analytical chemistry

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Luminometric Label Array for Counting and Differentiation of Bacteria

Milla Högmander,[†] Catherine J. Paul,[‡,§] Sandy Chan,[‡,∥] Elina Hokkanen,[†] Ville Eskonen,[⊥] Tapio Pahikkala,[#] and Sari Pihlasalo*[,†,‡,⊥]

[†]Department of Cell Biology and Anatomy, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, FI-20520 Turku, Finland

[‡]Applied Microbiology, Department of Chemistry, Lund University, P.O. Box 124, SE-22100 Lund, Sweden

[§]Water Resources Engineering, Department of Building and Environmental Engineering, Lund University, P.O. Box 118, SE-22100 Lund, Sweden

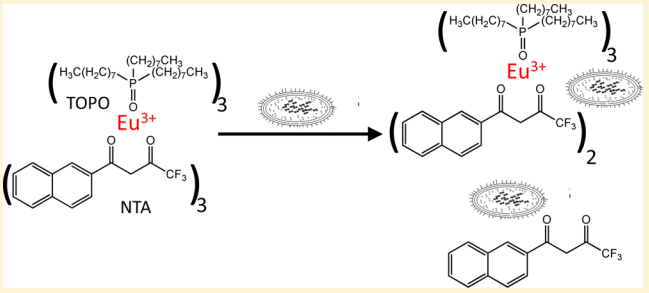
[∥]Sweden Water Research, Ideon Science Park, Scheelevägen 15, SE-22370 Lund, Sweden

[⊥]Laboratory of Materials Chemistry and Chemical Analysis, Department of Chemistry, University of Turku, Vatselankatu 2, FI-20500 Turku, Finland

[#]Department of Information Technology, University of Turku, Vesilinnantie 5, FI-20500 Turku, Finland

Supporting Information

ABSTRACT: Methods for simple and fast detection and differentiation of bacterial species are required, for instance, in medicine, water quality monitoring, and the food industry. Here, we have developed a novel label array method for the counting and differentiation of bacterial species. This method is based on the nonspecific interactions of multiple unstable lanthanide chelates and selected chemicals within the sample leading to a luminescence signal profile that is unique to the bacterial species. It is simple, cost-effective, and/or user-friendly compared to many existing methods, such as plate counts on selective media, automatic (hemocytometer-based) cell counters, flow cytometry, and polymerase chain reaction (PCR)-based methods for identification. The performance of the method was demonstrated with nine single strains of bacteria in pure culture. The limit of detection for counting was below 1000 bacteria per mL, with an average coefficient of variation of 10% achieved with the developed label array. A predictive model was trained with the measured luminescence signals and its ability to differentiate all tested bacterial species from each other, including members of the same genus *Bacillus licheniformis* and *Bacillus subtilis*, was confirmed via leave-one-out cross-validation. The suitability of the method for analysis of mixtures of bacterial species was shown with ternary mixtures of *Bacillus licheniformis*, *Escherichia coli* JM109, and *Lactobacillus reuteri* ATCC PTA 4659. The potential future application of the method could be monitoring for contamination in pure cultures; analysis of mixed bacterial cultures, where examining one species in the presence of another could inform industrial microbial processes; and the analysis of bacterial biofilms, where nonspecific methods based on physical and chemical characteristics are required instead of methods specific to individual bacterial species.



The detection and identification of bacterial species is of interest to many fields including medicine and industry. While the vast majority of bacteria are harmless or helpful for human health: participation to the recycling of nutrients; the fixation of nitrogen; putrefaction processes; conversion of dissolved hydrogen sulfide and methane to energy; and protection against diseases,[1] some bacteria are harmful pathogens. The rapid differentiation of particularly this last group of bacteria is required for safety, research, and quality control in biological, food, industrial, and medical laboratories. In clinical medicine, the rapid identification of bacterial species causing infections enables the selection of optimal antibiotic, fast decisions on treatments, efficient treatment, and reduction of antibiotic resistivity and health care costs. In the food industry, bacteria, such as *E. coli* O157:H7 and *Pseudomonas fluorescens*,[2,3] cause food poisonings and food spoilage, and early detection can prevent foodborne epidemics. However, the traditional plate count for detection of bacteria is time-consuming and laborious and often some knowledge about the type of bacteria suspected is required in order to select the appropriate assay conditions.[4] The results are based on microbial growth and are obtained after 24 h at the earliest. It is not suitable for the quantification of total bacteria, as the dead cells do not divide and part of the bacteria cannot be

Received: December 28, 2016
Accepted: February 9, 2017
Published: February 9, 2017

DOI: 10.1021/acs.analchem.6b05142
Anal. Chem. 2017, 89, 3208−3216

cultured. Thus, novel fast, specific, and cost-effective methods are needed.

Automated cell counters are commercially available. They are objective and less time-consuming compared to subjective hemocytometric count.[5] However, automated counters, such as automated hemocytometer,[6−9] Coulter counter,[10−15] and flow cytometers[7,16−19] can suffer from inaccuracy at low cell concentrations, low throughput, and high cost for both equipment purchase and routine operating.[20] Small cells with diameters <5 μm cannot be detected with automated hemocytometers, and thus, these instruments are not suitable for counting of bacteria. The Coulter counter requires an additional instrumental resetting for different cells.

