Rapid Characterization of Bacteria Using ClairScope™ and SpiralTOF™

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In many fields such as clinical diagnosis and food inspection, there is a demand for rapid, reliable and simple-to-use methods for characterizing bacteria. This paper explores the use of two new and innovative instruments called ClairScope™ and SpiralTOF™ for this rapid characterization. The JASM-6200 ClairScope™ integrates an optical microscope (OM) with a scanning electron microscope (SEM) where it is possible to observe samples in solution, in an open system, by the SEM at atmospheric pressure. This type of sample would typically require extensive sample pretreatment that would take a day or more with conventional SEM techniques. With the recently developed ClairScope™, fine morphological observation can be performed directly in solution with simple sample pretreatment of one hour or less. The JMS-S3000 SpiralTOF™, is a matrix-assisted laser desorption/ionization mass spectrometer (MALDI-MS) with a spiral ion trajectory. With sample pretreatment as fast as a few minutes, the SpiralTOF™ can characterize ribosomal proteins and phospholipids with high accuracy. Ribosomal proteins are biomarkers for phylogenetic classification, and phospholipids are used for chemotaxonomic analysis. The combination of ClairScope™ and SpiralTOF™ are found to be powerful instruments for the characterization of bacteria.

Introduction

Rapid characterization methods are in demand for bacteria, which are simple to use and reliable, in a variety of fields such as clinical diagnosis, food inspection, and in environmental energy industries. Bacterial characterization methods can be divided into two categories: analysis of phenotype and genotype. Phenotype analysis includes a morphological study and chemotaxonomy where analysis of bacterial cell components such as proteins and lipids are analyzed. Genotype analysis includes gel electrophoresis and DNA sequencing using a sequencer.

In many cases, a skilled operator can identify the species of bacteria using an optical microscope combined with supplemental information. However, optical microscope (OM) has limited resolution and with bacterial size typically smaller than a few micrometers, detailed structural information cannot be obtained. The use of scanning electron microscope (SEM) overcomes this resolution limitation but often requires long sample pretreatment such as dehydration and fixation that can take a day or even longer.

A chemotaxonomy, based on biochemical analysis, is effective in investigating characteristics of bacterial strains. Yet with current methodology, sample preparations are cumbersome. Also, these methods tend to be affected by differences in culture conditions, sample preparation techniques and measurement conditions which can lead to results that are not as reproducible as desired. The reduction in cost, improved throughput and larger databases are making DNA analysis methods more popular. However, in principle, errors in amplification and sequencing cannot be avoided. Therefore, using polyphasic methods to characterize bacteria is of prime importance.

In this paper, possibilities of new rapid characterization methods for bacteria were studied based on both phenotype and genotype. *Escherichia coli* NBRC 3301 was used as a model strain. The JASM-6200 ClairScope™ (atmospheric scanning electron microscope: ASEM) [1], was used to observe the morphology of the bacteria at atmospheric pressure. The JMS-S3000 SpiralTOF™, a matrix assisted laser desorption/ionization mass spectrometer (MALDI-MS) [2] with a spiral ion trajectory, was used to analyze the ribosomal proteins and phospholipids.

Morphological Observation of Bacteria with the JASM-6200 ClairScope™

The configuration of the JASM-6200 ClairScope™ is shown in **Fig. 1**. This microscope consists of an inverted SEM that incorporates a thin-film (silicon nitride, SiN)-windowed ASEM specimen dish on top. The film with a thickness of 100 nm allows transmission of the electron beam and also maintains a vacuum in the ASEM. These features enable SEM observation according to the following procedure: an ASEM specimen dish with the sample is placed on the ClairScope™, the sample is scanned with the electron beam from underneath through the thin SiN film, and then backscattered electrons emitted from the sample are detected. The ClairScope™ also includes a wide field OM just above the ASEM specimen dish for observation of the same field of view. This innovative microscope enables observation of samples such as bacteria, in solution at atmospheric pressure, using both optical and SEM imaging. Rapid and simple SEM observation can be achieved without the long dehydration and fixation sample treatments typically required with conventional SEMs.

