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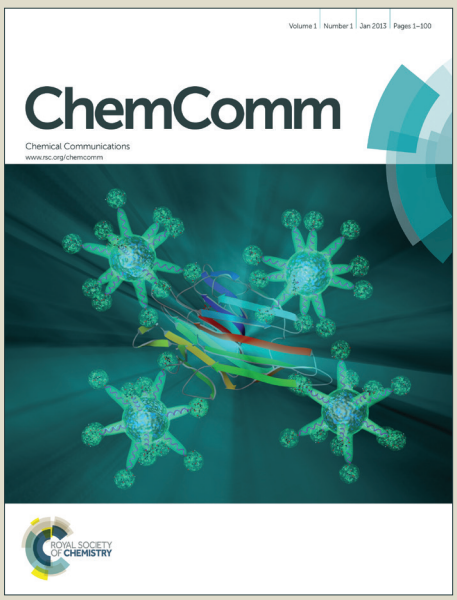

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Proteome reactivity profiling for the discrimination of pathogenic bacteria

Cite this: DOI: 10.1039/xoxxoooooox

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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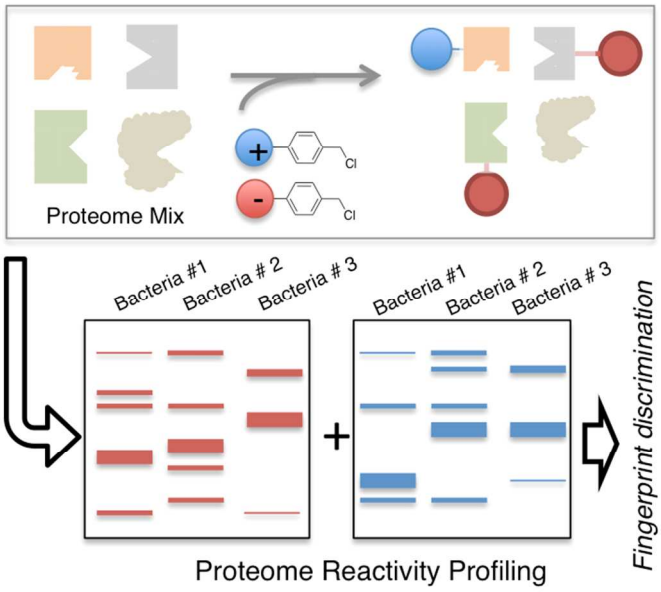
Diverse proteome reactivity profiles were obtained using small-molecule electrophiles. Based on cross-reactivity profile, each protein generated a unique reactivity fingerprint. Here, we report the first proteome reactivity signature-based discrimination of 11 bacteria. Perfect differentiation of 11 bacteria can be achieved using 2 benzyl-halide probes.

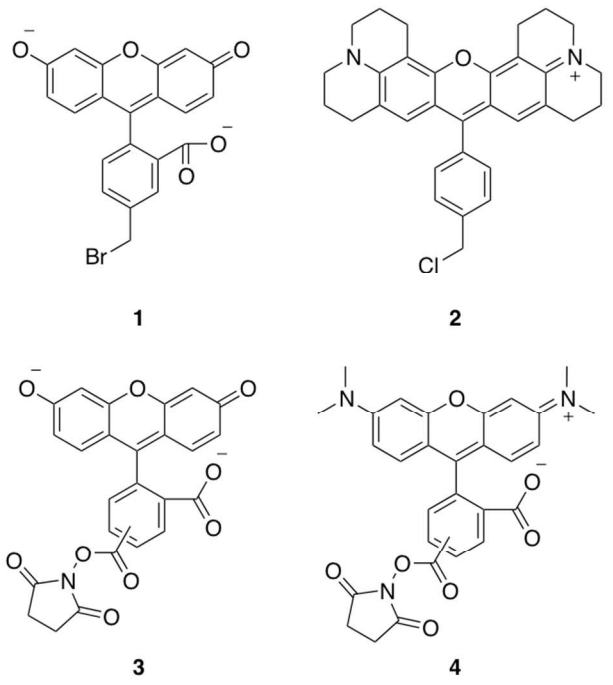
Activity-based protein profiling (ABPP) has been successfully applied to monitor the functional state of enzymes in complex proteome using reactive chemical probes.[1, 2] The basis of ABPP is chemical probes in which the electrophiles are connected to reporter tags. Chemical probes make a covalent bond at the active site of enzyme via its catalytic reaction, and the labeled enzymes can be further visualized or identified in high-throughput manner. Depending on the reactive functional groups, many kinds of enzymes can be targeted, including serine protease, cysteine hydrolase, and kinases.[3] As an alternative to conventional antibody-based techniques, ABPP has a merit in that it can provide intact functional status of enzyme instead of expression abundances. Furthermore, it is possible to profile multiple enzymes within superfamily from a single experiment since they share enzymatic intermediate status. Thus far, the focus has been the design of novel chemical probes for various enzyme superfamilies and the identification of target enzymes for each chemical probe.

