown organism in

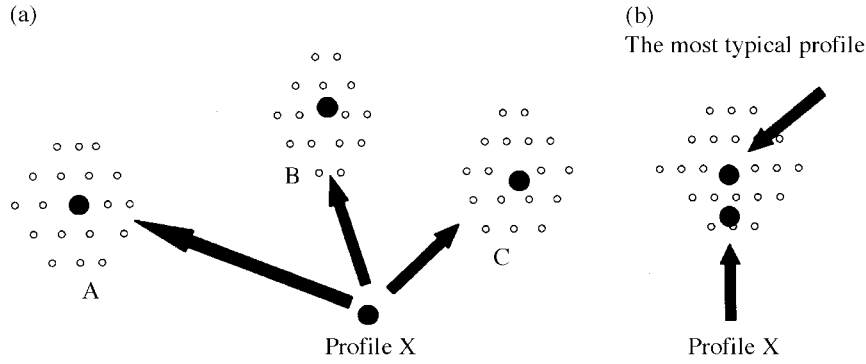

Fig. 15.1 Numerical identification. (a) Determination of the relative proximity of the phenotypic profile of X of the unknown organism to the profiles of the different established taxa A, B, C in the database and (b) determination of the relative proximity of the phenotypic profile X to the most typical profile of the closest taxon (Anon, 1988).

turn with each column of the identification table (table with the percentage of positive test reactions, a table of q taxa (species) for m diagnostic characteristics), and to calculate a distance to the centre of each taxon (species) hemisphere. A column representing a species defines, in effect, a region in hyperspace, and it is useful to think of a species as being represented by a hypersphere in that space, whose position and radius are specified by the numerical values of the percentages (Sneath, 1986b). To identify an organism the profile obtained has to be compared to the profiles of the species in the identification table. This comparison is done by calculating for the profile obtained (Fig. 15.1):

- its relative proximity to the different taxa of the data base (percentage identification). In this way it is possible to determine whether the profile obtained is close to a species.
- its proximity to the most typical profile in each of the species. The most typical profile is the one that has no tests against the identification in relation to the percentages shown in the identification table for the species in question. If a test does differ the profile observed moves slightly away from the most typical profile.

The numerical process allows a likelihood to be attached to an identification (Table 15.1), so that one can know to some order of magnitude the certainty that the identity is correct. The results are not affected by an occasional aberrant property of the unknown. However, the accuracy of such estimates depends critically on having a sufficiently large database including a large number of characteristics for a good number of strains of the species implied (both reference strains and strains of various origins). Also the quality of the determination of the characteristics is important. Microbiological data are prone to more experimental error than is commonly realized. Standardization of the test procedure is a necessity to obtain reproducible and reliable test results

Table 15.1 An example of numerical identification (Anon, 1988)

Step 1: Identification table (4 tests, 4 taxa)

	Test 1	Test 2	Test 3	Test 4
Taxon A	50	93	87	2
Taxon B	0	100	85	98
Taxon C	15	15	91	3
Taxon D	0	95	98	1

Step 2: Frequencies of occurrence of the reactions

	Unknown strain				Most typical profile			
	Test 1 −	Test 2 +	Test 3 +	Test 4 +	Test 1	Test 2	Test 3	Test 4
Taxon A	0.500	0.929	0.869	0.021	0.50	0.929	0.869	0.970
Taxon B	0.990	0.990	0.849	0.979	0.99	0.99	0.849	0.979
Taxon C	0.843	0.151	0.909	0.031	0.843	0.843	0.909	0.961
Taxon D	0.990	0.949	0.979	0.011	0.99	0.949	0.979	0.980

Step 3: Frequencies of occurrence of the profile

Observed profile

Taxon A	$0.500 \times 0.929 \times 0.869 \times 0.021 =$	0.00846
Taxon B	$0.990 \times 0.990 \times 0.849 \times 0.979 =$	0.81463
Taxon C	$0.843 \times 0.151 \times 0.909 \times 0.031 =$	0.00359
Taxon D	$0.990 \times 0.949 \times 0.979 \times 0.011 =$	0.01012
		0.83680

Step 4: Percentages of identification

Taxon A	$(0.00846/0.83680) \times 100 =$	1.01
Taxon B	$(0.81463/0.83680) \times 100 =$	97.35
Taxon C	$(0.00359/0.83680) \times 100 =$	0.43
Taxon D	$(0.01012/0.83680) \times 100 =$	1.21
		100.00

Step 5: Classification of taxa

1. Taxon B % id = 97.35
2. Taxon D % id = 1.21
3. Taxon A % id = 1.01
4. Taxon C % id = 0

(Sneath, 1986a). It should be noted that the numerical identification is based on typical profiles shown by the majority of the strains included in the species. However, strain variation is common and atypical strains, e.g. non-sorbitol fermenting *E. coli* O157:H7; non-haemolytic *L. monocytogenes*, slow lactose fermenting *E. coli*, may be misidentified or give rise to an unacceptable identification by this methodology.

Determination of the morphological, physiological and biochemical characteristics are the main routes for the identification of bacteria and lead to an accurate identification for the majority of the foodborne pathogens. For a number of bacterial groups this approach may not be sufficiently reliable for identification purposes. For example for taxa which are phenotypically and genotypically highly variable, e.g. *Bacillus* (Joung and Côté, 2002), or for taxa for which the taxonomy has recently evolved because of information acquired by molecular methods and for which no appropriate simple and unique morphological, biochemical or physiological characteristics have been established to differentiate the newly designed species e.g. *Aeromonas* (Millership, 1996). Also the paucity of phenotypic characteristics in particular bacterial groups can cause problems for identification, e.g. *Campylobacter* (Engvall *et al*., 2002). For these bacterial groups an alternative approach is recommended.

15.3 Identification based on chemotaxonomy

Chemotaxonomy is the application of chemical and physical techniques to elucidate the chemical composition of whole bacterial cells or parts of cells (Jones and Krieg, 1986). Amongst the techniques which can be applied for bacterial identification based on the comparison of patterns/fingerprints with a database are FAME and SDS-PAGE.