In this study, the *E. coli* NBRC 3301 was grown in Luria-Bertanimedium at 30°C for 8 h. The *E. coli* NBRC 3301 was harvested by centrifugation then washed twice with phosphate-buffered saline (PBS). They were re-suspended in the PBS and put on the ASEM specimen dish that was coated with poly-L-lysine. At this point, the sample was fixed with 2% paraformaldehyde/1% glutaraldehyde in PBS for 10 min. In addition, the sample was stained with 0.6% platinum blue [3] for 1 h. ASEM images of *E. coli* NBRC 3301 are shown in **Fig. 2**. Even with such a short sample pretreatment time, the ClairScope™ provided high resolution images where rod-shaped *E. coli* NBRC 3301 smaller than 2 μm were clearly visualized.

Although information obtained through imaging with the ClairScope™ can look different from that obtained by conventional SEM or transmission electron microscope, this new SEM not only allows for rapid observation but also enables much simpler and faster sample pretreatment prior to imaging for high throughput analyses. Also, with the recent advancement in fluorescent labeling, complimentary information can be obtained through the OM and correlating the information from both the OM and ASEM can play an important role in the characterization of bacteria.

Analysis of Ribosomal Proteins and Phospholipids with the JMS-S3000 SpiralTOF™

The JMS-S3000 SpiralTOF™ is shown in **Fig. 3** with its ion optical system. This instrument is an advanced MALDI TOFMS, for the analysis of a wide range of samples such as lipids, synthetic polymers, peptides, and pro-

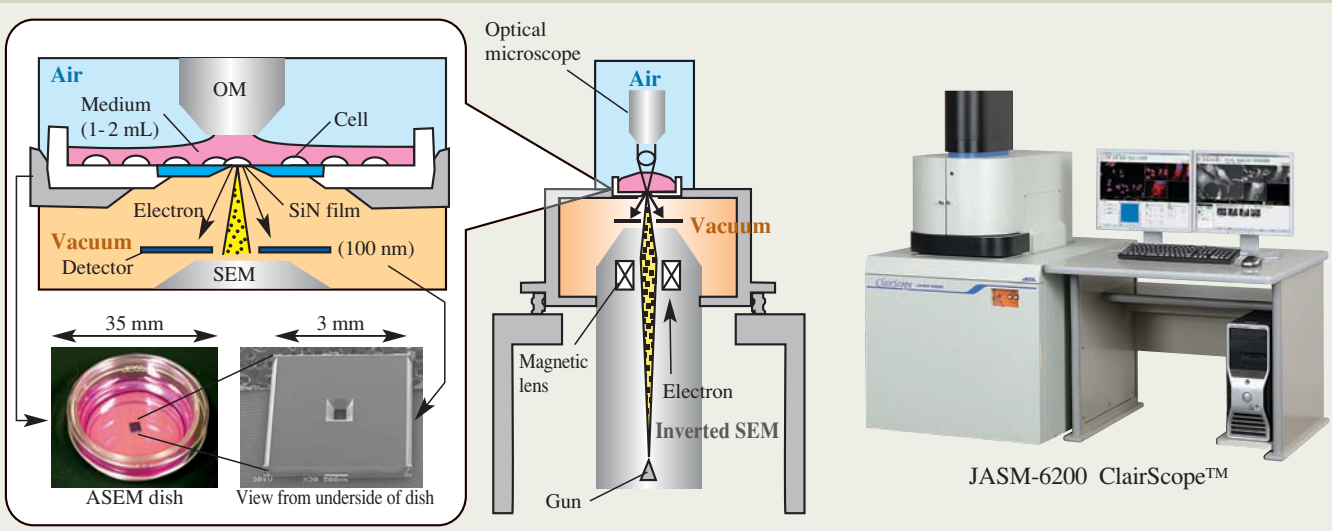


Fig. 1 The configuration of ClairScope™. Optical microscope (OM) is placed opposite to an inverted scanning electron microscope (SEM) (atmospheric scanning electron microscope: ASEM) and a film-windowed dish is placed between OM and ASEM.

