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Label-free Identification of Bacteria Species Based on Divergence Angle Distribution of Light Scattering Patterns

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In this paper, we propose a facile scheme for label-free bacteria discrimination by utilizing the divergence angles (DAs) of the light scattering patterns (LSPs) of bacteria colonies. We compared three species of bacteria, *Escherichia coli*, *Salmonella typhimurium*, and *Listeria monocytogenes*, in terms of the DAs of the forward LSPs of their colonies by using the proposed scheme. The sizes of the cultured bacterial colonies used for comparison ranged from 0.8 to 2.2 mm for all three species. First, to obtain their LSPs, a low-power green laser of 532 nm illuminated each bacterial colony. Then, the sizes of the LSPs were estimated from the acquired colony scattering patterns. The DAs of the LSPs were calculated using the measured colony sizes and LSP sizes. Experimental results show that there exists an optimal distribution range of the DA for each bacterial species. We believe that this new identification scheme of pathogens using the specific DA distribution range will be beneficially utilized for a simple verification of conventional bacteria discrimination methods.

1. Introduction

Pathogenic bacteria, such as *Escherichia coli*, *Salmonella typhimurium*, and *Listeria monocytogenes*, can cause foodborne illnesses, leading to persistent risks to food safety, and can be harmful or even deadly to humans.[1–4] The adverse effects of these bacteria are directly linked to social costs and public health issues. Therefore, quickly detecting pathogens and identifying contamination are essential for individuals and also public health. Accordingly, the detection and analysis of germs have become an indispensable part of our present society, and many studies have been continuously carried out. There are various methods of detecting bacteria, such as immunoassay based on the specific binding of antigen and antibody,[5–8]

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polymerase chain reaction (PCR) to amplify nucleic acids,[9–11] and fluorescence analysis based on biosensors using phosphors.[12,13] Recently, pattern analysis to identify a variety of genera has been carried out using optical-forward scattering patterns obtained by irradiating bacteria with low-power light. The light scattering patterns (LSPs) are acquired by observing the scattered patterns when light introduced to the surface of a bacteria colony is scattered forward. The LSP-based method has the advantages of being faster than the existing methods and nondestructive to bacterial colonies. In a related study, Bayraktar *et al.* proposed in 2006 a forward light scattering technology to identify bacterial colonies on agar plates without the processing of samples, and they analyzed colonies consisting of six strains that belong to the genus *Listeria*.[14] Then, using the Bacteria Rapid Detection using Optical Scattering Technology (BARDOT) system, Banada *et al.* analyzed the LSPs of *Listeria* species colonies in 2007[15] and performed bacterial classification by observing the characteristics associated with the LSPs of various species of *Listeria* in 2009.[16] Other researchers have also studied bacterial LSPs by employing this system. In 2008, Bae *et al.* also applied several theories to the LSPs of three *Listeria* species obtained using the BARDOT system[17] and presented an automated device to find bacterial colonies and capture forward scattering signatures. In 2010, Rajwa *et al.* analyzed the LSPs of bacterial communities formed by different *Salmonella* serotypes.[18] In 2011, Bae *et al.* identified bacterial species according to their growth time, the diameters of patterns observed in the LSPs of bacterial colonies, and the number of rings in the LSPs,[19] and in 2012, proposed a portable bacterial identification system based on LSPs.[20] In 2014, Marcoux *et al.* investigated the LSPs of four strains of *E. coli* colonies cultured with different incubation times.[21] In 2015, Jo *et al.* analyzed LSPs using Fourier transform light scattering and statistical classification,[22] and Minoni *et al.* compared LSPs for different types of bacteria according to the location of a charge-coupled device (CCD) camera, incubation time, and diameters of bacteria colonies.[23] However, the LSPs of the above-mentioned bacterial communities vary significantly, depending on the sizes of the bacterial colonies, the culture medium, and the incubation time; individual bacterial species do not have their own LSPs discernible with the naked eye. For example, depending on the culture medium or incubation time, completely different LSPs can be obtained for the same bacteria, and similar LSPs can be obtained for different bacteria. Here, we propose a new discrimination scheme for bacteria based on the divergence angles (DAs) of the LSPs of bacterial colonies. Our discrimination scheme was assessed for three bacterial genera, *E. coli*, *S. typhimurium*, and *L. monocytogenes*. When these three bacteria are cultured in a brain heart infusion (BHI) medium, their LSPs look similar, making it difficult to distinguish the patterns. To validate the proposed method in situations where it was difficult to discriminate between bacteria by the LSP alone, the DAs were measured and compared with each other for three bacteria cultured in the BHI medium. In Sect. 2, the preparation of bacteria samples and the measurement process of the DAs of the LSPs in the samples will be described. Then, experimental results on the DAs of the samples and some discussion on them will be provided in Sect. 3. Finally, a brief summary and conclusion will be given in Sect. 4.