Several microtiter plate methods have been developed and exploited for the counting of bacterial cells. Intracellular esterase,[21,22] AlamarBlue,[23−25] MTT,[26] neutral red,[23,27−32] and acid phosphatase[5,33−35] assays exploit enzymatic or cellular activities and have long incubation times, require incubation at elevated temperatures, and/or detect only viable cells. ATP detection[36−42] is a fast assay, however, it is sensitive to contaminating ATP and detects only viable cells. A total cell count, including all bacterial cells, can be obtained by detecting DNA with a DNA intercalator dye,[11,43−50] but these methods can be sensitive for free DNA.

Traditionally, bacteria have been identified with culture methods, which are time-consuming and laborious.[4] The existing genotypic and phenotypic and more advanced PCR methods[51−53] are complicated and time-consuming or they suffer from high cost, weak capability to detect new strains, and limited screening capacity.[54−58] The methods, such as gene microarray[59,60] and sequencing identification techniques,[61] exploit identification of specific genes and can miss new strains without the specific gene target or suffer from false-positive results due to inaccurate replication of DNA. Microscopy based methods, such as fluorescence in situ hybridization,[62] are useful but require complex procedures and user experience. Immunological techniques[63,64] are highly specific but can be limited to only known bacteria, as well as to the high cost of antibodies. Surface-enhanced Raman spectroscopy[65] requires a rugged surface that is hard to produce reproducibly, while methods based on mass spectrometry[66] often require advanced equipment and specialized personnel, as well as sample manipulation for isolation of target proteins or DNA. Biochemical arrays can be advantageous in that no antibodies are required. However, the reported arrays suffer from long testing time, detection of volatiles, poor limit of detection, complicated protocols, array format, and/or reading technique of the signal.[67−70] Moreover, nonspecific binding to nanoparticles, replacement of fluorescent polymers or enzymes, and fluorometric/colorimetric detection have been utilized for detection and identification.[71−75] However, these methods require the interaction of bacteria with solid surfaces, which limits the ability to detect small molecules, ions, and intracellular components.

Earlier, we reported on the label array method utilizing unstable lanthanide chelates for the analysis of honey and cacao brands[76] and for the detection and differentiation of metal ions.[77] The arrays were based on the interaction of the liquid sample with the chelates and additional chemicals. These interactions led to the sample-dependent modulation of the luminescence signal. Here, a novel label array for the counting, differentiation, and mixture analysis of bacterial species is presented. The lanthanide chelate interacts differently with the parts of the bacteria, such as their distinguishing surface and intracellular structure, providing the basis for signal modulation within the array (Figure 1). The presence of the bacteria is

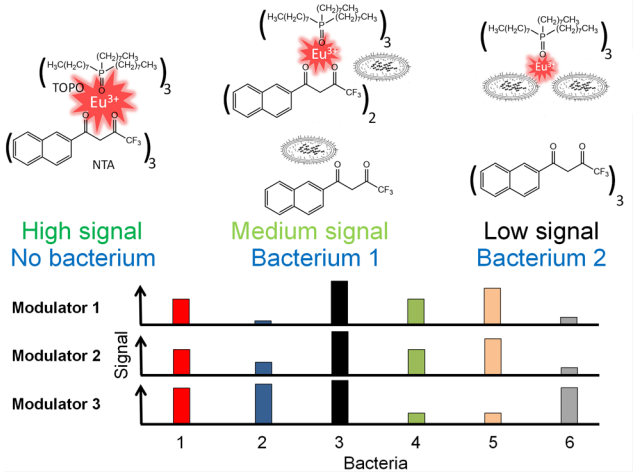


Figure 1. Principle of proposed nonspecific method for differentiation and quantification of bacterial species. The system is presented with one modulator example and with few possible interactions. The lanthanide chelate might break due to the bacterial sample, which leads to differences in luminescence signal and detection of bacterial species. Several modulators enable the differentiation of bacterial species.

detected as the increase or decrease in the luminescence signal depending both on the concentration and species of the bacteria and choice of modulators. The applicability was shown for the counting and differentiation of nine individual cultured bacterial species and mixtures of three bacterial species.

