Microbial Identification in the Pharmaceutical Industry

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ABSTRACT A review of the microbial identification methods that are available to support compendial testing was undertaken to determine the state of the art within the pharmaceutical industry and to stimulate the USP revision process. Emphasis was given to the preliminary screening of microbial isolates for cellular morphology, staining, and diagnostic biochemical reactions to either characterize the microorganisms or support decisions for using different microbial identification schema and the rapid microbial identification methods that are available. The relative advantages of phenotypic and genotypic microbial identification methods are discussed. Due to the complexity of the topic, the authors believe that treatment of alternative microbial identification systems warrants a guidance chapter separate from the proposed General Chapter ⟨1227⟩ *Validation of Alternative Microbiological Methods*.

INTRODUCTION

Microorganisms found in pharmaceutical ingredients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished products are frequently identified to assist in product investigations. This is especially common if their numbers exceed alert and/or action levels for the material or process environment tested. Routine monitoring might include characterization by colony morphology, cellular morphology (rod, cocci, etc.), gram reaction, and key enzyme activities. This information may be sufficient to confirm that the bacteria found in the material are typical for that material or process environment or indicative of no change in the level of environmental control in an aseptic processing area. However, there may be a need for more precise identification methods during an investigation to assign a species name to the microorganism. Microbiological identification systems are based on different analytical techniques, and each has restrictions due to method and/or database limitations and each has inherent shortcomings in terms of accuracy, reproducibility, technical complexity, rapidity, and cost. A decision must be made regarding the appropriate technology to use in the routine pharmaceutical microbiological testing laboratory with these limits in mind as well as a thought to the need for the level of identification (genus, species, strain) needed for the particular situation.

REGULATORY GUIDANCE ON MICROBIAL IDENTIFICATION

What is the status of microbial identification in the compendia and pharmaceutical regulations? There is no USP chapter that specifically addresses microbial identification. However, the need for microbial identification is specifically cited in USP General Chapter ⟨61⟩ *Microbial Limit Tests*, where it is recommended that microorganisms demonstrating characteristic cellular and colonial morphology on selective and/or diagnostic agar media in the absence of specified microorganisms tests be confirmed, if necessary, by other suitable cultural and biochemical tests.

The proposed *Microbial Limit Tests* harmonization document (*1*) does not include this specific text, but it does allow for the use of alternative microbiological tests. The Australian Therapeutic Goods Administration (TGA) recommends testing for the absence of all pseudomonads in topical preparations (*2*) but does not provide specific means for identification. FDA, in their 1993 *Guide to Inspections of Microbiological Pharmaceutical Quality Control Laboratories*, references the FDA *Bacteriological Analytical Manual* (*3*) for microbial identification techniques in reviewing microbial limits tests that relies heavily on selective and differential media.

USP General Chapter ⟨71⟩ *Sterility Tests* (harmonized with the JP and Ph. Eur. January 1, 2004) allows for invalidation of the test if "after determination of the identity of the microorganisms isolated from the test, the growth of this species (or these species) may be ascribed unequivocally to faults with respect to the material and or the technique used in conducting the sterility test procedure." The TGA document contains similar wording (*4*) but has gone on record that this identification must be performed at the level of DNA (*5*). FDA has also argued that invalidation of a sterility test requires identification of microorganisms at the DNA level in its draft aseptic processing guideline (*6*).

The manufacturing area is also a concern for microbial identification. USP informational chapter ⟨1116⟩ *Microbiological Evaluation of Clean Rooms and Other Controlled Environments* recommends that microbial isolates be identified at an appropriate level to support the environmental monitoring program. The recent FDA draft guidance document on aseptic processing (*6*) recommends the use of genotypic microbial identification methods because of the

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increased accuracy and precision of these methods. The authors believe that this statement should be critically reviewed.

There is obviously a great deal of interest in microbial identification but little guidance about precisely how to conduct it. Although the draft USP informational chapter ⟨1223⟩ *Validation of Alternative Microbiological Methods* provides some guidance (*7*), the treatment is not complete. There is very little guidance about precisely how to qualify different methods of microbial identification, and there are many methods to choose from.

CHARACTERIZATION OF BACTERIA IN THE PHARMACEUTICAL ENVIRONMENT

Historic Background

The pioneer in the classification of bacteria was the German biologist Cohn, who first suggested in 1870 the division of bacteria into groups based on their cellular morphology as determined by light microscopy. At the time it was a revolutionary idea that there are many different species of bacteria. Cohn recognized six genera without the advantages of pure culture and staining techniques, emphasizing that bacteria occur with different species. They were *Micrococcus* (ball or egg-shaped), *Bacterium* (short, rod-like), *Bacillus* (straight, fiber-like), *Vibrio* (wavy, curl-like), *Spirillum* (short, screw-like), and *Spirochete* (long, flexible, spiral).

