Microbial Identification in a GXP Environment—Which System is Best?

Scott Sutton

There are a variety of microbial identification technologies on the marketplace available to the quality control laboratory. The choice of which technology to use is one that must be approached with a firm grounding in the requirements of the facility for cost, turnaround time, capacity, level of identification, and many other considerations. Development of a user requirements specification document should be a priority to gain clarity in requirements and to build consensus among management. Once the appropriate technology is determined, qualification of that technology should ensure that it meets user requirements.

INTRODUCTION

Microbial identification plays a central role in the cleanroom control program (1). The method of identification, however, must be wedded to the need. For example, any organism isolated from the critical aseptic processing area must be identified to great detail, while those from class D/ISO 8/100,000 areas might only be characterized to the genus level. The key concern is providing sufficient detail to assist in the tracking of the state of control of the facility.

Most identification schemes still rely on the Gram stain, a differential staining technique developed in the late 1800s by Christian Gram (2). This differential counterstaining technique is good at distinguishing a real difference in cellular morphology. Unfortunately, this method is prone to a significant level of operator error, which has encouraged the development of alternate methods for showing the difference in cell structure (3, 4). Traditional methods of identification also consider a variety of phenotypic characteristics.

PHENOTYPIC METHODS

Phenotypic methods typically incorporate reactions to different chemicals or different biochemical markers. The API strip is basically a prepackaging of the standard method that required racks of test tubes into a convenient bubble-wrap. This method was further refined in the Vitek automated system, which miniaturized the process (5, 6). The Vitek system has recently been enhanced to provide greater resolution of microorganisms (7, 8).

Biolog, Inc. offers a second phenotypic system. The fundamental unit in this system is a 96-well plate that has different carbohydrate sources in each well, with a tetrazolium redox dye. If the microorganism is capable of utilizing the carbohydrate, the well turns dark indicating reduction of the dye (9, 10). The end-result is a pattern of wells (a "metabolic fingerprint") that allows the user to identify the unknown microorganism. This method has recently been extended to include the identification of molds and filamentous fungi with a proprietary software package.

The use of cellular fatty acid (FA) composition to identify the genus and species has been popular for several years (11, 12). The fatty acids are extracted from the cell cultures and then the patterns of fatty acid esters are determined by gas chromatography (13). A popular system for this application is the MIDI Sherlock Microbial Identification System.

There are some new methods under development for the pharmaceutical quality control (QC) lab.

These include Fourier-Transform Infrared (FTIR) microscopy (14) and Matrix-Assisted Laser Desorption Ionization–Time of Flight (MALDI-TOF) mass spectroscopy (15, 16). The MALDI-TOF system especially is making some headway, with two vendors marketing the technology into the pharmaceutical market. However, as of this writing, these technologies have not seen widespread use in the QC lab.

GENOTYPIC METHODS

The US Food and Drug Administration elevated the use of genotypic identification methods with the release of the revised aseptic processing guidance document late in 2004 (17). Section X.B. of the guidance document reads, "Genotypic methods have been shown to be more accurate and precise than traditional biochemical and phenotypic techniques. These methods are especially valuable for investigations into failures (e.g., sterility test; media fill contamination). However, appropriate biochemical and phenotypic methods can be used for the routine identification of isolates."

The Qualicon Riboprinter is fundamentally an automated Southern Blot apparatus using labeled ss-DNA probe from the 16sRNA codon. The resulting pattern is then used to identify the unknown microorganism (18,19). If the initial banding pattern is inconclusive, then the restriction endonuclease can be changed to provide an extraordinary level of strain discrimination (20).

Another genotypic identification system on the market is the MicroSeq 500 16S rDNA Bacterial Sequencing Kit offered by Applied Biosystems. As the name implies, it provides the materials needed to sequence the first 500 base pairs of the unknown microorganism's 16s ribosomal RNA codon (21). The technology involves amplification of the 16S codon by PCR, followed by automated sequencing.