EXPERIMENTAL SECTION

Materials. AcroWell 96-well filter plates with a PVDF membrane (pore size 0.45 μm), AcroWell 96-well filter plates with a GHP membrane (pore size 0.45 μm), AcroPrep Advance 96-well filter plates with a GHP membrane (pore size 0.2 μm), and AcroPrep 96-well filter plates with a glass membrane (pore size 1.0 μm) were from Pall Life Sciences (Ann Arbor, MI). Bacteria (*Serratia liquefaciens*, *Staphylococcus epidermidis*, *Hafnia alvei*, *Bacillus licheniformis*, *Escherichia coli* JM109, *Aeromonas hydrophila*, and *Lactococcus lactis*) were obtained from the culture collection at Applied Microbiology, Department of Chemistry, Lund University (Lund, Sweden). *Bacillus subtilis* spores were ordered from Merck KGaA (Darmstadt, Germany) and *Lactobacillus reuteri* ATCC PTA 4659 was obtained from BioGaia AB (Stockholm, Sweden). Tryptic soy agar, agar−agar, peptone from casein, yeast extract, MRS broth powder, sodium chloride, dipotassium hydrogen phosphate, disodium hydrogen phosphate dodecahydrate, potassium dihydrogen phosphate, magnesium sulfate heptahydrate, hydrochloric acid, sodium hydroxide, and acetone were ordered from Merck KGaA (Darmstadt, Germany). Triton X-100, Michler's ketone, sodium sulfate, sodium nitrate, magnesium chloride hexahydrate, potassium fluoride, copper(II) chloride, diethylenetriaminepentaacetic acid (DTPA), and ampicillin were from Sigma-Aldrich (St. Louis, MO), calcium chloride dihydrate from J. T. Baker (Deventer, Holland), tryptic soy broth powder from Becton, Dickinson and Company (Sparks, MD), D(+)-glucose and tris(hydroxymethyl)aminomethane (Tris) from VWR Chemicals (Leuven, Belgium), 95% ethanol from Solveco AB

DOI: 10.1021/acs.analchem.6b05142
Anal. Chem. 2017, 89, 3208−3216

(Rosersberg, Sweden), 1-hexadecylpyridinium chloride and nitriloacetic acid (NTA) from Acros Organics (Geel, Belgium), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) from Bio-Rad Laboratories (Hercules, CA), europium fluorescence intensifier from Kaivogen Oy (Turku, Finland), and dimethyl sulfoxide from Fisher Scientific (Loughborough, U.K.). Europium(III) chloride hydrate $EuCl_3 \cdot xH_2O$ was purchased from Alfa Aesar GmbH and Co. KG (Karlsruhe, Germany) and terbium(III) chloride hexahydrate $TbCl_3 \cdot 6H_2O$ from Sigma-Aldrich (St. Louis, MO). 4,4,4-Trifluoro-1-(2-naphthalenyl)-1,3-butanedione (β-NTA), 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron) disodium salt monohydrate, 4-hydroxy-6-(trifluoromethoxy)quinoline-3-carboxylic acid, nalidixic acid, and oxolinic acid were obtained from Acros Organics (Geel, Belgium) and tri-*n*-octylphosphine oxide (TOPO) from Sigma-Aldrich (St. Louis, MO). High purity Milli-Q water was used to prepare all aqueous solutions.

Methods. *Preparation of Culture Media.* For LB medium, 10 g of sodium chloride, 10 g of peptone from casein, and 5 g of yeast extract were dissolved in 1 L of water. For TSB medium, 30 g of tryptic soy broth powder was dissolved in 1 L of water. LL medium was prepared in three parts: 6.25 g of yeast extract, 3.13 g of dipotassium hydrogen phosphate, and 3.13 g of potassium dihydrogen phosphate were dissolved in 1 L of water, 10 g of D(+)-glucose in 100 mL of water, and 0.5 g of magnesium sulfate heptahydrate in 100 mL of water. These solutions were autoclaved at 121 °C, for 15 min. Before use, LL medium was prepared by mixing 8 parts of yeast extract/phosphate solution, 1 part of D(+)-glucose solution, and 1 part of magnesium sulfate solution. For MRS medium, 52.2 g of MRS broth powder was dissolved in 1 L of water, and it was sterile filtered with 0.2 μm syringe filter. For tryptic soy agar, 40 g of tryptic soy agar powder was dissolved in 1 L of water by heating. LB agar was prepared by dissolving 15 g agar−agar, 10 g of sodium chloride, 10 g of peptone from casein, and 5 g of yeast extract in 1 L of water by heating. Both broths were autoclaved before pouring into Petri dishes.

Culture of Bacteria and Preparation of Samples. *Bacillus subtilis*, *Serratia liquefaciens*, *Staphylococcus epidermidis*, and *Hafnia alvei* were cultured with shaking in TSB broth, *Bacillus licheniformis*, *Escherichia coli* JM109, and *Aeromonas hydrophila* in LB broth, and *Lactococcus lactis* in LL broth. *Lactobacillus reuteri* ATCC PTA 4659 was cultured in MRS broth without shaking. After overnight cultivation at 37 °C, all cultures were washed four times in 0.15 M NaCl and then concentrated by centrifugation (1500 g). The absorbance of the diluted samples was measured at 620 nm in disposable cuvettes.

Counting of Bacteria. Dilutions of bacteria washed in 0.15 M NaCl were prepared in 0.15 M NaCl. For the counting of bacteria, 300 μL of each dilution was pipetted onto filter plates with a prewetted PVDF membrane. The suspensions were filtered in a vacuum and modulator 1 containing a lanthanide ion, ligands, and additional modulators, was added to the wells (see Table S1 in the Supporting Information). The europium luminescence emission intensities were measured in a 2000 μs window after a 300 μs delay time with 340 nm excitation and 616 nm emission wavelengths using a Labrox plate reader (Labrox, Turku, Finland). All experiments were performed in triplicate, and an average of the normalized signals is presented in curves with standard deviation as the error bars.