In 1884, the Danish microbiologist Christian Gram developed the differential staining of bacteria that is universally used to classify them as negatively or positively reactive to what is now called the Gram's stain. The established system of bacterial classification based on colony morphology and color, cellular morphology, differential staining, motility, physiology, biochemical reactions, and substrate utilization used by American microbiologists was systematized in the 1923 Bergey's *Manual of Determinative Bacteriology*. The routine identification methods employed in the food, clinical, and microbiology laboratory continue to be based on the determination of the morphology, differential staining, and physiology of a microbial isolate by means of miniaturized and automated substrate utilization screening methods to speciate the isolate, e.g., API, Vitek, and Biolog microbial identification systems (*8–10*).

MICROBIAL ISOLATION

Microorganisms are present in a variety of milieux in the pharmaceutical manufacturing environment. The first step to identification is to isolate a pure colony for analysis. This purification is normally accomplished by subculturing one or more times on solid media to ensure purity, each time streaking for single colonies. This technique also allows full phenotypic expression and growth of sufficient inoculum for the identification.

It should be recognized that expressions of the microbial phenotype, i.e., cell size and shape, sporulation, cellular composition, antigenicity, biochemical activity, sensitivity to antimicrobial agents, etc. frequently depend on the media and growth conditions (*Table 1*). These conditions will include the better-known variables such as temperature, pH, redox potential, and osmolality and lesser-known variables such as nutrient depletion, vitamin and mineral availability, growth cycle, water activity of solid media, static or rotatory liquid culture, and solid versus liquid media culture, as well as colony density on the plate.

Table 1. Phenotypic characteristics that may be employed in microbial taxonomy

Categories	Characteristics
Cultural	Colony morphology, colony color and size, and pigment production
Morphological	Cellular morphology, cell size, flagella type, reserve material, gram reaction and spore, and acid-fast staining.
Physiological	Oxygen tolerance, pH range, temperature optimum and range, and salinity tolerance
Biochemical	Carbon utilization, carbohydrate oxidation or fermentation, and enzyme patterns
Inhibition	Bile salt-tolerance, antibiotic susceptibility, and dye tolerance
Serological	Agglutination
Chemo-Taxonomic	Fatty acid profile, microbial toxins, and whole cell composition

In contrast, the microbial genotype is highly conserved and is independent of the culture conditions, so the identifications may be conducted on uncultured test material—primary enrichments that increase the amount of nucleic acid available for analysis, the primary isolation cultures from microbial limit testing (⟨61⟩), or environmental monitoring plates. For example, a recent publication (*11*) confirmed the stability of repetitive-sequence PCR patterns of the bacteria *E. coli, P. aeruginosa, E. faecalis, S. epidermidis,* and *A. baumannii* with respect to both the age of the culture and 5, 10, and 15 subcultures. *Table 2* lists a number of genotypic characteristics that may be determined.

Table 2. Genotypic/phylogenic characteristics that may be employed in microbial taxonomy

Categories	Characteristics
Genotypic	DNA base ratio (G + C content), restriction fragment patterns, and DNA probes
Phylogenic	DNA–DNA hybridization, and 16S and 23S rRNA codon sequences

PRELIMINARY SCREENING OF MICROBIAL ISOLATES

Microorganisms isolated from pharmaceutical ingredients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished products on compendial media in all likelihood will be physiologically stressed. The microorganisms will go from a metabolic status suitable for slow or no growth for survival under adverse conditions to rapid growth under laboratory culture conditions. This transition can be managed by careful handling of the isolates. Individual representative colonies from the primary isolation media are streaked onto solid media as described above in preparation for identification. The first step is to determine the gram reaction and cellular morphology of the bacteria isolates. This is a critical step for phenotypic identification schemes. If the wrong gram reaction and/or cellular morphology are assigned to an isolate, subsequent testing may be conducted using the wrong microbial identification kit, resulting in an incorrect or nonsensical result. Several common preliminary screening tests are described below.

Gram Staining

Gram staining methods employed include the four-step method: Crystal violet (primary stain); iodine (mordant); alcohol (decolorizer); safranin (counter stain) or the three-step method in which the decolorization and counter-staining step are combined. Done correctly, Gram-positive organisms retain the crystal violet stain and appear blue; Gram-negative organisms lose the crystal violet stain and contain only the counter-stain safranin and thus appear red. Common pitfalls in this method are that heat fixation may cause Gram-positive cells to stain Gram-negative and older cultures may give Gram-variable reaction; using too much decolorizer could result in a false Gram-negative result and not using enough decolorizer may yield a false Gram-positive result. One variation that has advantages in some situations is to perform a methanol fix of the bacterial smear on the microscope slide; this may be preferable to a heat fix for consistent results.

Because the Gram-staining reaction must be read under a microscope, this method provides two pieces of information: the Gram-staining characteristics and the cell morphology (i.e., rods or cocci, single cells or chains or clumps or clusters, etc.). The use of a semi-automated cell-staining device such as the Aerospray Gram-staining instrument (Wescor, Inc., Salt Lake City, UT) to give more consistent results is recommended.