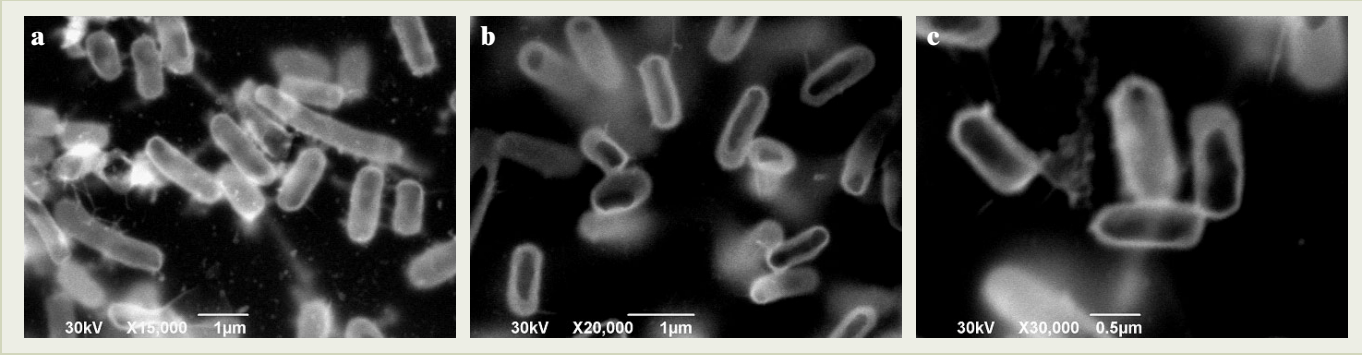
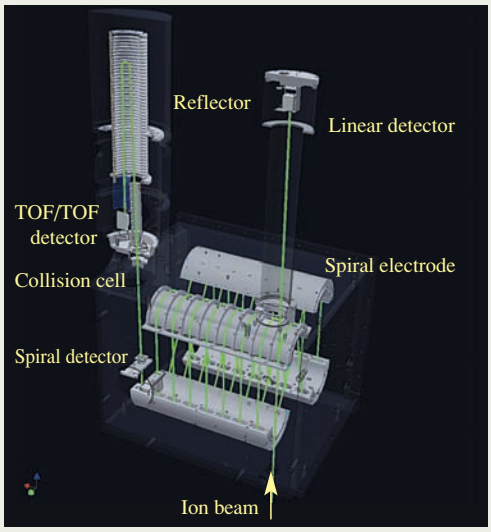


Fig. 2 ASEM images of *E. coli* NBRC 3301. ×15000 (a), ×20000 (b), and ×30000 (c)



JMS-S3000 SpiralTOF™

Fig. 3 The configuration of SpiralTOF™. Combined use of the spiral mode (flight length: 17 m) and the linear mode (flight length: 1.2 m) extends the application of this instrument to a wide range of fields.
Spiral mode: Mass resolution >60,000, Mass accuracy <1 ppm.

teins, through the combination of the spiral mode and the linear mode of operation. In particular, the spiral mode has an ion flight length of 17 m which is achieved by the ion optical system having a spiral trajectory. This suppresses beam broadening to prevent degradation of mass resolution and mass accuracy. Therefore, the SpiralTOF™ provides higher mass resolving power and higher mass accuracy than conventional MALDI TOFMS. This paper reports on the analysis of ribosomal proteins in the linear mode and the analysis of phospholipids in the spiral mode.

Analysis of ribosomal proteins with the SpiralTOF™ in linear mode

In recent years, mass spectral patterns obtained by MALDI-MS have been used for rapid identification and classification of bacteria. In this method, ribosomal proteins are observed and their spectra are compared with reference spectra from bacterial strains stored in a library database. This provides identification of bacteria at "species level" which can be obtained by 16S rRNA gene sequencing [4].

On the other hand, the characteristics of bacteria such as antibacterial activity and toxicity are frequently different at the "strain level". To discriminate bacteria at the strain level, and to classify the bacteria based on genotype, we have used observed masses of ribosomal proteins as biomarkers instead of the mass spectral fingerprints [5-7]. Observed masses of ribosomal proteins in MALDI mass spectra have been precisely assigned with calculated mass from DNA sequence. We have shown that it is possible to discriminate bacteria at

the strain level, using the number of identical peaks. Based on our method, the classification results were comparable to phylogenetic classification based on DNA gyrase subunit B gene sequencing [5, 6].