Differentiation of Bacterial Species. Bacteria washed in 0.15 M NaCl were diluted to concentrations corresponding to absorbance 0.0005 or 0.0015 in 0.15 M NaCl or 30 mM Tris, pH 8. For the differentiation of bacterial species, 300 μL of these dilutions were pipetted to filter plates having a glass, GHP, or prewetted PVDF membrane with 0.2, 0.45, and 1.0 μm pore sizes. The suspensions were filtered in a vacuum. The additional modulator was added to each well and filtered in a vacuum for modulators 11−13. The modulator solutions 1−17 containing a lanthanide ion, ligands, and an additional modulator (see Table S1 in the Supporting Information) were added to the wells. The lanthanide luminescence emission intensities were measured in a 2000 μs window after a 300 μs delay time with 340 nm excitation and 616 nm emission wavelengths for europium and in a 400 μs window after a 400 μs delay time with 340 nm excitation and 545 nm emission wavelengths for terbium using a Labrox plate reader (Labrox, Turku, Finland). All experiments were performed with four replicates.

Effect of Modulator Components on Ability of E. coli JM109 to Divide. To test the effect of the modulator components on the ability of *E. coli* JM109 to divide, the array reagents corresponding to modulators 1−17 were added to microcentrifuge tubes containing washed *E. coli* JM109 (final concentration corresponding to absorbance 0.0015). For modulators 11−13, ethanol was first incubated with *E. coli* JM109 in low volume after which the rest of the array reagents were added. After 35 min of incubation, 1:150 and 1:1500 dilutions were prepared and 100 μL of the dilutions were spread to tryptic soy agar plates. The colonies were calculated after 25 h and confirmed for new colonies after 67 h of cultivation at 37 °C.

Mixture Analysis of Bacterial Species. Different mixtures of *Bacillus licheniformis*, *Escherichia coli* JM109, and *Lactobacillus reuteri* ATCC PTA 4659 washed in 0.15 M NaCl were prepared and diluted to concentrations corresponding to absorbance 0.0015 in 0.15 M NaCl or 30 mM Tris, pH 8. For the mixture analysis of bacterial species, 300 μL of these dilutions were pipetted to filter plates with a GHP or prewetted PVDF membrane and suspensions were filtered in a vacuum. The additional modulator was added to each well and filtered in a vacuum for modulator 12. The modulator solutions 1, 12, and 14−17 containing a lanthanide ion, ligands, and an additional modulator (see Table S1 in the Supporting Information) were added to the wells. The lanthanide luminescence emission intensities were measured at several time points after the pipetting in a 2000 μs window after a 300 μs delay time with 340 nm excitation and 616 nm emission wavelengths for europium and in a 400 μs window after a 400 μs delay time with 340 nm excitation and 545 nm emission wavelengths for terbium using a Labrox plate reader (Labrox, Turku, Finland). All experiments were performed with three or four replicates.

RESULTS AND DISCUSSION

We developed a novel luminometric label array for counting and differentiation of bacterial species. The detection relies on the nonspecific interactions and competition between the target bacteria, unstable lanthanide chelates, and selected chemicals as additional modulators. The term modulator is used in this article for the combination of the lanthanide ion, the ligands, other chemicals, and the type of filter plate. The arrays were performed in filter microtiter plates to eliminate the removal of assay contaminants and matrices and to enable the analysis of different sample matrices, such as household water. The sample was filtered through the wells, and after the addition of the array components (modulators), the wells were measured with

DOI: 10.1021/acs.analchem.6b05142
Anal. Chem. 2017, 89, 3208−3216

a luminescence plate reader using time-gated measurements. The simplified principle of the array with the well-known Eu^{3+}:2-naphthoyltrifluoroacetone (β-NTA):tri-*n*-octylphosphine oxide (TOPO) chelate as an example is presented in Figure 1 and multiple other chelating structures, covering also other lanthanides, are known.[78] The lanthanides and ligands can also be varied in different concentrations and ratios. The Eu^{3+} chelate is added to the sample. In the absence of bacteria and under chelate stabilizing array conditions, β-NTA and TOPO form the chelating coordination bonds providing the high luminescence signal. In the presence of bacteria, the Eu^{3+} chelate interacts nonspecifically with different bacterial species, their metabolites, and other components added to the sample, which leads to the disruption of the chelating structure. For example, β-NTA or TOPO interact with the sample, are replaced by the sample, or Eu^{3+} ion binds to the bacteria and the chelating coordination bonds are lost. However, the bacteria might also serve as protection in the Eu^{3+} chelate destabilizing environment and result in an increase in the signal. These phenomena enable the detection and counting of bacteria.