15.3.1 Serology

Serotyping is a typing system based on the presence of variability in the antigenic constituents of the cells (Henriksen, 1978). It can be used to characterize strains at the infraspecific level. For example on the basis of the antigenic complexity of their surface antigenes (cell wall lipopolysaccharide, flagella and capsule constituents), the genera of the family *Enterobacteriaceae* can be divided into many serovars (Jones and Krieg, 1986). For example, *Salmonella* includes more than 2000 serovars. However, as was recently acknowledged, these serovars do not represent separate species. Based on biochemical and physiological tests, the genus *Salmonella* was originally differentiated into five and later seven subgenera, designated I, II, IIIa, IIIb, IV, V and VI. Currently after analysis by modern techniques, the genus is considered to consist of either one or two species. Evidence from DNA hybridization and numerical taxonomy supports the existence of only one

species, *S. enterica*, while sequence analysis of the ribosomal DNA suggests separation into two species, *S. enterica* and *S. bongori*, the former comprised of six subspecies (*enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), *indica* (VI)) and the latter representing *Salmonella* group V. Most serovars of significance in humans, including those responsible for foodborne disease, belong to subspecies I (*S. enterica* subsp. *enterica*). Within the former subgenus system, the serotype level of *Salmonella* has historically been afforded species status. The name of a particular serotype appeared in a typical italicized genus-species form; for example serovar typhimurium was described as *S. typhimurium*. Currently, only serovars of subspecies *enterica* (formerly subgenus I) are given names and the serovar name is not afforded species status and thus serovar typhimurium is fully described as *S. enterica* subsp. *enterica* serovar typhimurium, which is conveniently abbreviated to *S.* Typhimurium. Thus the serovar name is kept but distinguished from the species level. Serovars of subspecies other than enterica are simply described by their antigenic formula although some of the older and more common serovars are still often referred to by name; for example *S. enterica* subsp. *salamae* serovar 1,4,12,27:b:{e,n,x} is still commonly known as *S.* sofia (Cox, 2000).

Serotyping is of little value in classification and thus not considered as being part of the identification but is valuable in epidemiological studies. In addition, often antisera are not readily available and the serotyping can only be performed by a few reference laboratories (Jones and Krieg, 1986; Vandamme *et al*., 1996).

15.3.2 Cellular fatty acids

A variety of lipid-containing organelles are present in bacterial cells. Polar lipids are the major constituents of the lipid bilayer of bacterial membranes and have been studied frequently for identification purposes. Lipopolysaccharides present in the outer membranes of Gram negative bacteria have also be analyzed for identification purposes. Bacterial lipid determination, particularly cellular fatty acid analysis by gas-liquid chromatography, has long been recognized as a valuable chemotaxonomic tool for the classification and identification of mainly non-fermentative Gram negative bacteria (Osterhout *et al*., 1991). More than 300 different chemical structures of fatty acids have been identified. The wealth of information contained in these compounds can be estimated by considering not only the presence or absence of each fatty acid, but also by its relative percentage in the total fatty acid profile. Each fatty acid is characterized by its structural variability including chain length, double-bond position and the presence and location of substituent groups (Vandamme *et al*., 1996).

Gas-liquid chromatographic analysis of fatty acid methyl esters (FAMEs) is increasingly used for routine microbial identification. Due to the essential role of fatty acids in cell structure and function, the FAME content is a genetically stable parameter provided that highly standardized culture conditions are used. Fatty acid patterns obtained may be influenced by composition of growth medium, temperature of incubation, age of culture and the techniques employed

to analyze the sample (Jones and Krieg, 1986). Once a sufficient number of FAME profiles generated from taxonomically well characterized type and reference strains have been stored into a library, an identification database can be arranged for the rapid identification of unknown strains.

The Sherlock commercial microbial identification system (previously known as the Microbial ID Inc., Newark, Del.) for whole-cell fatty acid analysis provides databases containing species-specific FAME entries. For specific identification purposes, however, the user may need to construct a new database for reasons of taxonomic resolution or deviating growth requirements. The procedures for extraction, chromatographic separation and data analysis are relatively simple and have reached a high degree of automation (Vandamme *et al*., 1996). It is a fairly inexpensive and rapid alternative for the identification of bacterial groups that cannot be reliably speciated using phenotypic methods including *Bacillus* spp., *Clostridium* spp., *Aeromonas* spp., and members of the *Pseudomonadaceae*.

15.3.3 Whole-cell protein analysis

The basis of this concept is that closely-related organisms should have similar or identical kinds of cellular protein. One-dimensional polyacrylamide gel electrophoresis (PAGE) of cellular proteins can yield patterns of up to *ca*. 30 bands. The protein 'fingerprint' obtained for a strain is a reflection of the genetic background of that strain and can be used for identification purposes (Pot, 1994). However, in contrast to, for example, whole-cell fatty acid analysis, protein electrophoresis does not supply descriptive information, because in general, the identity of none of the protein bands is revealed. Numerous studies have revealed a correlation between high similarity in whole-cell protein content and DNA-/DNA hybridization.

In practice, whole cells or cellular membrane fractions are used, and the proteins are solubilized by means of a detergent such as sodium dodecyl sulfate (SDS). By use of rigorously standardized conditions, extremely reproducible protein patterns can be obtained which are amenable to rapid, computerized, numerical analysis and databases can be created for identification purposes (Jones and Krieg, 1986). The comparison of whole-cell protein patterns obtained by highly standardized sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has proven to be extremely reliable for comparing and grouping large numbers of closely-related strains. SDS-PAGE yields discriminative information at or below the species level. SDS-PAGE can be convenient for reliable identification of lactic acid bacteria. The large number of species in, for example, the genus *Lactobacillus*, are difficult to discriminate with a limited number of morphological and biochemical characteristics (Vandamme *et al*., 1996).

15.3.4 MALDI-TOF mass spectrometry, Fourier transform infrared (FTIR) spectroscopy, Raman spectroscopy

Different procedures have been developed in recent years that use mass spectrometry to characterize bacteria. The rapidity and accuracy with which data can be obtained by Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) make this an interesting tool for rapid identification of bacteria at the strain level (Lynn *et al*., 1999; Kridhnamurthy *et al*., 1996).