A final genotypic method that is being marketed into the QC pharmaceutical laboratory is the Bacterial Barcodes system (22) marketed by bioMerieux. This system is also based on PCR technology, using as a primer a sequence homologous to a repetitive sequence in the bacterial genome. The amplified sequence is then separated by gel electrophoresis and visualized to give the "barcode" specific to that strain.

Qualicon markets the BAX system to the food industry that contains primers for *Salmonella, Listeria,* or *E. coli* O157:H7 (23). This system has promise for determination of the absence of specified organisms in the product.

Other genetic methods have been published in the literature, although few are available to the pharmaceutical market (24).

CHOOSING A METHOD

There are a variety of identification technologies available. When choosing one for the lab, bear in mind the strengths, and weaknesses, of the various methodologies. For example, the aseptic processing guidance document strongly recommends the use of genotypically-based methods (17). However, if you choose technologically demanding methods, there is potentially an associated cost in facilities, labor (highly skilled technicians), and maintenance that is not present with the more bench-level methods. In other words, consider the difference in cost between the different methods—not only the difference in purchase price, but differences in validation costs, maintenance costs, certification and requalification costs, as well as consumables and labor. Generally speaking, the more elaborate the technology, the more expensive it will be to qualify and maintain it in a good manufacturing practice (GMP)-compliant state (totally aside from consumable costs).

Another consideration is if you will be able to use physiological information from the organism to your advantage. It is helpful in some situations to know the ability of the microorganism in terms of growth conditions and ability to use different nutrient sources (e.g., can it eat your product?). While many phenotypic methods provide such information, few others do and none of the genotypic methods will provide this. Literature research must be relied on for this information if it is not provided by the technology.

This does leave the question of using the appropriate method for your situation. Many situations benefit from having a relatively inexpensive test that

gives good quality identifications to the species level. Perhaps further precision for many applications is not warranted. In these situations, cost of consumables, throughput, and turn-around-time for reports are the primary considerations.

Useful information on considerations in the selection of technologies is presented in the new *United States Pharmacopeia* (USP) chapter <1113> "Microbial Characterization, Identification and Strain Typing" (25). In addition to providing some information on determining the needs of the organization for microbial identification technology, this chapter also provides some basic guidance on lab operational considerations to assist in reducing errors in the process.

One final aspect must be considered as part of the determination of the microbial identification needs. All techniques work by indirect means. That is to say, a series of tests are run on the unknown organism. Based on the organism's responses to the test, a match is found in a pre-existing database and the identity of the unknown organism is deduced by its similarity to the match. If the database does not have a match, then the technique is unable to assign an identity. The size, breadth, and appropriateness of the technology's internal database are critically important in determining its value to the lab.

How is the best identification method to use determined? The most direct approach to deciding the appropriate technology is to research the choices from the perspective of needs and expectations. This is not as easy as it may sound. The accurate determination of a company's needs may extend well outside the microbiology lab, including regulatory affairs, quality assurance, and perhaps manufacturing departments as well. The development of a user requirements specification (URS) document to drive this process is highly recommended (see Sidebar). This is a formal quality document, similar in concept to a design qualification document. Different companies will have different formats for these documents, but the essential features of the document will be that it has the essential requirements and that it has upper management sign-off (for a variety of reasons it is a good idea to document upper-management commitment).