An attempt to explain the interactions of the Eu^{3+} chelate with bacteria was performed with following tests. The binding of Eu^{3+} ion to bacteria was tested by incubating Eu^{3+} and β-NTA/TOPO mixture in the presence and absence of *B. subtilis*. After washing by centrifugation, the bound Eu^{3+} ion was quantified by utilizing the luminescence enhancement with europium fluorescence intensifier. The signal was approximately hundred times higher in the presence of bacteria compared to the absence of bacteria indicating the clear binding of Eu^{3+} ion to bacteria (data not shown). The binding of ligands to bacteria was tested by incubating β-NTA/TOPO mixture in the presence and absence of *B. subtilis*. These mixtures were centrifuged and Eu^{3+} ion was added to the supernatant. The signal was approximately 10× higher for the sample in the absence of bacteria compared to the presence of bacteria indicating also the binding of ligands to bacteria (data not shown). These tests showed that the binding of both Eu^{3+} ion and ligands can explain the loss of signal in the presence of bacteria and any interactions could not be excluded.

Different bacterial species can interact with the chelate with varying efficiencies, leading to differing signal levels, that is, the modulation of the signal. It is known from the literature that the Eu^{3+} ion chelated to thenoyltrifluoroacetone binds to nucleic acids, phospholipids, inorganic polyphosphates, and proteins.[79] Bacterial species interact differently with the Eu^{3+} chelate leading to the modulation of the luminescence signal, which then give a specific fingerprint for each sample or bacterial species. The application of several different chelates and additional modulators, such as salts, buffers, detergents, small molecules, antibiotics, and solvents, further improves the differentiation. The unique fingerprint for each distinct bacterial species is created using the array of different modulators which provide a multidimensional luminescence signal profile. The differing physical characteristics between bacterial species or types (for instance cell envelope structures, such as Gram negative vs Gram positive) are identified from the differences in luminescence profiles generated from their structure and cellular surface entities, such as fimbriae and S-layers. If the bacteria are partly broken with chemicals, differentiation can then be according to the sites, metabolites, and contents inside the bacteria and not only the surface structures outside. The differences in the susceptibility of bacterial cell walls to disaggregating agents are also exploited. The cellular contents, surface, and structures are typically very complex containing different compounds, polymers, lipids, and peptides in varying ratios, which provides a vast number of variables for the identification.

Counting of Total Bacterial Cell Numbers. The counting of nine different single strains of bacteria prepared in pure culture and with varying properties was demonstrated with the developed array. The properties of all bacteria, including their taxonomy and the result of Gram staining, are listed in Table S2 in the Supporting Information. Five Gram positive and four Gram negative bacteria were included, covering six families (*Bacillaceae, Aeromonadaceae, Enterobacteriaceae, Staphylococcaceae, Streptococcaceae*, and *Lactobacillaceae*) and eight genera (*Bacillus, Aeromonas, Escherichia, Staphylococcus, Serratia, Lactococcus, Lactobacillus*, and *Hafnia*). They represent a selection of bacteria associated with foodborne and waterborne illnesses, protective beneficial bacteria, natural human flora, and water environments. To demonstrate the performance of the method, calibration curves for washed overnight cultured bacteria were measured with a single modulator 1 (see Table S1 in the Supporting Information). Of the tested modulators, this modulator gave the highest sensitivity and similar response for all bacteria examined. The effect of the Eu^{3+} chelate constitution (varying ratios between β-NTA/TOPO concentration and Eu^{3+} concentration) of this modulator was tested (see Figure S1 in the Supporting Information). The results show that both β-NTA and TOPO are required to gain high sensitivity and performance for the method. In the absence of bacteria, the signal increases, as the β-NTA and TOPO concentrations increase. The signal levels out at excess concentrations of ligands (Figure S1a). High ratio between signals at high and zero concentration of bacteria can be obtained by utilizing Eu^{3+} chelates with slight excess number of ligands compared to the number of ligands determined by the coordination number of Eu^{3+} ion (Figure S1b). Moreover, the highest sensitivity for the detection of bacteria can be gained with modulator 1 containing 27 β-NTA and 27 TOPO molecules per one Eu^{3+} ion (data not shown).

For simplicity, we correlated the bacterial concentration to the optical density reading at 620 nm. For differently sized bacteria, our result might depend on the size of bacteria and thus, the luminescence signal might correlate better with the mass than the count of bacteria, as the optical density measures total mass or volume of bacteria rather than the count.[80] The calibration curves as a function of absorbance are shown in Figure 2. It should be noted that the shown absorbance values below 0.001 are not measurable, however, the absorbance of the stock was measured and the absorbance values of the diluted suspensions were computational. The calibration curves overlap well and the deviation from other curves is the highest for *L. reuteri* ATCC PTA 4659. The limit of detection is below absorbance of 2×10^{-5} for all bacterial species (based on the calculation of the standard deviation 2 × SD of the luminescence signal with the assumption of the linear relation at low bacterial counts). Assuming one absorbance unit corresponds to approximately 5.9×10^8 bacteria per mL (obtained by relating the absorbance at 620 nm and the standard plate count for *E. coli* JM109 overnight culture), this limit of detection corresponds to 10^4 bacteria per mL. However, the sample volume was 300 μL and, consequently, the limit of detection for a 3 mL sample is thus below 10^3