Sample preparation and data analysis of ribosomal proteins were carried out according to a previous paper [8]. Briefly, the cells were mixed directly with a matrix solution containing sinapinic acid in an organic solvent. Since *E. coli* NBRC 3301 is a gram-negative strain, cell walls are lysed by mixing with this matrix solution. About 1 μL of sample/matrix mixture was spotted on the MALDI target plate. The MALDI-MS measurements were performed using SpiralTOF™ in the linear mode. **Fig. 4** shows MALDI mass spectrum for *E. coli* NBRC 3301. In this spectrum, each peak was clearly separated with high intensity, and these observed peaks were assigned as ribosomal proteins by comparing with the calculated masses. In the bacterial characterization using DNA sequencing, in principle, the possibility of an error in amplification and sequencing processes cannot be excluded. In contrast such errors do not occur in our method because neither amplification nor a sequencing process is used. In addition, ribosomal proteins are always observed independent of culture conditions, because they are typical housekeeping proteins. Thus, our method is highly reliable. Moreover, our method can detect a subtle difference in an amino-acid sequence as a clear peak shift in the mass spectra. This capability provides high sensitivity to variations of the amino-acid sequence, which is related to DNA sequences. Therefore, this simple method provides highly reliable and sensitive information for bacterial characterization.

Compositional analysis of phospholipids with the SpiralTOF™ in spiral mode

The cell components of bacteria are much more diverse than those of eukaryote. Thus, analyzing the components such as lipids, peptideglycans, and proteins is effective for bacterial characterization at the strain level. This is known as chemotaxonomy. Lipids including fatty acids, phospholipids, sphingolipids, mycolic acids and the isoprenoid quinine group are often studied. In particular, glycerophospholipids are included in all bacterial cells so that analyzing them is valuable. The compositional distribution of hydrocarbon chains, especially carbon numbers and degree of double bonds, as well as the chemical structures of hydrophilic groups are important biomarkers for chemotaxonomy of bacteria.

Ishida *et al.* proposed the on-probe method where whole bacterial cells are deposited directly on to a MALDI target plate, and intact phospholipids from the cells are analyzed by MALDI-MS [9]. This simplified sample pretreatment significantly reduces sample preparation time to several minutes. Since bacteria normally contain several types of phospholipids with different chemical structures, mass spectra peaks for phospholipids frequently show overlaps that are not easily resolved. It is crucial to separate and identify these peaks clearly and precisely for accurate characterization of bacteria. For this purpose, the SpiralTOF™ is especially valuable, since it has high mass resolving power and high mass accuracy (at m/z ~1,000, peak of ΔM<0.04 Da can be separated).

The sample preparation for phospholipids from whole bacterial cells were carried out according to the method established by Ishida *et al.* [9]. Polymerix (Sierra Analytics Corporation) was used for data analysis.

Fig. 5 shows a MALDI mass spectrum of phospholipids extracted from the whole cells of *E. coli* NBRC 3301. Most of the peaks observed in the mass spectrum were assigned as phosphatidylethanolamine (PE) within 0.001 Da (ca. 1 ppm) error. These PEs contain unsaturated double bonds and have carbon numbers from 29 to 36 (**Fig. 6**). The results were found to be almost the same as those reported by Ishida *et al.* [9]. In this simplified sample pretreatment, separation techniques for samples are not used. This tends to give complex mass spectra consisting of many peaks from different components. Use of a mass spectrometer with insufficient mass resolving power and mass accuracy makes it impossible to separate adjacent peaks and can lead to errors in assigning peaks and with the final result. On the other hand, the SpiralTOF™, which has high mass resolving power and high mass accuracy, provides a clear separation of adjacent peaks. This yields reliable results, even with automatic assignment of peaks using software from complex mass spectral data. The SpiralTOF™ is expected to play a significant role in analyzing practical samples comprised of many components, since it provides accurate results with simple sample pretreatment methods and data analysis.