2. Materials and Methods

2.1 Sample preparation

Bacteria samples of *E. coli*, *S. typhimurium*, and *L. monocytogenes* were incubated for 24 h at 37 °C in a BHI broth medium. Following the bacterial incubation process, samples of 1 μL volume were taken and spread on a BHI agar medium and cultured for 12 h for *E. coli* and *S. typhimurium* and 24 h for *L. monocytogenes* at 37 °C. The three bacteria species were incubated on the same plates, yielding 10–100 colonies per plate. The sizes of the cultured bacteria colonies were in the range of 0.8–2.2 mm for all three species. To investigate over 200 colonies with respect to each bacterial species, we prepared 15 to 20 plates for incubation in the same culture environment and selected 10 to 15 colonies on average for each bacterial species satisfying the sizes in good condition among the colonies grown on one plate.

2.2 Measurement process of DA

Figure 1 shows a schematic diagram of the experimental setup for capturing the LSPs of bacterial colonies. Output light of a 532 nm laser was guided through a single-mode fiber, and guided light illuminated the bacterial colonies via a biconvex lens (Thorlabs) with a focal length of 150 mm, which was installed at the output terminal of the single-mode fiber. When the laser beam incident on a bacterial colony was larger than the size of the colony, spatial crosstalk occurred in the LSP owing to optical diffraction. To minimize this crosstalk, therefore, the biconvex lens was moved along the *z*-direction to adjust the size of the incident beam so that it almost matched the size of the colony. LSPs created from bacterial colonies were acquired