Spore Staining

Spore staining methods employed include a two-step method: malachite green (primary stain) and safranin (counter stain). The fixed bacterial smear is stained with 7.6% aqueous malachite green solution for 10 minutes, rinsed, and counter stained with a 0.25% aqueous safranin solution for 15 seconds, rinsed, and blotted dry. This procedure stains the spores green, but the rest of the bacterial cell is stained red.

Biochemical Screening

Key biochemical screening tests are the oxidase test to separate Gram-negative rod-shaped bacteria into nonfermenters (oxidase positive) and enteric (oxidase negative) bacteria, the catalase test to separate *Staphylococci* (catalase positive) from *Streptococci* (catalase negative), and the coagulase test to separate *staphylococci* into coagulase negative (presumptively nonpathogenic) and coagulase positive (more likely pathogenic) *staphylococci.*

For many types of investigations, these few tests may provide enough information to trend data. However, there are much more definitive bacterial identification schemes or systems available to the pharmaceutical microbiologist.

MICROBIAL IDENTIFICATION BY PHENOTYPIC METHODS

Phenotypic methods utilize expressed gene products to distinguish among different microorganisms. Generally, these require a large number of cells in pure culture. Disadvantages of culture methods for microbial enumeration and identification are well known and include the long incubation times, the inability of most environmental microorganisms to grow on artificial media, the specific growth requirements of many microorganisms, the unintentional selectiveness of culture methods, and the need to fully express phenotypic properties of recently isolated stressed microorganisms by subculture from primary isolation, selective, or diagnostic media prior to microbial identification. However, the carbon utilization and biochemical reaction patterns for microbial species in the database for a microbiological identification system are always based on inocula development for that identification system on specified culture media and incubation conditions to achieve consistency of identification. Despite these limitations, phenotypic microbial identification methods are successfully employed in the routine food, water, clinical, and pharmaceutical microbiological testing laboratory. An excellent overview of manual and automated systems for the detection and identification of microorganisms may be found in the 8th Edition of the *ASM Manual of Clinical Microbiology* (*12*).

Carbon Utilization and Biochemical Reaction Methods

A compendial example of this approach is ⟨61⟩ *Microbial Limit Tests,* which utilizes general and selective enrichment broth and diagnostic solid media to demonstrate the absence of specified microorganisms. These methods can be conducted in any microbiology laboratory.

There are several microbial identification kits based on carbon utilization and biochemical reactions that are readily available. The API strip takes what originally required racks of tubes and reduces them in size and complexity to several

small, prepackaged strips. The Vitek Microbial Identification System takes this concept a step further, miniaturizing the reaction tubes in cards and coupling the assay with automated incubation and reading. However, these traditional biochemical methods are not the only phenotypic test methods available to the pharmaceutical microbiologist.

The Biolog Microbial Identification System has an identification scheme based on carbohydrate utilization. A 96-well microtiter plate holds a variety of media containing specified carbohydrates and a redox indicator. If growth occurs in a particular well, the reduced conditions within that well result in the redox dye tetrazolium turning dark, signifying catabolism of the substrate. This reaction is easily read manually or by a photometric plate reader, and the result is a semi-automated identification system that complements the traditional methods.

In addition to the carbohydrate utilization method, Biolog has also developed a "Phenotype MicroArrays" system that utilizes the same basic technology but can monitor, either directly or indirectly, a large number of different aspects of cell function. The range of phenotypes monitored includes cell-surface structure and transport functions, catabolism of carbon, nitrogen, phosphorus, and sulfur compounds, biosynthesis of small molecules, cellular respiratory functions, and finally, stress and repair functions. This offers the small lab an opportunity to delve into greater detail during an investigation and to provide finer differentiation among isolates.

In addition to the phenotypic methods used to identify microorganisms based on their gram reaction, cellular morphology, carbon utilization patterns, and biochemical reactions are methods based on the cellular composition of the microorganisms grown on specified media. These include the MIDI (gas–liquid chromatographic fatty acid ester analysis) Microbial Identification System and the MicroMass MALDI TOF mass spectrometry with the MicrobeLynx pattern recognition software.

Fatty Acid Analysis

When bacteria are grown on defined and reproducible media, the fatty acid composition of their membranes is consistently expressed as a phenotypic characteristic. The MIDI Microbiological Identification System utilizes this characteristic. Fatty acids are extracted from cell cultures and esterified, and the patterns of fatty acid esters are then determined using gas–liquid chromatography. This system then identifies microorganisms based on the unique fatty acid pattern of each strain. The system uses gas chromatographs, proprietary microbial databases, and pattern recognition software to identify each strain.