DOI: 10.1021/acs.analchem.6b05142
Anal. Chem. 2017, 89, 3208−3216

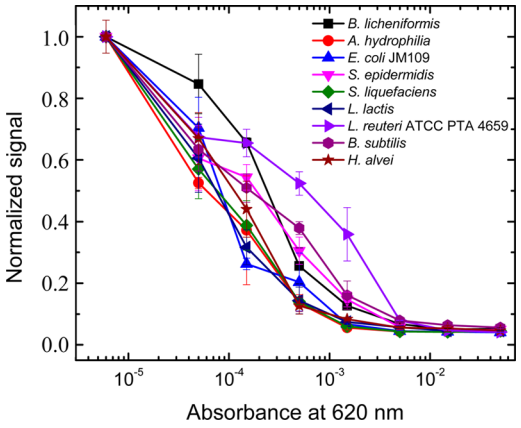


Figure 2. Counting of different bacterial species with a single modulator 1.

bacteria per mL. The dynamic range is reasonably wide, approximately 2 orders of magnitude, and the average coefficient of variation for signal using three replicates for all measured calibration curves was 10%. This sensitivity and dynamic range covers well the household water samples having typically 10^3–10^6 bacteria per mL[81−85] and suggests that the method presented could be applicable for the quick analysis of bacteria in drinking water samples. The achieved limit of detection of the developed array is higher than the limits of standard plate count[86] and ATP detection[36−42] methods possessing a limit of detection of single cells per sample. However, they are capable of detecting only the viable cell numbers (not total count). Moreover, ATP detection is sensitive for contaminative ATP and the plate count is time-consuming and requires culturable bacteria in culture conditions. The limits of detection for other methods, such as hemocytometry, flow cytometry, or Coulter counter,[6−19,87] are either higher or similar to the limit reached with the developed label array, however, the array presented here has the additional advantages for bacterial analysis in that it combines user-friendliness and high-throughput with low costs.

Differentiation of Bacterial Strains. We demonstrated the performance of the developed array for differentiation of nine single strains of bacteria utilizing 17 different modulators (see Table S1 in the Supporting Information). Different modulators have their characteristic tendencies to distinguish different targets. For example, as the Tb^{3+} chelates possess high water solubility, they possess high selectivity for ionic interactions, whereas Eu^{3+} chelates have a higher tendency for hydrophobic interactions. The additional modulators, such as detergents, ethanol, and antibiotics, are exploited, for example, for the (partial) breakage of bacteria. We studied the effect of the modulator components to the activity of bacteria by testing the ability of *E. coli* JM109 to divide after the exposure to the array components (see Table S3 in the Supporting Information), although the method is not only dependent on the effect of the modulators to the activity of bacteria, but instead, on all nonspecific interactions of modulator components with bacteria. The components corresponding to modulators 4, 9, and 11−13 containing 1-hexadecylpyridinium chloride or ethanol inhibited the growth completely. The effect of other components was less significant. The tested cultured and washed bacteria presented here were diluted to the same absorbance values. The prepared dilutions of the samples were applied to all 17 modulators and the luminescence signals were measured to identify the bacterial species present in the sample. Luminescence signal profiles were obtained for each bacterial species enabling the differentiation of all nine distinct bacterial species. This was performed via training a MTGRLS model (see the Supporting Information for the details) and the models ability to perform differentiation also on samples not used to train it was confirmed with LOOCV. The heatmap containing the model predictions obtained from LOOCV showed that each individual bacterial species generated a different and unique signal profile (Figure 3a). All four replicates measured for each species could be separated from the replicates of the other species including members of the same genus (*B. licheniformis* and *B. subtilis*) and as all nine bacterial species could be separated from each other, suggests that the species could be predicted by applying linear models. This data was used as a training data set to show the performance in blindfolded analysis and the repeatability. The measurements for the differentiation of bacteria were repeated for the modulators 1 and 2 having the highest differentiation ability and the data analysis was performed with the blinded test samples to distinguish same set of bacterial species (see Figure S2 in the Supporting Information). High ability for the differentiation of five bacterial species was obtained for the training data set obtained with modulators 1 and 2. Similarly, five bacterial species could be well distinguished in the blind test and four of these were same bacterial species than five species that were distinguished in the training set.

Our results suggest that the array could be a useful tool for the differentiation of bacterial species in mixtures with low diversity, such as monitoring strategic cocultures being developed for food and biotechnology applications including biofuels.[88,89] Other biotechnology applications could include routine monitoring of single cultures as deviation of that signal could indicate unwanted growth of other organisms that could derail the biotechnological production process, including controlled fermentations for food products, biofuel, and industrially relevant enzymes.[90,91]

Principal component analysis was further performed to examine and highlight differences, similarities, and relations between the strains of bacteria. The two first principal components are shown in Figure 3b. Gram negative bacterial species clustered together, while Gram positive bacterial species were more widely dispersed, with the exception of *L. lactis*, and separated from the Gram negatives. While all Gram negative bacterial species (*A. hydrophilia*, *E. coli* JM109, *S. liquefaciens*, and *H. alvei*), had quite similar fingerprints and principal components, *L. lactis* was a Gram positive bacterium that partly overlapped with the analysis of Gram negatives, suggesting that it is not only the membrane properties contributing to the Gram staining character that are the basis for the separation in the assay. At species level, although *B. licheniformis* and *B. subtilis* are differentiated, a similar value for the second principal component could reflect the taxonomic similarity for these two bacterial species and *B. subtilis* and *S. epidermidis*, both order *Bacillales*, have a similar fingerprint according to the analysis. Additional testing of diverse members of species *Bacillus* and order *Bacillales* would confirm if this pattern could be consistent for these types of bacterial strains. The three species tested from family *Enterobacteriaceae*: *E. coli* JM109, *S. liquefaciens*, and *H. alvei*, showed similarities in their luminescence signal profiles and in the first principal component. The differences observed according to the Gram staining properties show a link between the luminescence