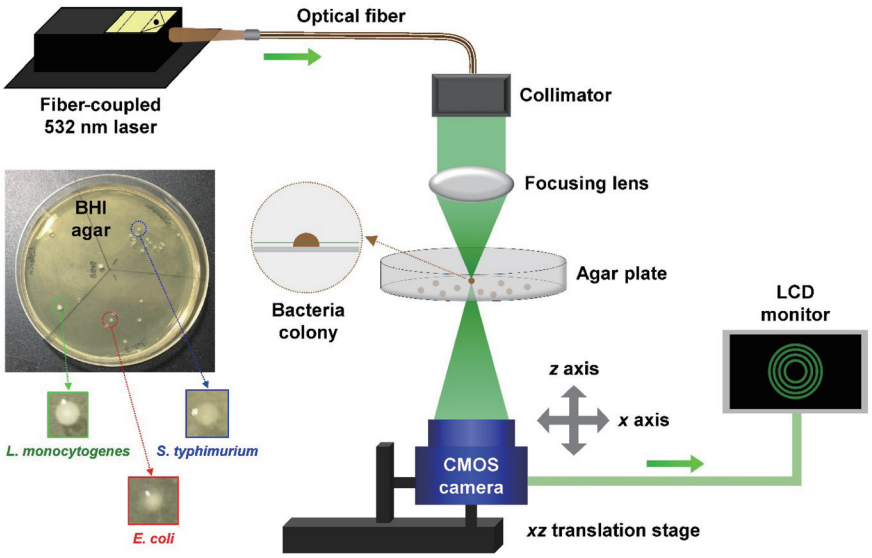


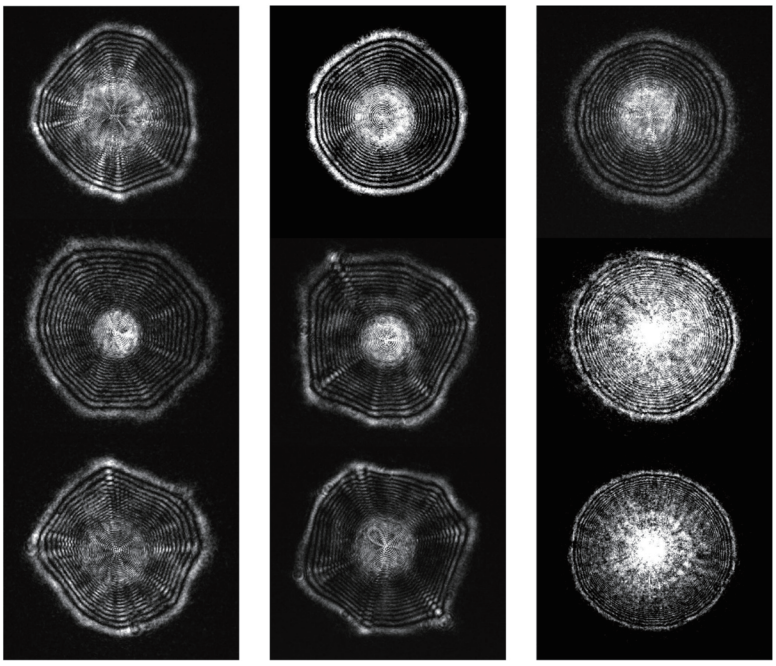
Fig. 1. (Color online) Schematic diagram of the experimental setup for acquiring the LSPs of bacterial colonies.

using a complementary metal-oxide semiconductor (CMOS) camera (Thorlabs), and graphical information obtained by the CMOS camera was transferred to a monitor connected with a PC for visual observation and storing image information. The position of the camera attached to an *xz*-axis translation stage was minutely adjusted to collect clear and distinct LSPs. The *xz*-axis stage can be finely tuned in units of μm, and the *z*-axis adjustment enables the selective capture of LSPs.

3. Experimental Results and Discussion

3.1 LSPs

Figures 2(a)–2(c) show the LSPs of the three bacteria species of *E. coli*, *S. typhimurium*, and *L. monocytogenes*, respectively, obtained by using the setup shown in Fig. 1. The sizes of all bacteria colonies shown here are ~1 mm. As mentioned in the introduction, many studies on the analysis of LSPs have focused on the effect of the colony diameter, the incubation time, the camera position, and the number of rings in the LSP. However, as can be seen in Fig. 2, since the LSPs of bacteria are similar when bacteria are cultured in the BHI medium, the discrimination of bacteria species is elaborate work requiring sophisticated image comparison and mapping processes. Basically, the size of an LSP depends on the size of the bacterial colony irradiated by the laser beam. Moreover, the LSP size varies slightly among bacteria



(a) (b) (c)

Fig. 2. LSPs of bacterial colonies of (a) *E. coli*, (b) *S. typhimurium*, and (c) *L. monocytogenes*.

species even in similarly sized colonies. Because the LSP size is mainly determined by the size of the bacterial colony and the DA, the DA is presumed to be affected by the unique structural characteristics of the bacterial colonies, which will differ among bacteria species. Thus, in this study, we will investigate the DAs of some bacterial colonies and then identify bacterial genera by analyzing their differences.