Mass Spectrometric Methods

Less well known than the fatty acid analysis (and far less utilized), MALDI TOF mass spectrometry has promise as a technique for the rapid identification of microorganisms. Whole bacterial cell analysis has yielded unique mass spectra from charged macromolecules from common species of bacteria. The absence of sample preparation, coupled with rapid analysis and high throughput make the technology attractive as a rapid microbial identification method. The method involves applying the bacterial cultures to the instrument plate wells, overlying the whole cells with a solvent matrix of alpha-cyano-4-hydroxycinnamic acid for Gram-negative bacteria and 5-chloro-2-mercaptobenzothiazole for Gram-positive bacteria. The wells are then bombarded with a nitrogen laser that causes desorption of ionized cellular components that travel down a tube toward a detector. The time for the charged components to reach the detector operated in a positive ion detection mode using an acceleration voltage of +15 kV is a function of their kinetic energy, i.e., mass and charge. The detector signal is captured as a unique fingerprint for different species/stains of microorganism in the acquisition mass range of 500 to 10,000 Daltons. The MicroMass MALDI TOF mass spectrometry—MicrobeLynx database currently has approximately 3500 spectral entries covering more than 100 genera and more than 400 different species. The time to process a sample is on the order of 3 minutes, so the equipment could run at least 100 microbial identifications a day.

Survey of Phenotypic Methods

Representative phenotypic microbial identification systems that are widely used in the pharmaceutical industry are listed in *Tables 3* and *4*. With the substrate utilization methods, the incubation time may be shortened from overnight to 2–4 hours by using higher inoculum levels.

Table 3. Representative phenotypic microbial identification methods for members of the family *Enterobacteriacae*

Product	Manufacturer	Method	Incubation Time
API 20E	bioMérieux, Durham, NC	Substrate utilization and biochemical reactions	Overnight
BBL Crystal Enteric/ Nonfermenter	BD Diagnostics, Sparks, DE	As above	4 hours
GN Microplate	Biolog, Hayward, CA	As above	As above
Vitek GNI Plus	bioMérieux, Durham, NC	As above	2 hours and/or overnight

Table 3. Representative phenotypic microbial identification methods for members of the family *Enterobacteriacae* (Continued)

Product	Manufacturer	Method	Incubation Time
MIDI Sherlock	MIDI, Newark, DE	Fatty acid ester analysis	Overnight
MALDI TOF Mass Spectrometry with MicrobeLynx software	Waters Corp., Milford, MA	Spectral analysis of ionized cellular components	3 minutes

Table 4. Representative phenotypic microbial identification methods for *Staphylococcus* spp. and other Gram-positive cocci

Product	Manufacturer	Method	Incubation Time
API Staph	bioMérieux, Durham, NC	Substrate utilization and biochemical reactions	Overnight
BBL Crystal Gram-Positive ID System	BD Diagnostics, Sparks, DE	As above	4 hours
Vitek GPI	bioMérieux, Durham, NC	As above	2–15 hours
Biolog GP	Biolog, Hayward, CA	As above	Overnight
MIDI Sherlock	MIDI, Newark, DE	Fatty acid ester analysis	Overnight

NUCLEIC ACID-BASED (GENOTYPIC) MICROBIOLOGICAL METHODS

Historic Background

In 1953, Watson and Crick reported the chemical structure and the base pairing of the DNA molecule. Their Nobel prize-winning publication in the British scientific journal *Nature* was the beginning of the new field of molecular biology that revolutionized biology and medicine.

An important milestone in the development of molecular biology-based microbiological methods was the discovery by Kornberg in 1958 of the enzyme DNA polymerase, which is responsible for the replication of nucleic acid. This was followed by the use of DNA–DNA hybridization to show the DNA relatedness between enteric bacteria in 1969, as well as the discovery of restriction enzymes to cleave DNA into analyzable fragments (the basis of ribotyping) that same year. The first use of ribosomal 16S RNA gene homology in bacterial taxonomy was described by Woese in 1977, and that same year DNA sequencing was used in taxomony by Sanger. Eight years passed before polymerase chain reaction (PCR) was developed as a tool to rapidly amplify a DNA segment (Mullis in 1985), and the first complete DNA sequencing of the genome of a bacterium, *Haemophilis influenzae,* was achieved ten years later by Venter and his team in 1995. Additional information on these milestones may be found in the American Society for Microbiology website article titled "Significant Events of the Last 125 Years" (*13*).

By the end of 1999 there were 4313 validly named prokaryotic species and 849 validly named genera of Bacteria and Archaea reported in the second edition of Bergey's *Manual of Systematic Bacteriology* (*14*) based on 16S rRNA DNA sequence analysis. The phylogenetic organization, i.e., DNA relatedness at the species level of the manual is notable with references to the information contained in the species descriptions. The revolution in microbial taxonomy started by Woese in 1977 would seem to be complete.

The key to the whole field of nucleic acid-based identification of microorganisms was the introduction of the concept of molecular systematics using proteins and nucleic acids in 1965 by the American Nobel laureate, Linus Pauling. Because the sequence of nucleic acids in a particular microorganism is extremely conserved or constant, even over geological time, and the DNA and RNA molecules are relatively stable, they are excellent materials for the detection and identification of microorganisms. However, this is complicated by the fact that a single bacterial cell weighs 10^{-13} g and the nucleic acid sequences that are amplified by Polymerase Chain Reaction (PCR) may represent 100 to 100,000 times less than the weight of the bacterial cell. To analyze the nucleic acid from a microorganism one needs to culture the organism and/or amplify the nucleic acid.