Conclusion

There is no complete single method which can fully cover the characterization of bacteria. With this in mind, there is a demand for the development of simple, rapid and reliable methods. In this paper, *Escherichia coli* NBRC 3301 was characterized based on phenotype and genotype using the ClairScope™ and the SpiralTOF™. The ClairScope™ pro-

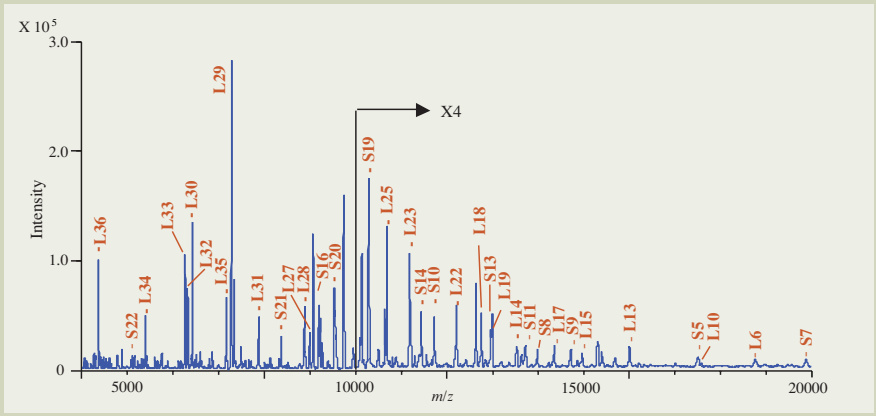


Fig. 4 MALDI mass spectrum of *E. coli* NBRC 3301 (linear mode).

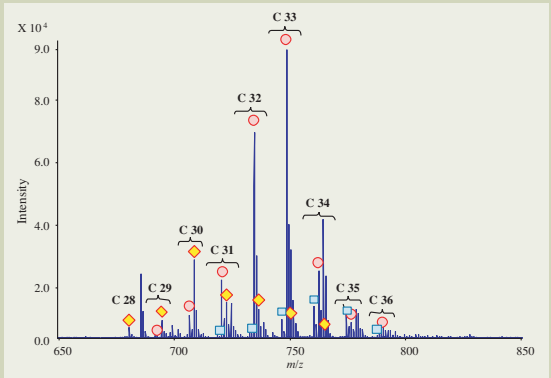


Fig. 5 MALDI mass spectrum of phospholipids extracted from whole cells of *E. coli* NBRC 3301 (spiral mode). ◇ PE (saturated), ○ PE (monoene), and □ PE (diene).

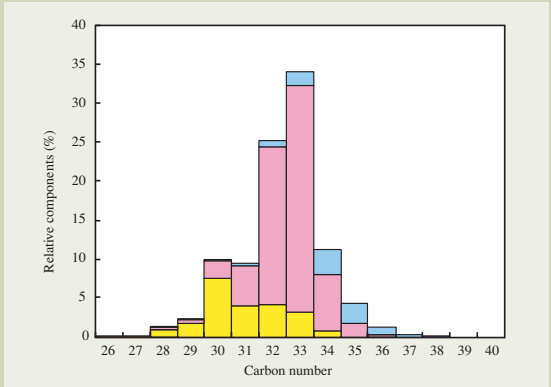


Fig. 6 Composition of PE extracted from whole cells of *E. coli* NBRC 3301.
□ PE (saturated), □ PE (monoene), and □ PE(diene).

vided morphological observation with simple sample pretreatment methods requiring only one-hour (fixation and staining), versus the treatment methods required with conventional SEM which can take up to ten times longer. The advantage here is rapid morphological observation at high magnifications with very simple and fast sample pretreatment. The ClairScope™ is expected to be especially valuable for slow-growing bacteria including pathogenic ones, since it also requires a small amount of sample.

In addition, the SpiralTOF™ enabled highly reliable analyses of bacteria with pretreatment times that take only a few minutes. The linear mode was used for the analysis of ribosomal proteins. These proteins act as biomarkers for phylogenetic classification of bacteria at the strain level. The spiral mode was used for the analysis of phospholipids, which are biomarkers for chemotaxonomy.

In many cases, the reliability of analyses that use rapid and simple methods is not high. However, the use of these two new and innovative instruments are not only simple to use, but also provides fast and highly reliable results.

The new methods described in this paper are well suited for high throughput characterization of bacterial samples. First, morphological observation with the ClairScope™ can provide insight such as whether a sample contains bacteria or sometimes whether they are pathogenic combined with some other information. Second, using the SpiralTOF™ in linear mode, ribosomal proteins which are used as biomarkers to distinguish and classify the bacterial samples at the strain level, can be analyzed. This also determines appropriate pre-treatment for chemotaxonomy of relevant strains. Third, components of bacterial cells such as phospholipids and mycolic acids, can be analyzed using the SpiralTOF™ in spiral mode. The combination of these methods will give a broad range of information (**Fig. 7**) and can provide complementary information for other methods such as sequence analysis of 16S rRNA gene.