3.2 Relationship among bacterial colony size, LSP size, and DA

Figure 3 shows a schematic diagram of the calculation of the DA in the LSP using the relationship among the bacterial colony size, LSP size, and DA. When an LSP of a bacterial colony is formed, the path of light propagation is similar to the way that light travels after passing through a lens. In other words, an optical beam incident on the bacterial colony passes through the colony, is concentrated toward a focal point, and then diffuses widely, as if light propagates through a lens. In Fig. 3, red-dotted lines indicate the path of beam propagation, and a, b, c, f, and θ are the size of the bacteria colony, the LSP size, the distance from the colony to the focal point, the distance from the focal point to the boundary of the LSP, and the DA, respectively. a and b are parameters that can be measured, and the distance from the plate where the bacteria colony is located to the LSP is fixed at 27 cm. Hence, it is possible to calculate the DA using Eqs. (1)–(3). The DA calculated with Eq. (3) is $\theta/2$, which is half the actual DA, but this value ($\theta/2$) is regarded hereafter as the DA for convenience.

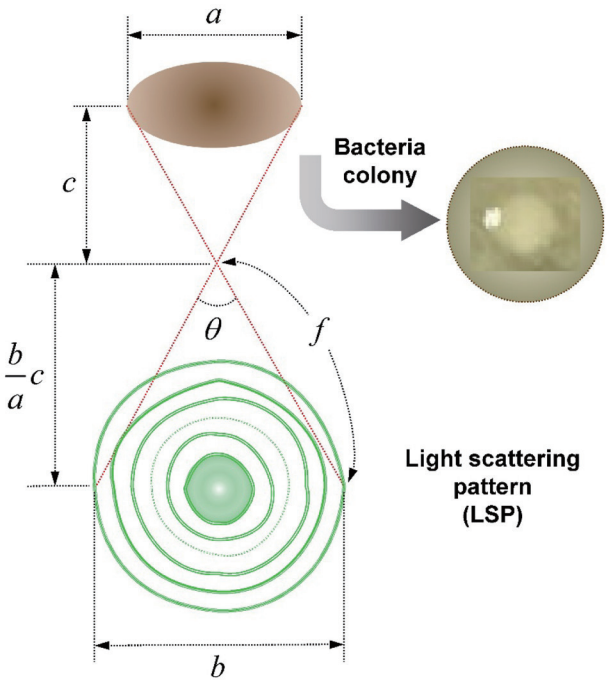


Fig. 3. (Color online) Relationship among the bacteria colony size, LSP size, and DA.

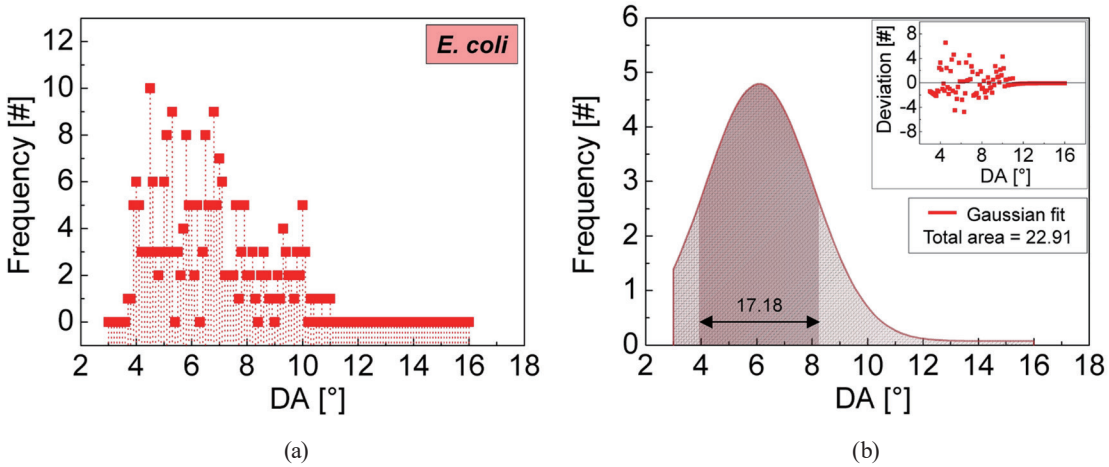
$$c + \frac{b}{a}c = 27 \Leftrightarrow c = \frac{27}{1 + b/a} \tag{1}$$