More details of the historic development of microbiological methods can be found in a chapter by one of the authors in the book *Rapid Microbial Methods in the Pharmaceutical Industry* (*14*).

Absence of Specified Microorganism Screening

To detect with a high probability a single *Salmonella* cell in 10 g of a pharmaceutical product as required by absence of specified microorganism screening, an enrichment culture would need to obtain at least 10^3 cells per mL. The cells would be lyzed, the nucleic acid extracted, and a thermostable polymerase used to copy a nucleic acid strand using oligonucleotide primers specific for *Salmonella.* This is achieved using a set of cycling temperatures to denature the nucleic acid, hybridize it with the primers, and use polymerase-mediated complementary-strand synthesis to produce more nucleic acid. Twenty to thirty thermocycles can increase the amount of target nucleic acid over a million times so it can be detected by its electrophoretic pattern after treatment with reverse transcriptase, reaction with chemiluminescent DNA probes, or rRNA base sequence. This technology has been commercialized for the detection of *E. coli* O157:H7, *Listeria monocytogenes,* and *Salmonella* spp. by Qualicon as the BAX Microbial Detection System; for the detection of *E. coli* O157:H7 by Applied Biosystems as the TaqMan Pathogenic *E. coli* Detection System; and for *Salmonella* spp. as the Roche LightCycler PCR Salmonella Detection System (see *Table 5*). With PCR technology it will be possible to develop a multiplex PCR system for the simultaneous detection of the USP indicator organisms *E. coli, S. aureus, P. aeruginosa,* and *Salmonella* spp. for absence of specified microorganism testing to meet microbial limits requirements for nonsterile pharmaceutical drug products as indicated in specific monographs.

BAX Microbial Identification System

The BAX Microbial Detection System takes microbial enrichments according to standard protocols for the type of food to be examined. A heated lysis reagent solution ruptures the bacterial cell wall, thereby releasing the DNA that is added to a lyophilized PCR reagent, and the DNA is amplified in the thermocycler/detection instrumentation.

Ribotyping

With the RiboPrinter Microbial Characterization System marketed by duPont Qualicon, DNA is extracted from the bacterial cell lysate of pure colonies picked from an agar plate; the cells are lyzed, and the DNA is cut into fragments by a restriction enzyme. The DNA fragments are separated by molecular size and charge using gel electrophoresis and are transferred to a membrane where they are hybridized with a labeled DNA probe. A chemiluminescent agent is added, and the emission of light is captured by a digitizing camera. The RiboPrint is compared to other patterns in a database, and the bacterium is identified or a stain comparison is made from the ribotyping patterns. The RiboPrinter has the capacity to identify on the order of 20 organisms daily.

Bacterial Barcodes

Bacterial Barcodes developed a repetitive-sequence and PCR-based DNA fingerprinting (rep-PCR) technology that is combined with the Agilent 2100 Bioanalyzer and Caliper Technologies LabChip and the DiversiLab software to identify a range of bacterial stains of clinical significance. The rep-PCR technology is based on noncoding repetitive sequences that are found interspersed through bacterial DNA. When PCR is performed using primers complementary to these repetitive sequences, the DNA sequences lying between the repetitive sequences are amplified. These amplified sequences were initially separated by electrophoresis. The stained gel contains a banding pattern or barcode that is distinctive for the bacterial stain. Computer-based analyses of the digitized images of the banding generated dendrograms demonstrating the relatedness of the stains. The Bioanalyzer processes the LabChips DNA chips, replacing the standard gel electrophoresis with automated lab-on-a-chip microfluids. These components are capable of preparing samples, capturing DNA fingerprints, and analyzing the results within 5 hours.

Ribosomal RNA Base Sequencing

The MicroSeq 500 16S rDNA Bacterial Sequencing Kit marketed by Applied Biosystems allows bacterial identification using the DNA sequence of the first 500 base pairs of the bacterium's 16S rRNA gene, and the full-gene kit enables full characterization of the entire 16S rRNA gene of the bacterium. The MicroSeq Bacterial Sequencing Kits have been validated for use on PCR thermal cyclers from Applied Biosystems. The nucleic acid is extracted from a loop of a bacterial colony and amplified. The amplification products are run on the capillary electrophoresis-based ABI PRISM 310 Genetic Analyzer for maximum automation or on the slab gel-based ABI PRISM 377 DNA Sequencer for maximum throughput. After sequencing, the MicroSeq system identifies a bacterium or its closest genetic relatives through dedicated software that compares the gene sequence of the sample against the MicroSeq 16S rDNA Database. This database includes the 16S rDNA gene sequences of more than 1200 known organisms, and the microbial identification daily throughput ranges from 8 to 128, depending on the sequencing instrumentation configuration.

PCR Kit for the Detection of *Salmonella* in Food

The LightCycler Salmonella Detection Kit (RocheDiagnostics) was developed to detect low levels of *Salmonella* in food. Using sequence-specific primers in a PCR, the LightCycler amplifies and detects in real time, employing fluorescence hybridization probes, a fragment of the *Salmonella* DNA derived from an enrichment culture. This procedure, including PCR set and 36- to 48-cycle run, can be completed with 75 minutes with 30 samples included in the LightCycler run.