The sample preparation is easy to perform by MALDI-TOF MS. The bacteria are grown on culture media (liquid or solid) and the cells are collected and washed with water or buffers to eliminate salt. The bacterial sample is suspended in an extraction solvent like trifluoroacetic acid, acetonitrile (ACN) and different mixtures of solvents which solubilize the biomarkers (Domin *et al*., 1999). A sonication can be added to increase the protein extraction (Easterling *et al*., 1998). Finally, the preparation is mixed with a 'MALDI matrix' like α-cyano-4-hydroxycinnamic acid, ferulic acid or 3,5-dimethoxy-4-hydroxycinnamic acid (Madonna *et al*., 2000), spotted on MALDI target plate and dried before analysis.

The comparison of MALDI-TOF MS spectrum with a database of bacterial characteristic profiles allows a rapid identification of new unknown samples. However, the sample preparation is a critical step in the analysis of bacterial cells by mass spectrometry. Some factors can strongly affect the general appearance of the observed spectra: incubation (time/temperature), bacterial protein extraction, salt content, MALDI target plate preparation, etc. (Saenz *et al*., 1999; Wang *et al*., 1998). In order to obtain the result reproducibility, it is important to use the same procedures of bacterial sample preparation every time.

Some of the difficulties involved in MALDI-TOF/MS of bacteria have been the complexity of the spectra, the large mass ranges used, and the subtle differences that may be observed in spectra from related strains. However, although related strains share many peaks in common, each strain reveals one or several mass peaks which appear to be genus-, species- or strain-specific. These biomarkers can be used for bacterial diagnostics. In addition, a mathematical approach was developed to compare MALDI-TOF mass spectra. This mathematical fingerprint-matching technique eliminated the need for subjectivity in comparing spectra visually to determine whether or not they matched or were different. MALDI-TOF/MS is an attractive alternative to SDS-PAGE to differentiate bacterial strains.

This MALDI-TOF approach was successfully applied to differentiate pathogenic and non-pathogenic bacteria. Some studies also reported the detection of spore-forming bacteria. Unique patterns of biomarkers (diagnostic peaks) were reproducibly found to characterize and differentiate *Bacillus* species. In addition MALDI-TOF/MS could be applied for the analysis of spores. Spectra obtained directly from several bacilli, including *B. anthracis*, *B. cereus* and *B. thuringiensis* showed many spectral similarities, whereas *B. mycoides* was quite different. However, despite these similarities, the spectra were sufficiently unique to allow

the differentiation of the different *Bacillus* species as well as *B. cereus* and *B. thuringiensis* strains (Lay, 2001; Ryzhov *et al*., 2000).

FTIR spectroscopy involves the observation of vibrations of molecules that are excited by an infrared beam, and generates a characteristic infrared absorbance spectrum which is associated with specific chemical groups of the different components in the bacterial cells. Raman spectroscopy is a light-scattering technique in which the frequency and intensity of the scattered light after exposure of the whole cell suspension or microcolony to a laser beam is analyzed. The Raman scattering patterns obtained are an indication of the type and concentration of chemical compounds or structures present in the cells. Both FTIR spectroscopy and Raman spectroscopy have been used to discriminate and classify bacteria at the strain level by producing complex, yet distinct and reproducible biochemical fingerprints of bacteria. Cluster analysis of the fingerprints obtained enabled differentiation of strains of *Pseudomonadaceae*, *Bacillus*, lactic acid bacteria, and *Enterobacteriaceae* (Harhay and Siragusa, 1999; Amiel *et al*., 2001; Mariey *et al*., 2001; Irudayaraj *et al*., 2002). The ability of FTIR spectroscopy and Raman spectroscopy for identification of foodborne pathogens needs to be studied further.

15.4 Identification based on genetic information

Determination of the morphological, physiological and biochemical characteristics are the main routes for the routine identification of bacteria. Chemotaxonomic methods use biochemical fingerprints for identification of bacteria. They rely on patterns of biochemical cellular components (e.g. proteins, fatty acids, whole cell components) and are thus considered as phenotypic methods. Obviously, large numbers of phenotypic data reflect a considerable amount of genetic information. As shown numerical analysis of phenotypic traits was indeed taxonomically relevant. Nevertheless, it is impossible to classify bacteria phylogenetically by these criteria. In the last decade, it became generally accepted that bacterial classification should reflect as closely as possible the natural relationships between bacteria. Phylogenetic information has increased tremendously mainly through the introduction of molecular methods enabling the determination of genetic relatedness (DNA-DNA hybridization, 16S and 23S rRNA gene sequencing) (Stackebrandt, 2002b). Nowadays a bacterial species is defined on the basis of genetic information combined with a phenotypic description. According to Vandamme *et al*. (1996) a bacterial species is an assemblage of isolates which originated from a common ancestor population in which a steady generation of genetic diversity resulted in clones with different degrees of recombination, characterized by a certain degree of phenotypic consistency and by a significant degree of DNA-DNA hybridization and over 97 per cent of 16S rDNA sequence homology. Identification of unknown isolates by genetic information thus goes back to the basis of classification of unknown organisms.

15.4.1 16S rRNA gene sequencing

Ribosomal RNAs are molecules with universal, constant and highly constrained functions which are not affected by changes in the organism's environment. They are composed of highly conserved as well as more variable domains. It is generally accepted that ribosomal RNA (rRNA) or DNA sequences encoding rRNA (rDNA) are the best targets for studying phylogenetic relationships, as changes in sequence must happen more or less randomly in time at a more-or-less steady pace and quasi-independent of the sporadic real evolutionary changes happening in the phenotype (Woese, 2002). Moreover, rDNAs are easy to isolate in relatively large quantities from bacterial cells grown under any conditions. Nowadays they can be sequenced directly by using the PCR technique and a selection of appropriate primers. International databases comprising 16S rDNA sequences covering more than 95 per cent of the current described species are publicly available. Identification based on 16S rDNA sequences is performed by searching for the closest relative using the available 16S rDNA sequence data in these databases assisted by the BLAST or FASTA electronic system available on the Internet. A 16S rDNA sequence similarity of at least 97 per cent indicates that strains may belong to the same species (Stackebrandt, 2002a). However, this is only an indication of species identity because different validly described species may show > 97 per cent 16S rDNA sequence similarity to each other. Precise species identity must be confirmed by DNA-DNA hybridization or (for routine laboratories) by performing appropriate differentiating phenotypic tests between closely-related species showing > 97 per cent 16S rDNA sequence similarity with the unknown strain. Although, 16S rDNA sequencing is one of the basic modern techniques for identification of unknown isolates, these facilities are reserved to dedicated molecular biology laboratories. In addition, it is not an adequate technique to identify large numbers of strains, unless partial sequencing of the 16S rRNA gene up to about 500 bp is performed using one or two sequencing primers. 16S rDNA sequencing is especially useful to determine the position of an unknown isolate which cannot be allocated by simple and rapid phenotypic testing or chemotaxonomic methods to a defined species.