Partial list of topics covered in any URS designed for an identification system

- Assay throughput
  - How many samples a day need to be processed?
- Assay time-to-completion
  - How quickly are results obtained?
- Cost of consumables
  - How much? Frequently, the cost of consumables can soon dwarf the capital expense.
- Labor requirements
  - Including the technological sophistication of the operators—can your technicians actually operate the equipment reliably?
- Size of microorganism identification database
  - A major consideration. If you purchase two systems to cover identifications of unknowns, it is imperative to ensure that the databases are large and complementary; that is they both don't have the same organisms in them, but that they include many different ones as well.
- Facility requirements
  - This section should be devoted to obvious considerations such as electrical requirements, water plumbing requirements, air handling requirements, and floor/bench space. This should also address less obvious questions such as the access dimensions (is the machine bigger than any available door?), will it require segregated and protected facilities to minimize extraneous contamination, etc.
- Compatibility with existing systems
  - LIMS, workflow, etc.
- Need for physiological information
  - Do you need to know if the organisms are capable of degrading your product components? You may want to use a system that will help determine this.
- Purpose
  - Routine ID
  - Investigations
  - The use of the system may be different for different systems. A good system for routine work may not be the best for investigations, and vice versa.

In short, there are a wide variety of choices available to help with the identification of unknown organisms. It is important to define specific requirements and to purchase the appropriate system to meet those needs.

QUALIFICATION OF THE TECHNOLOGY

Once brought in-house, microbial identification equipment should be evaluated by the same type of process used for other analytical equipment. It is common for the vendor to supply the installation and operational qualification. This is an acceptable approach provided that the plans are approved through the company's quality assurance group and that all raw data are reviewed by responsible and knowledgeable individuals. The vendor is in the best position to confirm that the equipment is correctly installed and operating correctly. However, the vendor should not conduct the performance qualification (PQ) portion of the new technology for several reasons.

First of all, the main purpose of the PQ is to determine if the analytical equipment meets the requirements spelled out in the user requirements, which is normally documented as a controlled document (see USP <1058> "Analytical Instrument Qualification" for a more comprehensive treatment of this topic). Secondly, the user is missing a useful opportunity to train lab personnel without affecting product release or investigations if it is done in cooperation with the vendor's installation technicians as part of the instrument qualification. Finally, this is a clear opportunity to confirm that the technology brought in-house meets the expectations of the company as determined when the purchase was approved.

Some informational guidance is available in USP chapter <1113> to assist in designing the qualification protocol (described as the "Verification" in the chapter's text) (25). The interested reader is directed to USP for more information on this topic.

CONCLUSIONS

There is no single technology that is the "best" for all labs in all situations. It is important to research the available identification options with a clear idea of the company's needs in mind. Once a technology has been identified as a potential candidate, evaluation of the technology against the prepared user requirements document should culminate in a fully qualified analytical instrument for microbial identification.

REFERENCES

1. Sutton, SVW and AM Cundell, "Microbial Identification in the Pharmaceutical Industry," *Pharm Forum.* 30(5):1884-1894, 2004.
2. McClelland, R., "Gram's Stain; the Key to Microbiology," *Med Lab Observer,* pp. 20-31, April 2001.
3. Mason, DJ et al., "A Fluorescent Gram Stain for Flow Cytometry and Epifluorescence Microscopy," *Appl Environ Microbiol.* 64(7):2681-2685, 1998.
4. Powers, EM., "Efficacy of the Ryu Nonstaining KOH Technique for Rapidly Determining Gram Reactions of Food-Borne and Waterborne Bacteria and Yeasts," *Appl Environ Microbiol.* 61(10):3756-3758, 1995.
5. Aldridge, C et al., "Automated Microbiological Detection/Identification System," *J Clin Microbiol.* 6(4):406-413, 1977.
6. Odlaug, TE et al., "Evaluation of an Automated System for Rapid Identification of Bacillus Biological Indicators and Other Bacillus Species," *PDA J Parenteral Sci Tech.* 36(2):47-54, 1982.
7. Funke, G and P Funke-Kissling, "Evaluation of the New VITEK 2 Card for Identification of Clinically Relevant Gram-Negative Rods," *J Clin Microbiol.* 42(9):4067-4071, 2004.
8. Funke, G and P Funke-Kissling, "Performance of the New VITEK 2 GP Card for Identification of Medically Relevant Gram-Positive Cocci in a Routine Clinical Laboratory," *J Clin Microbiol.* 43(1):84-88, 2005.
9. Klingler, JM et al., "Evaluation of the Biolog Automated Microbial Identification System," *Appl Environ Microbiol.* 58(6):2089-2092, 1992.
10. Miller, JM and DL Rhoden, "Preliminary Evaluation of Biolog, a Carbon Source Utilization Method for Bacterial Identification," *J Clin Microbiol.* 29(6):1143-1147, 1991.
11. Abel, KH et al., "Classification of Microorganisms by Analysis of Chemical Composition. I. Feasibility of Utilizing Gas Chromatography," *J Bacteriol.* 85:1039-1044, 1963.
12. Eerola, E and O-P Lehtonen, "Optimal Data Processing Procedure for Automatic Bacterial Identification by Gas-Liquid