Table 5. Representative genotypic microbial identification methods

Product	Manufacturer	Method	Processing Time
RiboPrinter Microbial Characterization System	DuPont Qualicon, Wilmington, DE	Ribotyping, i.e., restrictive enzyme cleavage, electrophoretic separation, and chemiluminescent probes	8 hours
BAX Microbial Detection System	DuPont Qualicon, Wilmington, DE	Nucleic acid extraction, PCR amplification, and chemiluminescent probes	Overnight
MicroSeq 16S and 23S rRNA Gene Sequence Analysis	Applied Biosystems, Foster City,-CA	Nucleic acid extraction, PCR amplification, and rRNA base sequencing	2–4 hours
LightCycler PCR Salmonella Detection System	Roche Diagnostics, Indianapolis, IN	Nucleic acid extraction, PCR amplification, and chemiluminescent probes	1–2 hours

IDENTIFICATION OF FUNGI

Phenotypic Methods

Interest in fungal taxonomy has increased due the growing clinical importance of opportunistic fungal pathogens associated with immunocompromised patients due to the AIDS epidemic, leukemia, organ transplant, and invasive medical procedures. Fungi are classified and identified by their morphological features rather than their nutritional and biochemical differences. Fungal taxonomy is complicated by the existence of teleomorphs (sexual states) and anamorphs (asexual states) for the same fungus that develop at different times under different nutritional conditions, leading to dual species names (*16*). Clinical mycologists as well as pharmaceutical microbiologists are most familiar with Deuteromycetes (anamorphs) or, to use an older terminology, fungi imperfecti. Unfortunately, fungal taxonomy is technically difficult and is usually limited to mycological specialists. Although classical identification methods based on morphology are generally applied to fungal identification, carbon utilization and biochemical reaction patterns have been successfully used to identify yeast, e.g., API 20C and the Biolog FF System. These methods are suitable for the routine microbiological testing laboratory. *Table 6* presents a number of commercially available systems for phenotypic identification of fungi.

The Biolog Filamentous Fungi system employs redox chemistry similar to Biolog's other technology. Based on reduction of tetrazolium in response to metabolic activity, the reaction occurs in a 96-well plate that allows the analysis of fungal growth via turbidimetric means. There is an option for workers to add their own patterns of carbon usage produced by new cultures outside of the Biolog database.

Table 6. Representative microbial phenotypic identification methods for fungi

Product	Manufacturer	Method	Incubation Time
API 20C (Yeast)	bioMérieux, Durham, NC	Substrate utilization and biochemical reactions	24–48 hours
YT Microplate (Yeast)	Biolog, Hayward, CA	As above	As above
Vitek YBC (Yeast)	bioMérieux, Durham, NC	As above	As above
Biolog FF System (Filamentous Fungi)	Biolog, Hayward, CA	As above	4–14 days
Microbial Identification System (MIS)	MIDI, Newark, DE	Fatty acid ester analysis	24 hours to 7 days

Genotypic Methods

rRNA gene sequencing and Ribotyping may also be applied to fungal identification. For example, recent studies using MicroSeq D2 ribosomal DNA sequencing to identify molds and yeasts found in a clinical setting determined that 58 and 93.9% of the molds and yeasts, respectively, that could be identified with those isolates were neither identified nor included in the MicroSeq or API microbial identification system libraries (*17* and *18*). Clearly the fungal MicroSeq library is inadequate for mold identification.

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THE RELATIVE MERITS OF PHENOTYPIC AND GENOTYPIC MICROBIAL IDENTIFICATION METHODS

Application in Clinical Microbiology

Whereas rRNA gene sequencing has had a profound effect on microbial taxonomy, it may be asked what are the appropriate applications of nucleic acid-based methods to pharmaceutical microbiology? In clinical microbiology genotypic identification methods have been successfully applied to the identification of fastidious organisms, e.g., *Clamydia trachomatis* and *Neisseria gonorrhoeae,* slow-growing organisms, e.g., fungal pathogens, and *Mycobacterium tuberculosis,* rapid screening for pathogens e.g., group A Streptococcus confirmatory testing, and unculturable organisms, e.g., the Whipple's bacillus *Tropheryma whippelii* (*19*).

Because of medical reimbursement requirements, clinical microbiology is subject to greater cost controls than is pharmaceutical microbiology, and it is unlikely that rRNA gene sequencing will be a routine test method. However, in some instances the cost-effectiveness of the use of these methods can be demonstrated in better clinical outcomes, reduced hospital stays, or the prevention of future illness compared to conventional clinical microbiology testing. In contrast, it is difficult to justify genotypic identification methods for routine pathogen screening and identification, especially when the relatively few pathogens isolated from the screening of clinical samples that are usually sterile may be readily identified using phenotypic methods.