DOI: 10.1021/acs.analchem.6b05142
Anal. Chem. 2017, 89, 3208−3216

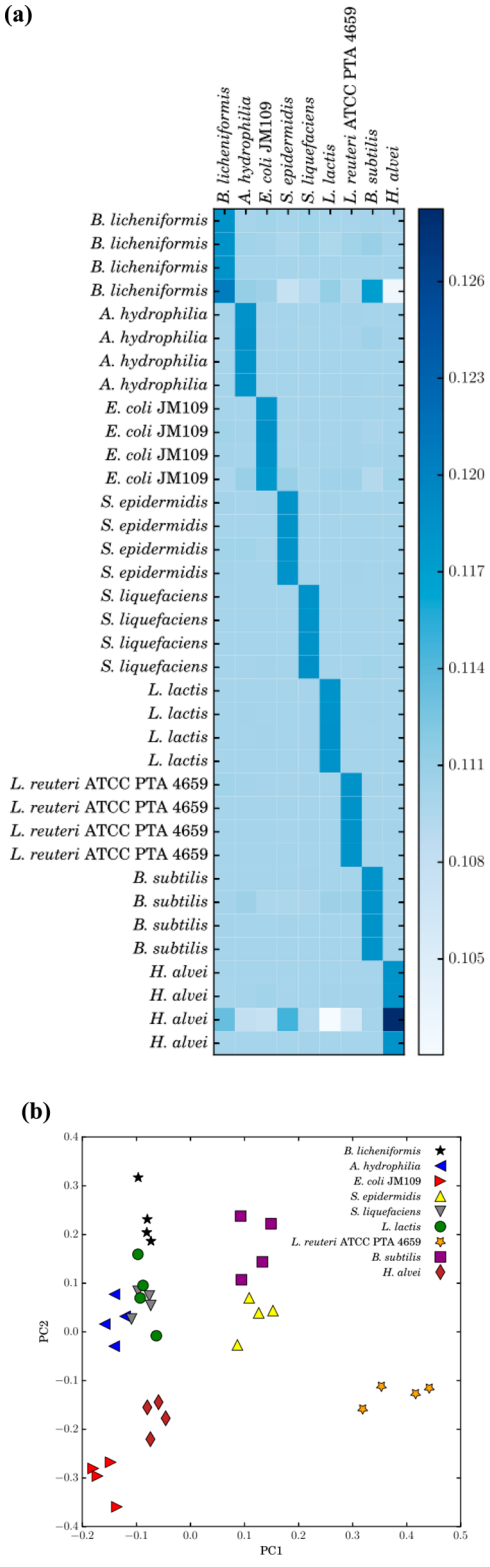


Figure 3. Differentiation of bacterial species with modulators 1−17 by a (a) linear separation (heatmap) and (b) principal component (first two principal components) analysis.

profiles and physical properties of the bacteria strengthening the argument that the modulators are responding to the properties of the cells. Therefore, the array might find use also in the classification of bacteria. However, wider study is required to reveal the specific physical and/or biological explanations for the observed relationships between the modulators and the properties and taxonomy of bacteria. Certain surface features of bacteria which could be participating in the observed results could include lipopolysaccharide, which decorates Gram negative bacteria with an outer shell of carbohydrates, capsular structures, or motility organelles such as flagella.[92,93]

Other components found in the samples, such as (in)organic matter originating from environmental samples, may additionally affect the signal and result in an incorrect analysis. However, the developed label array can be taught to distinguish or mask different contaminants and matrices and the detection of a large range of contaminants can be regarded even as an advantage for the versatility of the array. In this study of the application for bacterial analysis, total bacteria were separated from the matrix by the filtration in filter microtiter plates to further reduce the matrix effects. To study the effect of the sample matrix, a part of the modulators were added to both fresh and previously frozen drinking water samples containing bacteria. The bacteria could be detected only from the fresh sample (data not shown), which suggests that the developed array reacts mainly to intact bacteria and not for other contaminants or bacteria with broken cell walls. This is similar to limitations encountered with flow cytometry and plate counts. Moreover, the effect of different ions and metal chelators to two modulators of the method were tested (see Table S4 in the Supporting Information). No significant interference could be observed or the interference is low enough that it could be taken into account by utilizing similar sample matrices.