For a well-studied genus or species, genus or species-specific primers from the 16S rDNA sequence may be selected to construct a PCR for identification purposes with the advantage that it is feasible to examine many strains. In addition these universal and variable bacterial sequences in the 16S rRNA molecule are under various conditions present in the viable cell and thus allow *in situ* detection and identification of individual cells without cultivation.

15.4.2 DNA-DNA hybridization

To fully determine the inclusion of an unknown organism to a defined species or to establish the isolate as a new species, comparisons of the total genomic DNA of the isolate and two or more type strains of related species (showing >97 per cent 16S rDNA sequence similarity) should be performed. Description of a new species involves the determination of the mean mole GC content of the genome

and the study of the total genomic sequence similarities by DNA-DNA hybridization. The value of 70 per cent or greater DNA/DNA homology has been proposed as the borderline of species designation (Wayne *et al*., 1987). However, it seems to be only indicative rather than absolute. Phenotypic characteristics have to support this definition. Preferentially a number of simple and straightforward tests should support species determination based on DNA-DNA hybridizations (Vandamme *et al*., 1996). As recently proposed, taxonomists are encouraged to also use other genomic methods in species descriptions provided that there is sufficient degree of congruence between the technique used and DNA-DNA reassociation (Stackebrandt *et al*., 2002c).

Phenotypically identical but genotypically distinct groups of strains are referred to as genospecies. As a typical example, the taxonomy of the genus *Aeromonas* has evolved from a complex mixture of phenotypic and genotypic data. For this reason, biochemically distinct *Aeromonas* species are referred to as phenospecies, whereas distinct DNA hybridization groups (HGs) are usually called genospecies. Several of the genospecies are difficult to separate biochemically or have not yet been named because of lack of phenotypic characteristics that correlate well with DNA/DNA hybridization data (Huys *et al*., 1994; Carnahan and Altwegg, 1996).

In other cases, DNA-DNA hybridization values conflicted with data from other analyses. Strains of *Escherichia coli* and *Shigella dysenteriae* are extremely closely related and exhibit DNA-DNA hybridization values as high as 89 per cent. Nevertheless for epidemiological purposes the two taxa are not considered strains of the same species and even represent two different genera in the *Enterobacteriaceae*. On the other hand, certain strains of *Clostridium botulinum* are remotely related only by 16S rDNA analysis, they share less than 10 per cent DNA homology as measured by DNA-DNA hybridization and they are more closely related to other *Clostridium* species than they are among themselves. However, as all of them express a botulinum toxin they are classified in one species (Stackebrandt, 2002a).

15.4.3 DNA-based typing methods

The introduction of molecular biological techniques into microbiology yielded a large variety of DNA-based typing methods. Compared to the classical phenotypic typing techniques, molecular typing techniques have several advantages such as general applicability and a high discriminatory power. The currently available molecular techniques can be classified according to their working principles as follows.

- PCR-mediated typing techniques (random amplification of polymorphic DNA or RAPD, rep-PCR)
- typing techniques combining PCR with restriction analysis of a gene sequence showing variation (amplified ribosomal DNA restriction analysis or ARDRA, *fla*A typing of *C. jejuni*)

- typing techniques based on chromosomal restriction fragment length polymorphisms (e.g. ribotyping, pulsed field gel electrophoresis or PFGE)
- typing techniques combining restriction digestion with selective amplification (amplified fragment length polymorphism or AFLP)
- plasmid analysis (Heyndrickx *et al*., 2001; Vandamme *et al*., 1996).

Although most of these methods are particularly useful for discriminating strains of the same species and determining infraspecific relationships, some of these molecular typing methods can be applied to identify strains at the species level. For example, the results of the AFLP technique applied to *Aeromonas* species suggests its potential usefulness at the species and subspecies levels, as well as for the fine typing of individual strains (Huys *et al*., 1996). Also an extended database of ARDRA fingerprints of *Bacillus* sp. was constructed which can be used for identification purposes (Heyndrickx *et al*., 1996). Several molecular typing techniques have been shown to provide the possibility of *Salmonella* serovar identification and thus can replace the classical serotyping (Heyndrickx *et al*., 2001). Obviously the potential for identification of an unknown organism by DNA-based typing methods is restricted to a particular combination of 'taxon-typing method' and sufficient fingerprinting data of strains belonging to this taxon are needed to assess the usefulness of the typing method for reliable identification.

PCR-based typing methods with random or repetitive elements as primers have been applied to strain characterization in a wide variety of bacteria. In several of these studies species-specific DNA fragments were generated. These specific DNA fragments may be useful as probes to rapidly screen and identify other isolates (Vandamme *et al*., 1996). Analysis of the PCR fingerprints obtained for thermophilic campylobacters revealed that besides isolate-specific fragments, species-specific DNA fragments were generated which could be used as species-specific probes for *Campylobacter jejuni*, *C. coli* and *C. lari* (Giesendorf *et al*., 1993). Recently Gevers *et al*. (2001) demonstrated that rep-PCR fingerprinting using the $(GTG)_5$ primer is a valuable tool for differentiation of a wide range of food-associated lactobacilli at the species, subspecies and potentially up to the strain level.