Genotypic Methods

Genotypic microbial identification methods based on nucleic acid analyses may be less subjective, less dependent on the culture method, and theoretically more reliable because nucleic acid sequences are highly conserved by microbial species. These methods would include DNA–DNA hybridization, PCR, 16S and 23S rRNA gene sequencing, and analytical ribotyping. However, these methods are more technically challenging for the pharmaceutical microbiologist and are more expensive in terms of both equipment and current testing costs, and they often rely on a technology marketed by a single company. Their current use may be better suited to critical microbiological investigations associated with direct product failure or with the identifications conducted in a specialized research-oriented laboratory within a pharmaceutical company or sent to a contract testing laboratory. The increased accuracy of identification with rRNA base sequencing and the ability to determine the strain of microorganism with ribotyping may be seen as molecular epidemiology tools that are best deployed to definitively determine the origin of the microbial contamination in media fill and sterility test failures and environmental monitoring excursions exceeding established action levels.

Genotypic versus Phenotypic Methods

Phenotypic methods rely on the more subjective determination of cellular morphology, gram reaction, catalase, coagulase or oxidase activity, other biochemical reactions, and carbon utilization patterns for identification, which introduces some disadvantages in consistent identification. However, these are mature technologies that are marketed by multiple companies as consistent, prepackaged kits with well-established quality control procedures and mature instrumentation with extensive databases to identify the most commonly encountered microorganisms found in the pharmaceutical industry. Furthermore, their underlying technologies are familiar to microbiologists working in routine microbiological testing laboratories in both large and small pharmaceutical companies. In contrast, the technologies deployed in genotypic methods are not as well established and are better suited to a research environment. Typically a single company currently markets some technologies, and, in some cases, there is a lack of commercially available reagents, primers, and probes. In addition, the technology is unfamiliar to most pharmaceutical laboratories, and the tests may be subject to nucleic acid contamination if they are not run in an environment with suitable controls in place to eliminate such contamination. Also, the nucleic acid can be difficult to reliably extract from some organisms, and many of the systems lack validated and/or appropriate databases for environmental microorganisms encountered in the pharmaceutical industry. Additionally, the instrumentation is expensive, and the unit testing cost is greater than that for phenotypic methods, so the implementation of the technology may be limited to larger pharmaceutical companies. An important consideration with a microbial identification technology is the number and types of genera and species in the database compared to those in the public domain. A comparison of the database size of representative phenotypic and genotypic microbial identification methods is presented in *Table 7*.

Table 7. Comparison of the database size of representative phenotypic and genotypic microbial identification methods

System	Classification	Database Size	Comments
Vitek Microbial Identification Systems	Phenotypic	800+ species of bacteria and yeast	Stronger in clinical organisms
Microlog Microbial Identification Systems	Phenotypic	1900 species of bacteria, yeast, and molds	Stronger in environmental organisms
Sherlock MIDI Microbial Identification System	Phenotypic	2000 entries, including aerobic and anaerobic bacteria and yeasts	Wide applicability
MALDI TOF MS plus MicrobeLynx	Phenotypic	Approximately 3500 spectral entries covering more than 100 genera and more than 400 different species	Developed by a collaboration between Manchester Metropolitan University and NTTC
RiboPrinter	Genotypic	6000 ribotypes consisting of 197 bacterial genera and 14,000 species	Type organisms and submitted isolates
MicroSeq 16S rRNA gene sequencing bacterial identification system	Genotypic	Bacterial full-gene and 500 base-pair libraries have 1400+ entries	Includes an extensive coverage of Gram-negative nonfermenters, Bacillus, Coryneforms, Mycobacteria, and *Staphylococcus*
Microseq D2 LSU rDNA fungal identification system	Genotypic	900 entries for yeast and filamentous fungi	Weak in both clinical and environmental fungi
Accugenix rRNA Gene Sequencing Systems	Genotypic	Accugenix bacterial database: 1607; Accugenix fungal database: 544	Propriety database
Bergey's *Manual of Systematic Bacteriology,* 2nd ed.	Genotypic and Phenotypic	849 genera and 4313 validly named prokaryotic species	Taxonomic authority
Ribosomal Database Release 2/06/04	Genotypic	87,860 aligned and annotated bacterial and archeal rRNA gene sequences	Extracted from primary sequence databases (DDBJ, EMBL, and GeneBank)
GeneBank (NCBI)	Genotypic	1244 genera and 13,088 bacterial species	Public-domain database

Driving the technology is the undisputed fact that these genotypic microbial identification methods, based on nucleic acid analyses, are less subjective, less dependent on the culture method, and theoretically more reliable because nucleic acids are highly conserved by microbial species. However, the microbiology laboratory cannot just buy the equipment, plug it in, and transfer a technician from performing other duties to running these methods. A significant investment in training, validation, and database development is required to successfully implement these methods.

The genotypic methods have some significant advantages; however their use should be limited to critical microbiological investigations associated with direct product failure, and identifications should be conducted in a specialized research-orientated laboratory within a pharmaceutical company (or sent to a qualified contact testing laboratory). The increased accuracy of identification with rRNA codon base sequencing and the ability to determine the strain of microorganism with ribotyping in some respects is a type of molecular epidemiology that seeks to more definitively determine the origin of the microbial contamination in media fill and following sterility test failures and action level excursions during environmental monitoring.