$$f = b\sqrt{\left(\frac{c}{a}\right)^2 + \frac{1}{4}} \tag{2}$$

$$\frac{\theta}{2} = \sin^{-1}\left(\frac{b}{2f}\right) \tag{3}$$

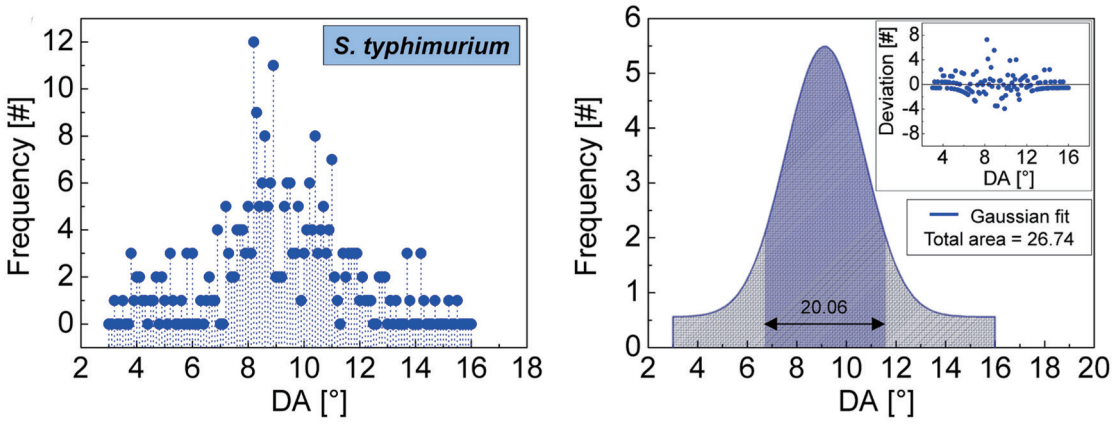
3.3 Measurement results of DAs and discussion

Figures 4(a), 5(a), and 6(a) show the acquisition frequencies of the DAs for *E. coli*, *S. typhimurium*, and *L. monocytogenes*, respectively. It is found from the figures that the DA acquisition frequencies are distinctly different among the bacteria species. Figures 4(b), 5(b), and 6(b) show the results of nonlinear regression analyses based on the Gaussian distribution for the DA acquisition frequencies plotted in Figs. 4(a), 5(a), and 6(a), respectively. The Gaussian distribution is the most important distribution in statistics and the most widely used probability distribution because countless natural phenomena take its form. When we model this Gaussian distribution of the DA acquisition frequency using a probability density function, the DA acquisition frequency can be stochastically analyzed as a Gaussian random variable. In our study, the distribution range of the DAs for the three bacterial species was calculated according to the ratio of a certain area centered on the mean (i.e., the DA having the maximum acquisition frequency) of the Gaussian distribution to the total area, that is, the acquisition probability of the DA. For example, when assuming a DA acquisition probability of 70%, the distribution range