Although DNA-based typing methods may be a valuable tool in identification of microorganisms with a difficult taxonomic position, they are primarly applied for infraspecific characterization and comparison and grouping of large numbers of strains at the genetic level. A merit is that the dendogram generated by cluster analysis of the DNA fingerprints allows the selection of representative strains for further characterization. Ultimately further phenotypic testing of well-chosen strains can be used to determine the physiological, ecological or pathogenic relevance of these strains. In addition these selected strains can be subjected to 16S rDNA sequencing or DNA-DNA hybridization to determine their identity or phylogenetic position.

15.5 Applications: identifiying the genus *Aeromonas*

Microbiological analysis of foodborne pathogens usually involves selective culture media. These media are frequently complex mixtures of nutrients, selective inhibitors (e.g. antibiotics), chemicals (bile salts, azide, selenite, tellurite) and dyes (brilliant green, crystal violet) and indicator systems, designed to inhibit unwanted organisms while permitting the sought organisms to grow. Colonies of sought organisms are sometimes distinguished by use of colour reactions, such as pH indicator to show acid production from a sugar, or production of a coloured product from a colourless one (Bolton, 1998). Usually classical phenotypic characteristics such as morphological, physiological and biochemical features are implied as confirmation tests. However, sometimes the development of simple and restricted confirmation schemes may not be feasible to provide a reliable identification of the foodborne pathogen, especially for particular genera or species that are not susceptible to being characterized by traditional phenotypic tests, e.g. *Aeromonas* sp., *Bacillus* sp., *Campylobacter* sp., pathogenic strains of *Escherichia coli* and other foodborne pathogenic species. The following will illustrate the application of information and techniques used in systematic studies to reach accurate identification of these foodborne bacterial pathogens.

Aeromonas is a Gram negative facultatively anaerobic rod-shaped organism that is ubiquitous in virtually all types of freshwater worldwide but which has also been isolated from human and animal clinical specimens and from food including fish, raw meat and vegetables (Neyts *et al*., 2000; Palumbo, 1996). There is evidence that *Aeromonas* species can grow and produce different virulence factors not only at optimal growth temperature (28C), but at refrigeration temperatures as well (Merino *et al*., 1995). This may be of importance to raw food products that are kept at refrigeration temperatures and have an extended shelf-life at this temperature. Although still controversial, some strains of *Aeromonas* are considered as opportunistic pathogens causing gastrointestinal diseases in immunocompromised individuals (Joseph, 1996).

Modern *Aeromonas* taxonomy is based on the so-called 'four-species-concept' described by Popoff in *Bergey's Manual of Systematic Bacteriology* (1984) including the mesophilic motile *A. hydrophila* complex, the *A. caviae* complex, the *A. sobria* complex and the psychrophilic, non-motile species *A. salmonicida*. Each of these mesophilic complex groups were found to contain two or more DNA hybridization groups (HGs), some of which have meanwhile obtained the species status as a result of extensive phenotypic characterization and the introduction of chemotaxonomic and genotypic methods. Currently, the genus *Aeromonas* comprises at least 15 *Aeromonas* species including *A. hydrophila* (HG1 and HG3), *A. bestiarum* (HG2), *A. salmonicida* (HG3, including five subspecies), *A. caviae* (HG4 and HG5), *A. media* (HG5), *A. eucrenophila* (HG6), *A. sobria* (HG7), *A. veronii* biovar sobria and biovar veronii (HG8/10), *A. jandaei* (HG9), *A. encheleia* (HG11), *A. schubertii* (HG12), *A. trota* (HG13), *A. allosaccharophila*, *A. popoffii*, and *A. culcicola*.

However, the taxonomy of the genus is continuously changing because of new findings. As such, the *Aeromonas* genus is a good example of a taxon where classical phenotypic testing lacks the potential for accurate identification. The application of advanced more discriminative methods can aid in addressing the current taxonomic and pathogenic problems in the genus of *Aeromonas*.

Rapid phenotypic testing to identify a suspected *Aeromonas* isolate leads to misidentification because the current morphological, physiological and biochemical tests lack the taxonomic resolution compared to chemotaxonomic, genotypic and phylogenetic methods currently used to construct the taxonomic framework of the genus *Aeromonas*. Application of molecular methods showed several genospecies which are difficult to separate biochemically or have not been named yet because of lack of phenotypic characteristics that correlate well with DNA-DNA hybridization data. For instance, the motile *A. hydrophila*-like members of HG3 still await proper naming and can still be genotypically confused with the physiologically distinct subspecies of *A. salmonicida*.

Data by biotyping (Valera and Esteve, 2002) and multilocus enzyme electrophoresis (Tonolla *et al*., 1991) correlated well with DNA-DNA hybridization data. However, these techniques are time-consuming and require too many reagents to be of practical use in routine analyses. FAME profiles are rather similar between *Aeromonas* species, although minor quantitative variations could be used to differentiate the majority of the phenospecies and/or hybridization groups in the genus *Aeromonas*. Hybridization groups that constituted the *Aeromonas hydrophila* complex, the *Aeromonas caviae* complex, and the *Aeromonas sobria* complex were basically grouped into distinct FAME clusters. Rapid identification of unknown aeromonads can be performed by using the database of FAME fingerprints constructed for *Aeromonas* and related genera. It should be noted that comparison of FAME data with the established database can only be performed if these were achieved by highly standardized cultivation and extraction procedures and specialized software like the Microbial Identification System (MIDI, Microbial ID, Inc., Newark, Del.) (Huys *et al*., 1994). From the molecular methods used in *Aeromonas* taxonomy, the genomic fingerprinting technique AFLP (Amplified Fragment Length Polymorphism) can be considered as one of the most powerful techniques to differentiate all currently recognized taxa in the genus. Numerical analysis of the digitized AFLP fingerprints revealed AFLP clusters which, in general, clearly supported the current *Aeromonas* taxonomy derived from DNA homology data (Huys *et al*., 1996).