LITERATURE SURVEY

As stated earlier, in clinical microbiology genotypic identification methods have been successfully applied to the identification of fastidious organisms (e.g., *Clamydia trachomatis* and *Neisseria gonorrhoeae*), slow-growing organisms (e.g., fungal pathogens and *Mycobacterium tuberculosis*), rapid screening for pathogens (e.g., group A Streptococcus confirmatory testing), and unculturable organisms (e.g., the Whipple's bacillus *Tropheryma whippelii*) (*19*). Grazier et al. (*20*) looked at the ability of ribotyping to discriminate among *Enterobacter cloacae* isolates in comparison to a battery of conventional serological and biochemical assays and concluded that there was good agreement between identification by the genetic method and the combination of conventional methods. Clabouts et al. (*21*) performed a similar study with *Clostridium difficile,*

Martin et al. (*22*) with *E. coli,* Nielsen et al. (*23*) for *Campylobacter jejuni,* and Nadon et al. (*24*) for *Listeria monocytogenes*—all with similar results.

To date few publications have directly compared phenotypic and genotypic microbial identification methods. A study conducted at the Mayo Clinic reported using 72 unusual aerobic Gram-negative bacteria comparing the results achieved with the Sherlock MIDI system (cellular fatty acid profiles), MicroLog System (carbon utilization), and MicroSeq System (16S rRNA gene sequencing) against their clinical laboratory's conventional system (*25*). The agreement with the conventional system at the genus level was MicroSeq (97.2%), MicroLog (87.5%), and Sherlock (77.8%). Drancourt et al. (*26*) demonstrated the need to accurately determine the gram reaction, oxidase and catalase activities, growth requirements, and appropriate biochemical profile determination to achieve accurate phenotypic identification; they used 16S rDNA sequence analysis to identify previously unidentified environmental and clinical bacterial isolates. They also found that 10% of the environmental isolates could not be identified because they may be new species, and the sequencing could not separate *Enterobacter* and *Pantoea* isolates into species. In a poster at a recent ASM Annual Meeting, Waddington et al. compared genotypic and phenotypic methods for bacterial identification (*27*). They reported that with 18 ATCC cultures representing common environmental isolates the accuracy/reproducibility using MicroSeq DNA sequencing, Ribotyping, MIDI fatty acid ester analysis, Microlog substrate utilization, and Vitek 2 substrate utilization and biochemical reactions was 100/100%, 81/97%, 50/81%, 65/62%, and 33/89%, respectively. The selection of cultures readily identified by the DNA sequencing system may have unintentionally biased the outcome of the study.

Other studies using rRNA gene sequencing support the promise of the system for microbial identification but highlight the need to expand the database of the MicroSeq 500 16S rRNA gene sequencing system. Furthermore, many microorganisms readily recognized by the phenotypic system do not require nucleic acid-based systems (*28* and *29*). However, a recent study by the National Reference Center for Mycology (Canada) shows that for difficult-to-cultivate mycobacteria these genetic approaches are actually more cost effective than conventional methods (*30*).

DECISION MATRIX

Whereas rRNA gene sequencing has had a profound effect on microbial taxonomy, the appropriate role of nucleic acid-based identification methods in pharmaceutical microbiology is not yet clear. When companies are evaluating the suitability of microbial identification methods they are encouraged to consider a range of factors objectively in a decision matrix. Factors that must be considered and weighed are safety issues, breadth of application of the method, history of regulatory approval, equipment cost, unit-test cost, rapidity of the method, number of identification runs per day, number of vendors who can supply the equipment, complexity of the method, ease of validation, training requirements, and potential cost savings. In addition, the use of the resultant information is a consideration. For example, there is no point to having highly detailed, expensive-to-obtain information to establish environmental trends in an aseptic manufacturing facility when identification to the genus level is sufficient to establish that the environment is in a state of control.

These considerations are common to all new microbiological methods. Recent publications discussing the adoption of new technology include the PDA Task Force Report (*31*), a recently published book on this topic (*32*), a USP *Stimuli* article discussing the relationship between rapid microbial methods and process analytical technology (*33*), and a recent *In-Process Revision* (*7*).

VALIDATION OF MICROBIAL IDENTIFICATION METHODS

An excellent document that addresses both the selection and validation of microbial identification methods is Cumitech 31: Verification and Validation of Procedures in the Clinical Microbiology Laboratory (*34*). Another option is the AOAC International Official Methods program, which qualifies microbiological methods as official methods using interlaboratory collaborative studies (*35*). Additionally, USP has proposed a validation scheme (*7*). However, the proposed USP chapter is unsatisfactory in several regards, not least of which is that in an effort to address all aspects of the problem it has become so stilted and unwieldy that it is difficult to apply. The authors propose that the issue of microbial identification be removed from the draft general chapter⟨1223⟩ and be developed as a separate chapter utilizing available reference material.

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