Chromatography of Cellular Fatty Acids," *J Clin Microbiol.* 9:1745-1753, 1988.
13. Olson, W.P. et al., "Identification of Bacterial Contamination in a Pharmaceutical Manufacturing Facility by GC FA Analysis," *Pharm Technol.*, pp. 32-36, February 1990.
14. Maquelin, K. et al., "Identification Of Medically Relevant Microorganisms By Vibrational Spectroscopy," *J Microbiol Meth.* 51:255-271, 2002.
15. Fox, A., "Mass Spectrometry for Species or Strain Identification after Culture or without Culture: Past, Present, and Future," *J Clin Microbiol.* 44(8):2677-2680, 2006.
16. Mazzeo, MF. et al., "Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for the Discrimination of Food-Borne Microorganisms," *Appl Environ Microbiol.* 72(2):1180-1189, 2006.
17. FDA, *Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing— Current Good Manufacturing Practice*, Sept 29, 2004.
18. Bruce, JL., "Automated System Rapidly Identifies and Characterizes Microorganisms in Food," *Food Technol.* Jan. pp77-81, 1996.
19. Jeffrey, M et al., "Validation of an Enhanced Method of Bacterial Ribotyping for Improved Efficiency and Identification of Stressed Isolates," *Pharm Technol.* 28(3):156-166, 2004.
20. Brisse, S et al. "Distinguishing Species of the Burkholderia cepacia Complex and Burkholderia gladioli by Automated Ribotyping," *J Clin Microbiol.* 38(5):1876–1884, 2000.
21. 7. Fontana, C et al., "Use of the MicroSeq 500 16S rRNA Gene-Based Sequencing for Identification of Bacterial Isolates that Commercial Automated Systems Failed to Identify Correctly," *J Clin Microbiol.* 43(2):615-619, 2005.
22. Healy, M et al., "Microbial DNA Typing by Automated Repetitive-Sequence-Based PCR," *J Clin Microbiol.* 43(1):199-207, 2005.
23. Jimenez, L., "PCR Detection of Salmonella typhimurium In Pharmaceutical Raw Materials and Products Contamination with a Mixed Bacterial Culture Using the BAX System," *PDA J Pharm Sci Tech* 55(5):286-289, 2001.
24. Olive, DM and P Bean, "Principles and Applications for DNA-Based Typing of Microbial Organisms," *J Clin Microbiol.* 37(6):1661-1669, 1999.
25. USP, <1113> "Microbial Characterization, Identification and Strain Typing" *USP* 35 vol 1 pp. 694-697, 2012. **GXP**

ARTICLE ACRONYM LISTING

FA	Fatty Acid
FTIR	Fourier-Transform Infrared
GMP	Good Manufacturing Practice
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization-Time of Flight
PQ	Performance Qualification
QC	Quality Control
URS	User Requirements Specification

ABOUT THE AUTHOR

Scott Sutton is the founder and principal consultant of Microbiology Network, Inc (http://bit.ly/n62yFG). He is a recognized consultant and trainer with emphasis in GMP, investigations, environmental monitoring, and contamination control as well as microbiology laboratory audits and operations. He may be reached by e-mail at scott.sutton@microbiol.org.