Mixture Analysis of Ternary Bacterial Samples. Mixtures of *B. licheniformis*, *E. coli* JM109, and *L. reuteri* ATCC PTA 4659 pure cultures were analyzed to demonstrate the applicability for real samples where the presence of several different species is known or desired. Different mixtures containing 0, 33, 67, or 100% of each bacterial species, based on the optical density reading at 620 nm, were prepared from washed bacteria and the array measurements were performed with six modulators (1, 12, and 14−17: see Table S1 in the Supporting Information). A MTGRLS model (see the Supporting Information for the details) was trained with the luminescence signals of the samples and the means and standard deviations of the predicted fractions of each bacterial species obtained via LOOCV are shown in Figure 4. Luminescence signal profiles for most of the mixtures could be explained as compilations of the profiles generated by those of the single bacterial strains, and their percentages in the samples. The fractions for mixtures containing *L. reuteri* ATCC PTA 4659 and *B. licheniformis* were accurately predicted, while mixtures containing *E. coli* JM109 were not as clear. This suggests that the analysis of mixtures may depend on the individual strains being examined, however the result is a promising demonstration of the developed array for the analysis of more complicated mixture samples. Conducting biotechnological transformations using bacteria can benefit from using two different types of bacteria that can co-operate in coculture. This includes biohydrogen,[88,94] lipid moieties,[95] and bioethanol.[96] The array developed here could be applied for optimization and routine monitoring of these next-generation biotech applications.

DOI: 10.1021/acs.analchem.6b05142
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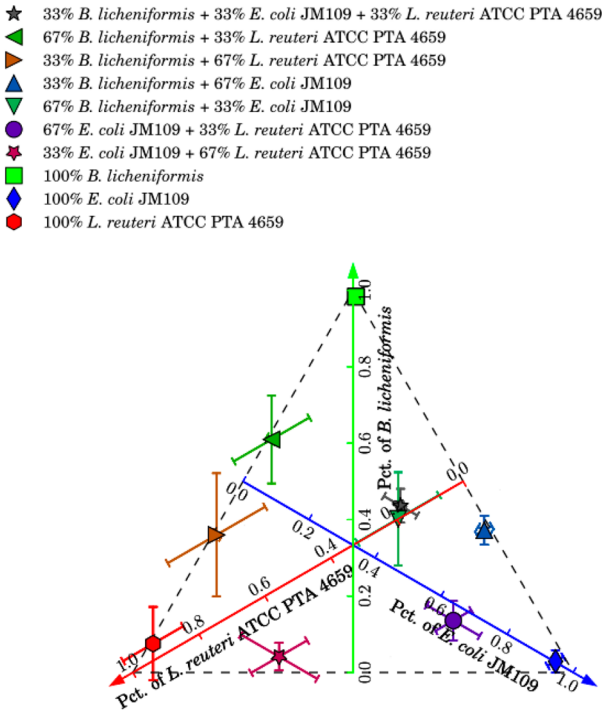

Figure 4. Mixture analysis of bacterial species (*B. licheniformis*, *E. coli* JM109, and *L. reuteri* ATCC PTA 4659) with modulators 1, 12, and 14−17.

CONCLUSIONS

In this article, we developed a novel and user-friendly label array for counting and differentiating pure cultures and mixtures of bacterial species. It was performed with a simple mix-and-measure approach for liquid samples pipetted and filtered into the filter microtiter plate wells. The array was created by adding the chemical entities to the microtiter wells containing the samples. The detection limit below 10^3 bacteria per mL can be reached, and thus, it covers well the quantities generally desired for analysis of bacteria isolated from household water samples and it is similar to the ones obtained with automatic cell counters. The label array allowed the differentiation of nine species of bacteria, including those within the same order or genus. The mixture analysis was demonstrated with ternary bacterial mixtures, which supports that the developed array could be exploited even for complicated real samples, including cocultures in biotechnology applications. The array in high-throughput format requires no special expertise or costly instruments, because the modulator components are simply added to the filtered samples in microtiter wells, and the luminescence is measured with microtiter plate readers commonly found in biological laboratories. The developed array could potentially be utilized in the future, for example, for quality control of food and water where specific strains of bacteria need to be monitored, and for nonspecific detection of groups of bacteria, for example, in the aggregates formed in biofilms. Furthermore, it could potentially be widened to address needs in the food industry, clinical laboratories, and environmental samples containing bacteria and even for eukaryotic cells. The array could be utilized as a high-throughput system for processing samples where the rapid and generalized detection of a change in the luminescence signal profile could identify and prioritize suspicious samples for more extensive examination.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b05142.

The details of the data analysis and the results for the repetition test for differentiation of bacteria performed as blindfolded data analysis, constituents of modulators, effect of the Eu^{3+} chelate constitution for the performance of the method, features of bacterial species, ability of bacteria to divide after the exposure to the array components, and effect of possible interfering agents (PDF).

AUTHOR INFORMATION

Corresponding Author

*E-mail: sari.pihlasalo@utu.fi. Phone: +358 29 450 3172.

ORCID

Sari Pihlasalo: 0000-0002-2411-6724

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the funding from the Academy of Finland (Grant 258617), the Finnish-Norwegian Medical Foundation, the Magnus Ehrnrooth Foundation, the Finnish Food Research Foundation, and the Ahti Pekkala Foundation.

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