15.6 Applications: identifying the genus *Bacillus*

15.6.1 *Bacillus* spoilers

The classical genus *Bacillus* encompasses the Gram positive spore forming rod-shaped aerobic bacteria and comprised, in 1993, more than 60 validly described species (Priest, 1993). The classical genus *Bacillus* is phenotypically

heterogeneous, with members exhibiting a wide range of nutritional requirements and growth conditions and occupying a variety of habitats. An extensive list of morphological, physiological and biochemical properties of most members of the genus have been compiled and procedures for isolation and tentative identification of typical strains of *Bacillus* species are available. However, identification of *Bacillus* species using classical phenotypic testing is not an easy task. Indeed DNA hybridization studies and 16S rRNA gene sequencing have revealed the genetic heterogeneity of the genus, and in fact intrageneric heterogeneity is as great as exists in most bacterial families, e.g. *Enterobacteriacae* (Slepecky and Hemphill, 2002). It is thus understandable that the genus has been subjected to reclassification. Reclassification of the genus *Bacillus* started in 1991 with the work of Ash *et al*. (1991). By comparative analysis of rRNA gene sequences of a collection of 51 *Bacillus* species they were able to group them into five distinct clusters. These studies, followed by others, have led to the creation of several new *Bacillus*-derived genera: *Alicyclobacillus*, *Paenibacillus*, *Aneurinibacillus*, *Brevibacillus*, *Virgibacillus*, *Gracilibacillus*, *Salibacillus* (Joung and Côté, 2002) and it is expected that more new genera and species will be described in the near future.

A miniaturized rapid identification system wherein many standardized biochemical assessments can be made along with some supplementary classical determinants (spore shape, presence of swollen sporangium, etc.) can be used for identification of the commonly encountered *Bacillus* species provided the corresponding correct species names are included in the database. A variety of techniques have been employed to find a simple and rapid approach to identify *Bacillus* species with a difficult taxonomic position. A first simple technique to assign *Bacillus* strains to groups is fatty acid methyl ester (FAME) analysis although it is not useful for exact species identification (Kämpfer, 1994). Also, a standardized method for amplified ribosomal DNA restriction analysis (ARDRA) has been described and numerical analysis of the ARDRA patterns allows the strains to be grouped. In general, the clustering of the *Bacillus* strains corresponds well with the known species delineations (Heyndrickx *et al*., 1996). Alternatively, SDS-PAGE of whole cell proteins can be performed. Preferably two or more methods should be used for identification. When large sets of isolates have to be identified, a first classification of the isolates can be performed with, for example, ARDRA, FAME or PAGE, after which one representative of each cluster or group in the classification (usually represented in a dendrogram) can be identified by 16S rDNA sequencing (Heyndrickx *et al*., 1998). This polyphasic approach integrating different kinds of techiques and information was used for identification of aerobic spore-forming *Bacillus* species in feed concentrates for dairy cattle (Vaerewijck *et al*., 2001). Feed concentrate for dairy cattle contains known as well as yet unknown species of *Bacillus* and related genera with properties which might be relevant to the dairy sector. Indeed, in the last decade a highly heat-resistant sporeformer has been reported to cause spoilage problems in UHT milk. The above-mentioned approach needed to be applied to determine the *Bacillus* species responsible. In

1996 the predominant thermoresistant spoiling sporeformer was taxonomically described as a new species *Bacillus sporothermodurans*. Besides *B. sporothermodurans*, other highly heat resistant *Bacillus* species have been found to contaminate milk. Often these species cannot be identified (or classified if unknown species are implied) by the normal classical phenotypic methodology. PCR assays were developed for rapid and simple detection of *Bacillus sporothermodurans* (Herman *et al*., 1997; Scheldeman *et al*., 2002).

15.6.2 *Bacillus cereus*

Bacillus cereus is a foodborne pathogen and part of the *B. cereus* group including also *B. anthracis*, *B. mycoides* and *B. thuringiensis*. Most procedures for the isolation and enumeration of *B. cereus* involve direct plating on mannitol-egg yolk-polymyxin agar followed by a restricted number of confirmation tests. The members of the *B. cereus* group can be differentiated only on the basis of highly mutable characteristics. *B. anthracis* can be separated from *B. cereus* based on non-haemolytic activity and non-motility. For *B. mycoides*, rhizoid growth is the definitive characteristic. On the other hand, *B. thuringiensis* can be distinguished from *B. cereus* by its ability to produce an insecticidal crystal inclusion inside the cell during sporulation. As these definitive characteristics are often located in plasmids which are transferable, they become indistinguishable when strains lose plasmids. In fact, recent studies suggested that *B. cereus* and *B. thuringiensis* should be regarded as one species. For this reason, molecular methods are preferred for the specific identification of strains of the *B. cereus* group. In the case of differentiation of *B. cereus* from *B. thuringiensis* specific primers targeting the variable region of 16S rDNA or the *gyrB* genes can be used to design a discriminatory PCR reaction (Chen and Tsen, 2002).

Apart from the problem of identification there is also discussion concerning the risk that the species belonging to the *B. cereus* group pose for food safety. Only *B. cereus sensu stricto* is an established foodborne pathogen. From epidemiological studies it is known that *B. cereus* is one of the major causes of bacterial foodborne gastroenteritis but because of the acute but self-limiting character of the disease it is underreported. *B. cereus* is a Gram positive sporeformer which is often (in limited numbers) found in numerous pasteurized foods, e.g. pasteurized milk, ready-to-eat pasteurized meals, rice dishes and pastas. In the case of temperature abuse the pathogen grows rapidly to high numbers ($> 10^5$/g) and produces toxin. *B. cereus* is the most important microbiological hazard in these types of food. Nevertheless, *B. thuringiensis* strains have also recently been found to produce enterotoxin profiles similar to those of *B. cereus* and may be present in pasteurized vegetable purées. It is thus recommended to determine, apart from the identity of the strain, also the toxic potential of the isolate. Standard procedures to detect the toxic potential are cytotoxicity studies (cell tissue cultures). However, these procedures are laborious and demand a dedicated infrastructure. Furthermore, the demon-

stration of the toxic potential uses either PCR detection or an ELISA kit, but these are focused on the diarrheal toxins and not available for the emetic toxin (Batt, 2000; Beattie and Williams, 2000). PCR detection is simple and easy to perform, but only indicates the presence of the toxin genes in the *B. cereus* strains without revealing any information regarding the expression of these genes during growth.