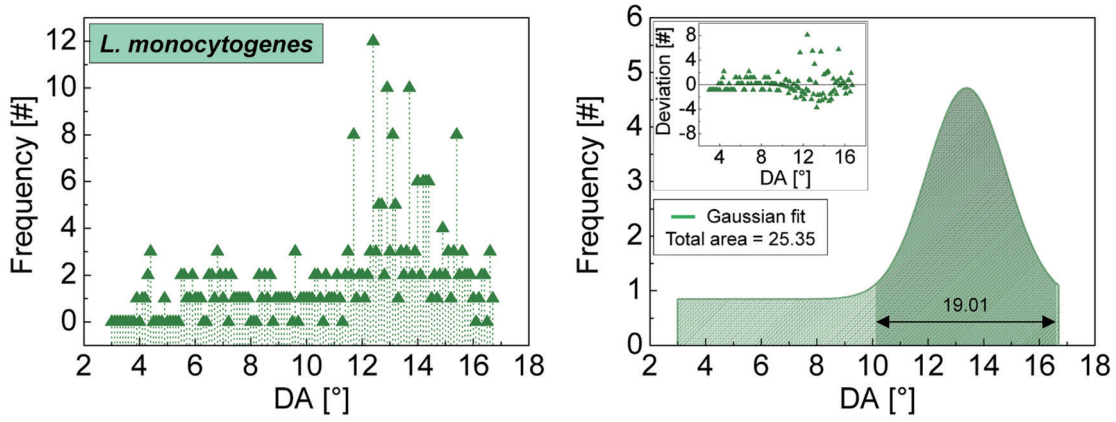
(a) (b)

Fig. 4. (Color online) (a) Acquisition frequency of the DAs of *E. coli* and (b) its nonlinear curve fit with a Gaussian distribution. The central shaded area in (b) indicates the ODR, and the inset of (b) shows the regular residuals between the measured acquisition frequency and the Gaussian fit.



(a) (b)

Fig. 5. (Color online) (a) Acquisition frequency of the DAs of *S. typhimurium* and (b) its nonlinear curve fit with a Gaussian distribution. The central shaded area in (b) indicates the ODR, and the inset of (b) shows the regular residuals between the measured acquisition frequency and the Gaussian fit.



(a) (b)

Fig. 6. (Color online) (a) Acquisition frequency of the DAs of *L. monocytogenes* and (b) its nonlinear curve fit with a Gaussian distribution. The central shaded area in (b) indicates the ODR, and the inset of (b) shows the regular residuals between the measured acquisition frequency and the Gaussian fit.

of the DA is determined by adjusting the shaded central area to be 70% of the total area of the Gaussian distribution for the three bacteria species. Specifically, at a DA acquisition probability of 70%, the DA distribution ranges for *E. coli*, *S. typhimurium*, and *L. monocytogenes* are defined by adjusting the shaded area to be 16.04 for the total area of 22.91, 18.72 for the total area of 26.74, and 17.75 for the total area of 25.35 in the Gaussian distribution of the DAs, respectively. As a result, the DA distribution ranges become 4.21° to 8.09° for *E. coli*, 6.96° to 11.31° for *S. typhimurium*, and 10.60° to 16.06° for *L. monocytogenes*. In the same way, at a DA acquisition probability of 80%, the central shaded area is modified to be 18.33 for the total area

of 22.91 for *E. coli*, 21.39 for the total area of 26.74 for *S. typhimurium*, and 20.28 for the total area of 25.35 for *L. monocytogenes*, and the DA distribution range is determined to be 3.77 to 8.57° for *E. coli*, 6.30 to 12.04° for *S. typhimurium*, and 9.07 to 16.70° for *L. monocytogenes*. The numbers of bacterial colonies used for these measurements of DAs were 230 for *E. coli*, 268 for *S. typhimurium*, and 249 for *L. monocytogenes*.