References

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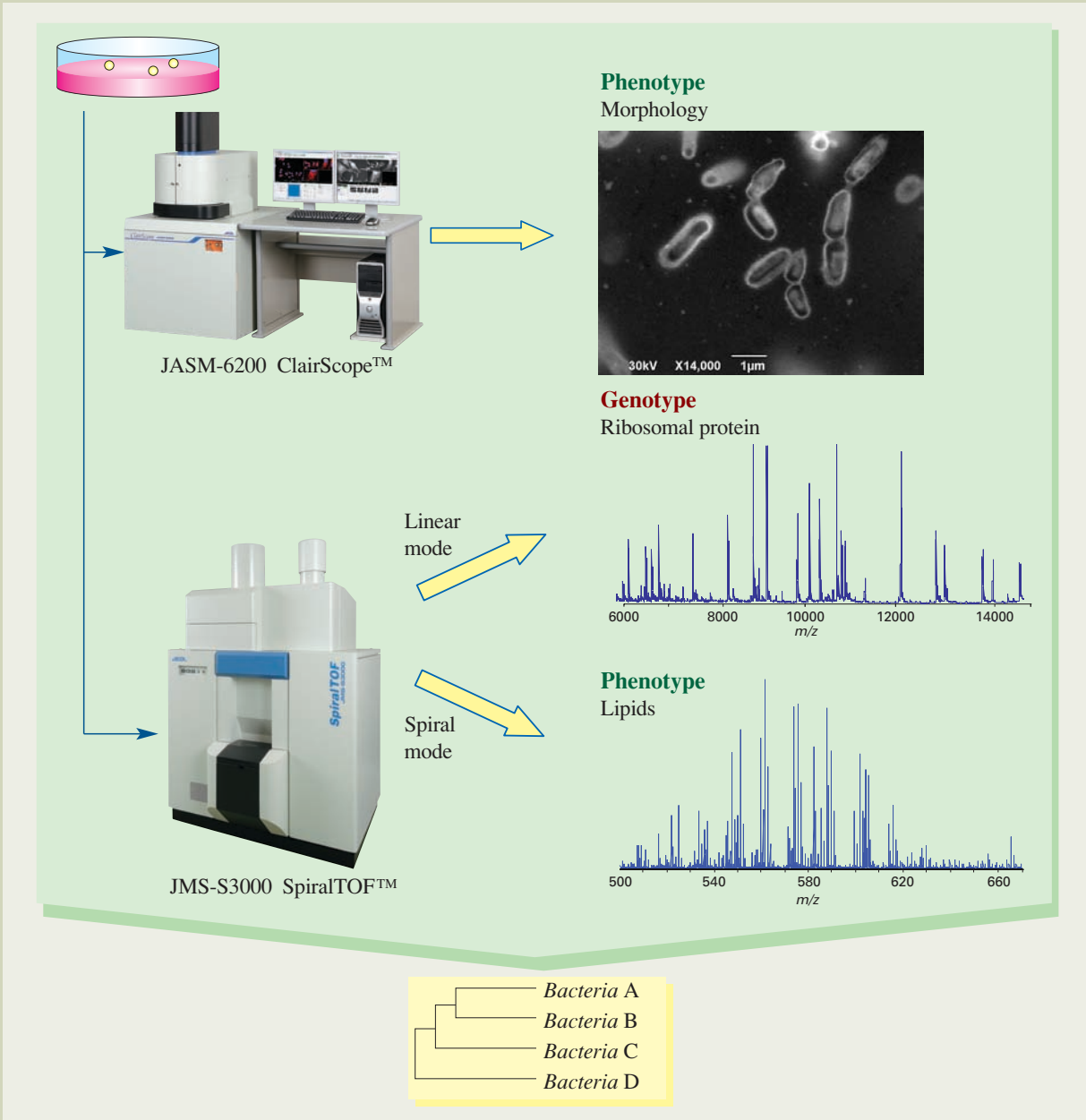

Fig. 7 Conceptual image of rapid characterization of bacteria using both ClairScope™ and SpiralTOF™.