15.7 Applications: identifying the genus *Campylobacter*

The *Campylobacteriaceae* comprises a group of Gram negative, non-saccharolytic bacteria with microaerobic growth requirements and a low GC content. A slender spiral 'corkscrew-like' morphology is typical. However, it has been documented for the thermophilic campylobacters (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) that they readily lose this morphology upon aging and cells incubated longer than 24 h will transform into bent rods or straight rods and finally become virtually completely coccoïd and non-culturable (Vandamme *et al*., 1996).

The genus *Campylobacter* initially included a wide assembly of species which were characterized by their biochemical inertness. Indeed, classical phenotypic tests routinely used for the identification of clinically significant campylobacters often yield negative results or yield variable results within species and do not allow a reliable differentiation of the species, especially for phenotypic aberrant strains. The biggest problems are found in the identification of *C. jejuni* and *C. coli*. The single discriminatory test is hippurate hydrolysis. Strains which were identified biochemically as *C. coli* (hippurate hydrolysis negative) have been shown by PCR to possess the hippuricase gene unique to *C. jejuni*. Detection of the hippuricase gene by PCR provides a more useful test for confirmatory identification of *C. jejuni*. Also problems are encountered for objective evaluation of the sensitivity to cephalothin and nalidixic acid. Reliable identification of *Campylobacter* spp. should be supplemented with a molecular method (Steinhauserova *et al*., 2001; Engvall *et al*., 2002). Sequence comparison of 16S rRNA gene sequences revealed a considerable genotypic heterogeneity among the species of the initial *Campylobacter* genus described, and three major clusters (rRNA homology groups) were recognized. Subsequently, the initial genus *Campylobacter* was divided into three distinct groups, each corresponding to a separate genus: *Campylobacter*, *Arcobacter* and *Helicobacter.* Information derived from 16S or 23S rDNA sequencing has been used successfully to design species- or genus-specific primers and probes. Applied in a PCR assay these primers and probes offer valuable alternatives for the identification of these bacteria belonging to the family of the *Campylobacteriaceae* (Vandamme *et al*., 1996).

Whole-cell protein analysis by SDS-PAGE has proved to be a valuable tool to identify unusual or atypical campylobacters. As with other bacteria, strains with highly similar protein patterns share high DNA hybridization values and therefore belong to the same species. The suitability of cellular fatty acid

analysis for the differentiation of the species is genus dependent; poor discriminatory power was found in *Campylobacter* species, whereas most *Arcobacter* species were easily differentiated (Vandamme *et al*., 1996).

15.8 Detecting virulence factors in foodborne pathogenic bacteria

15.8.1 Pathogenic *Escherichia coli*

Escherichia coli is a commensal bacterium in the human and animal gastrointestinal tract. However, some *E. coli* strains show pathogenic potential because of the acquisition of virulence factors. These are known as pathogenic *E. coli* (EC) and there are six major groups: enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteroaggregative (EAEC), and diffusely adherent (DAEC) *E. coli*. Like typical *E. coli,* strains in most pathogenic groups ferment lactose and are not affected by the elevated incubation temperature of 44ºC normally applied for *E. coli*. Hence, they can still be distinguished from other enteric bacteria using the standard methods for *E. coli*. Exceptions, however, are EIEC, which do not ferment lactose, and EHEC O157, which can be inhibited by the elevated incubation temperature (Feng, 2001).

E. coli O157 is an example of an organism that probably became virulent through the acquisition of a novel genetic element. Over time, genetic changes take place in a population at a regular rate leading to genetic diversity among specific cells in the population. Many types of genetic variability have been documented for *E. coli* by molecular typing methods. There have been claims that some *E. coli* strains are significantly more acid tolerant (McClure and Hall, 2000). This may be clinically important, since the capability to overcome the acid barrier of the stomach is facilitated. The first outbreak of infections caused by *E. coli* O157 was observed in 1982. This *E. coli* lineage is probably an example of a strain that acquired a Shiga-like toxin gene from an enteric organism that normally harbours this toxin (such as *Shigella dysenteriae*) as a secondary virulence factor, possibly through bacteriophage transduction into an already cytoadherent type of *E. coli*. Gene sequence comparisons have illustrated that insertion of foreign DNA into the *E. coli* chromosome occurs regularly (Finlay and Falkow, 1997; Van Belkum *et al*., 2001).

Some type strains in these pathogenic *E. coli* groups, such as O157:H7, are widely recognized by their serotype, but mainly the pathogenic *E. coli* group are recognized solely on the basis of their pathogenicity and no unique test is available to differentiate pathogenic *E. coli* from normal intestinal *E. coli*. The detection of pathogenic *E. coli* clearly demands the combination of classical phenotypic methods for isolation and identification of the *E. coli* species and molecular-based methods for the demonstration of potential pathogenicity of the *E. coli* strain. There appears to be a clear association between the presence or absence of certain genetic elements and bacterial virulence. DNA-based

methods for analysis of their virulence characteristics is required for definitive identification of each group of pathogenic *E. coli*. In the case of *E. coli* O157:H7, the most notorious foodborne pathogenic *E. coli*, the search for virulence factors includes the detection of the *stx*-genes (encoding cytotoxins known as Shiga-like toxins or verotoxins), the chromosomal *eaeA*-gene (encoding the intimin protein involved in attachment) and the *ehxA* gene (encoding for enterohemolysin). At present virulence factors are detected either by colony hybridization studies with specific probes or PCR tests with specific primer pairs. Recently DNA chips were introduced to map virulence gene patterns of pathogenic *E. coli* (Call *et al*., 2001; Chizhikov *et al*., 2001). As an alternative cytotoxicity for cells of tissue cultures can be determined or immunological detection of the Shiga-like toxins can be performed.

15.8.2 Pathogenic *Yersinia enterocolitica*, *Vibrio parahaemolyticus* and *Listeria monocytogenes*

In food microbiology numerous taxonomic species are indicated as foodborne pathogens although only a limited number of strains, carrying virulence factors have the potential to cause infections. As well as *E. coli*, as mentioned above, some commonly known foodborne pathogenic species also include non-virulent as well as virulent strains, e.g. *Yersinia enterocolitica, Vibrio parahaemolyticus* or *Listeria monocytogenes*. The practice of judging food, based on total counts or detection of the taxonomic species as such without consideration of the virulence factors being present, is not always appropriate. As mentioned above virulence genes can be detected by DNA-based methods. However, if virulence factors are associated with phenotypic traits testing these can also be included as confirmation tests in the classical procedure. Historically, this has been included in some of the classical pathogen detection schemes, although at the time of their introduction the association between the phenotypic characteristic and the responsible virulence gene was not established.