From the calculation results on the DA distribution range, it can be seen that the higher the DA acquisition probability, the wider the DA distribution range is for each bacterial species, and thus the overlapping areas among the DA distribution ranges of the three bacteria species increase with the acquisition probability. However, this overlapping area should be minimized for the identification accuracy of bacteria species. Considering this trade-off between the acquisition probability and the identification accuracy, we selected a standard acquisition probability as 75%, which is the maximum value among the DA acquisition probabilities that can keep the width of the above overlapping area below 1.5°. The DA distribution range obtained from the selected DA acquisition probability was designated as the optimal distribution range (ODR) of the DA for each bacterial species. The reason for setting the overlapping width of the DA to less than 1.5° is to choose an effective DA distribution range for bacteria classification by sacrificing a slight identification error that may occur in the boundary areas of the DA distribution ranges of the three bacteria species while maintaining a high DA acquisition probability. The ODRs derived from the selected DA acquisition probability (75%) were 3.94 to 8.23° for *E. coli*, 6.75 to 11.63° for *S. typhimurium*, and 10.14 to 16.57° for *L. monocytogenes*. In the above ODR of DAs, the DA overlapping widths were ~1.48° between *E. coli* and *S. typhimurium* and ~1.49° between *S. typhimurium* and *L. monocytogenes*. The actual acquisition probabilities of the DA, which can be evaluated by using the measured acquisition frequency of the DA based on the predetermined ODR, were ~80.9% for *E. coli*, ~74.3% for *S. typhimurium*, and ~76.7% for *L. monocytogenes*. The insets of Figs. 4(b), 5(b), and 6(b) show the regular residuals (i.e., deviations) between the measured DA acquisition frequency and the acquisition frequency fitted using the Gaussian distributions for *E. coli*, *S. typhimurium*, and *L. monocytogenes*, respectively. This deviation implies the frequency difference between the measured and fitted data, and the black solid line located at a value of 0 on the *y*-axis indicates the regression line. At a certain DA, the deviation is positive or negative when the measured acquisition frequency is greater or smaller than the fitted value, respectively. On the basis of these regular residuals, the average deviation of the measured DA acquisition frequency from the Gaussian distribution obtained by regression analysis was evaluated as ~1.05 for *E. coli*, ~1.12 for *S. typhimurium*, and ~1.10 for *L. monocytogenes*. The difference between the actual acquisition probability and the acquisition probability chosen for the ODR mainly results from the abovementioned deviation of the measured DA acquisition frequency from the Gaussian distribution. This frequency deviation is considered to originate from the variation of the DA due to changes in the colony height and shape, which may occur in the bacterial culture process according to the culture environment. In other words, since the characteristics of the bacterial colonies are very sensitive to the cultivation conditions, it should be considered that the DA distribution range may slightly vary and the resulting deviation may increase as the culture environment (i.e., the number of cultures) changes. Also, the maximum DA acquisition

frequencies of *E. coli*, *S. typhimurium*, and *L. monocytogenes* are 10, 12, and 12, respectively, and it can be confirmed from Figs. 4–6 that the DA having the maximum acquisition frequency for each bacterial species belongs to the ODR of the corresponding bacterial species. As described above, it is confirmed that it is convenient to distinguish among bacteria species when the DA distribution range is modeled by utilizing the probability density function, and it is possible to identify bacteria species with an acquisition probability similar to or higher than that selected for the ODR (here, 75%).

Figure 7 shows the DA distribution ranges of the bacterial colonies of *E. coli*, *S. typhimurium*, and *L. monocytogenes*, indicated as red, blue, and green symbols, respectively, according to the size of the colony (0.8–2.2 mm). Red, blue, and green solid arrows indicated as E, S, and L, respectively, represent the ODRs of the DAs for three bacterial species of *E. coli*, *S. typhimurium*, and *L. monocytogenes*, previously determined with the selected acquisition probability of 75%. As can be seen from the figure, the measured DAs of the bacterial colonies are concentrated in the ODR of the corresponding bacterial species (indicated as E, S, or L). Because two ODRs indicated as E and L, associated with *E. coli* and *L. monocytogenes*, respectively, have no intersection, i.e., no common ODR, and the measured DA of each bacterium is also rarely present in the ODR of the opposite side, these two bacteria species are easily and clearly distinguished. However, since the ODR indicated as S has overlapping DAs with the ODRs denoted by E and L, *S. typhimurium* may be difficult to separate from *E. coli* and *L. monocytogenes* in the DA ranges of 6.75 to 8.23° and 10.14 to 11.63°. Therefore, to increase the reliability of bacterial identification, it is necessary to derive the distribution range of the DAs, obtained by measuring the DAs of the LSPs for a large number of bacterial

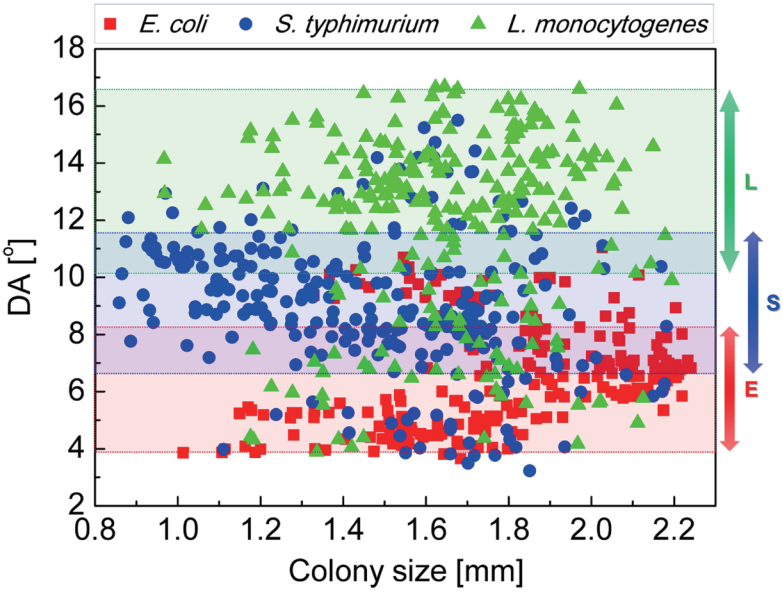


Fig. 7. (Color online) Measured DAs according to the colony size of *E. coli*, *S. typhimurium*, and *L. monocytogenes*.

colonies, and make a judgment on the bacteria species based on this distribution range. As mentioned above, the three bacteria species studied here have their own DA distribution ranges (or ODRs), and it is expected that these unique distribution ranges can be usefully harnessed for discriminating among these bacteria. In particular, this study can serve as a fundamental basis for the research of classifying various bacterial species through the exploration of the DAs of LSPs for other bacterial species. In brief, by analyzing the DA acquisition frequency of LSPs for three bacteria species, it was confirmed that a unique ODR could be found for each bacterial species. This dissimilarity in the ODR is presumed to be associated with the growth characteristics of each bacterial species. Our discrimination scheme based on this ODR of the DAs provides an alternative pathway to effective bacterial identification.

4. Conclusion

After capturing the LSPs of *E. coli*, *S. typhimurium*, and *L. monocytogenes* colonies, we calculated the DA by comparing the colony size with the LSP size for each bacterial colony and analyzed the acquisition frequency of the DAs for each bacterial species. Then, the ODR of the DA was derived for each bacterial species by performing nonlinear regression analysis based on the Gaussian distribution with respect to the acquisition frequency of the DAs and choosing a standard acquisition probability of 75%. The resultant ODR was determined as 3.94 to 8.23° for *E. coli*, 6.75 to 11.63° for *S. typhimurium*, and 10.14 to 16.57° for *L. monocytogenes*. Because *E. coli*, *S. typhimurium*, and *L. monocytogenes* have their own ODRs, it is expected that the classification reliability and reproducibility of bacterial species in the bacterial detection can be improved by obtaining the distribution range of the DAs through the analysis of DAs measured for sufficiently large numbers of bacterial colonies. Consequently, this study suggests a new way of classifying bacterial species using the DA distribution range of label-free bacteria unlike the conventional method. In particular, because numerous bacterial colonies are procured with only a single proliferation of bacteria, our discrimination scheme can be a cost- and time-effective means of identifying bacteria species. It is considered that the proposed identification method can be beneficially used as a subsystem for the secondary verification of existing bacteria detection/discrimination methods.

Acknowledgments

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