For *Yersinia enterocolitica* the initial event subsequent to infection is invasion of intestinal epithelial cells. This is connected with the presence of chromosomal genes termed the *inv* and *ail* loci in *Y. enterocolitica*. In addition, the presence of plasmid-encoding determinants play a major role in the overall virulence of pathogenic *Yersinia*. All pathogenic strains of *Y. enterocolitica* possess a 70 kbp plasmid carrying essential virulence genes, e.g. genes for the temperature inducible *Yersinia* outer membrane proteins (yop), and the gene for the adhesin (YadA). The virulence plasmid is well conserved among the pathogenic species; however, loss of the plasmid results in the loss of pathogenicity. Virulence of *Y. enterocolitica* can be determined by detection of the above-mentioned virulence genes and can be linked to phenotypic classical tests such as Congo red binding (Bhaduri, 2001).

A thermostable direct haemolysin (tdh) and tdh-related haemolysin (trh) are considered to be the major virulence factors of *Vibrio parahaemolyticus* and respectively encoded by *tdh* gene and *trh* gene. They are very common in

clinical strains and less so in environmental strains – estimated to be present in approximately 1 per cent of environmental isolates and up to 99 per cent of clinical isolates. Presence of tdh is correlated with the Kanagawa reaction on agar containing fresh human red blood cells, known as Wagatsuma agar (production of tdh causes beta-hemolysis). The presence of the trh correlated with a positive urease test (Tamplin, 2001).

Not all strains of *L. monocytogenes* are pathogenic. Wiedmann *et al*. (1997) characterized 133 isolates of *L. monocytogenes* according to ribotype and virulence gene analysis. These authors found that *L. monocytogenes* strains could be clustered into three distinct lineages. Within lineage 1 resided all strains isolated during outbreaks of listeriosis. In contrast, lineage 3 contained no human isolates, indicating that strains in this grouping may have reduced virulence. The production of sulfhydryl-activated hemolysin, listeriolysin O (encoded by the *hlyA* gene), is associated with the pathogenic potential of *L. monocytogenes*. The loss of hemolysin production was shown to be associated with loss of virulence in a mouse model.

An extracellular protein (p60) may be involved in the process of attachment and invasion of *L. monocytogenes* (encoded by the *iap* gene) and has been shown to be produced by all virulent *L. monocytogenes* strains (Donnelly, 2001). The potential use of fragments of these genes as a *L. monocytogenes* species-specific probe was demonstrated, and a multiple PCR assay was developed for rapid detection of *L. monocytogenes* targeting the *hlyA* or *iap* gene (Scheu *et al*., 1998).

15.9 Future trends

In the last ten years spectacular developments have been made in the field of sequencing of rRNA and genes coding for rRNA (rDNA). Sequence analysis of rRNA has become a rapid standard technique and the sequences generated have a very low error rate. These techniques revolutionized the insights in the phylogeny and taxonomy of bacteria and led to reclassification of numerous bacteria (Stackebrandt, 2002a). The molecular approach has facilitated classification and subsequently identification of unknown organisms and speeded up comprehension of the complex bacterial relations. But it has also raised questions about the approach to classification and subsequently identification using morphological, biochemical and physiological characteristics. However, although it is generally accepted that bacterial classification should reflect as closely as possible the natural relationships between bacteria as determined by their genetic relatedness, a taxon should be characterized by a certain degree of phenotypic consistency. Although genomic and chemotaxonomic methods may allow on some occassions a more accurate identification than the classical phenotypic identification tests, there is a need for phenotypic characterization of the isolates as well to evaluate their physiological and ecological functions.

It is interesting that increased automation and democratization of these advanced chemotaxonomic and molecular-based techniques facilitated access to these methodologies for identification purposes. Classical phenotypic methods will remain internationally the reference methods and first approach in identification of foodborne microorganisms. Nevertheless several of the methods mentioned here may be valuable tools for reliable identification particularly of genus or species with a difficult taxonomic position. They represent a list of methods which can provide information on the unknown isolate on a different level. Whereas classical identification tests supply information on a wide range of expressed features, chemotaxonomic and genomic methods supply information on DNA base composition and heterogeneity, RNA sequence, protein or lipid composition. These advanced techniques should be regarded as complementary to the current classical phenotypic identification schemes in use. Primarily, it is of interest to understand at which level these methods carry information, to realize their technical complexity and to know whether it is applicable to a particular taxon (Vandamme *et al*., 1996). For all of these techniques it is necessary to obtain a pure culture. This requires preliminary isolation steps (sample preparation techniques and manipulation of culture media) according to a rationale which is primarily defined by the food matrix and the level and type of microorganism under consideration and needs the expertise of a food microbiologist.

It is expected that these advanced techniques will be introduced increasingly in food microbiological laboratories. PCR methods and DNA-based typing methods are already accepted as rapid, simple and reliable methods for identification and typing of foodborne pathogens and rDNA sequencing has proven to be useful in identification of problematic isolates. It is of increasing importance to use a multidisciplinary approach in the identification of foodborne microorganisms in order to acquire a reliable and accurate identification. However, interpretation and application of these advanced methods should be supported by classical phenotypic characteristics and sustained ecologically.

15.10 Sources of further information and advice

For more details on systematics and defining a taxonomic rank the reader is referred to *Bergey's Manual of Systematic Bacteriology Volume 2* (Sneath P H A, Mair N S, Sharpe M E, Holt J G, eds), Williams & Wilkins, Baltimore, and *The Prokaryotes* (Dworkin M, (ed.)), Springer-Verlag, New York (www.prokaryotes.com). The concept of polyphasic taxonomy and overview of the multiple techniques which can be used for classification and/or identification of bacteria is explained by Vandamme *et al*. (1996). More details on molecular detection and typing of foodborne bacterial pathogens can be found in a review by Heyndrickx *et al*. (2001).

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