Series of electrophiles have been extensively investigated as chemical probes to unveil target proteins and their preference for labeling amino acid residues.[4, 5] Based on the previous reports, small-molecule electrophiles often lack selectivity toward a single class of enzyme superfamily,[6] and they can target distinct classes of enzymes instead.[7] Moreover, few hyper-reactive residues can also make a covalent bond, though such residues are not enzymatic active site.[8] We envisioned that all kinds of such cross-reactivity could be a valuable property to generate fingerprints of individual pathological status of proteome. Although reactivity profile of chemical probe has been utilized for enzyme inhibitor screening and identification of functional cysteine residues for each protein recently,[8, 9] its application for multi-analyte sensing is largely unexplored. Herein, we describe an alternative application of reactive chemical probes for discrimination of pathogenic bacteria (Scheme 1).



Scheme 1. Reactivity profile based fingerprinting

The idea was inspired by our taste and smell sensory organs. We use combinatorial signals from array of sensory receptors to identify and differentiate taste and smells.[10] Instead of designing ultra-selective sensors for a target molecule, array-based sensor utilizes distinctive response signatures. The cross-reactive sensor array has been applied using colorimetric dyes or synthetic receptors that differentially change color responses depending on the analytes, such as organic amines,[11] volatile organic compounds (VOC),[12] amino acids,[13] carbohydrates,[14] metal ions,[15] and beverages.[16-18] More recently, aggregation pattern of nanoparticle polymer has also been utilized to discriminate biological samples.[19-22] In the present work, reactivity signature of proteome was utilized for differential sensing.



Scheme 2. Structures of electrophiles for proteome reactivity profiling.

Most of chemical probes for ABPP are neutral electrophiles covalently linked to reporter tag with aliphatic linker. In order to maximize diversity of reactivity profile for chemical probes, we choose charged aromatic fluorescent compounds that contain either benzyl halide or *N*-hydroxysuccinimide (NHS) (Scheme 2). These two functional groups differ in their electrophilicity, therefore different reactive residues can be labeled depending on the nucleophilicity profile. Additionally, engaged target proteins can be diversify based on the charge state and binding pocket structure of protein.

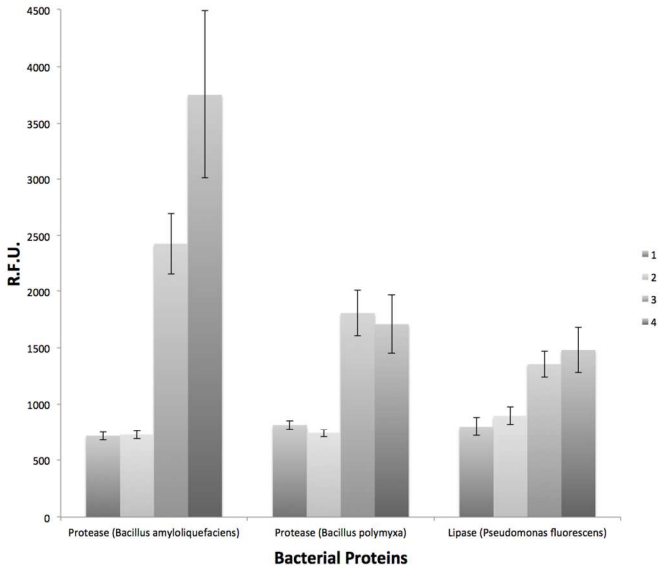


Fig 1. Reactivity profile of chemical probes towards three proteins; Protease from *Bacillus amyloliquefaciens* and *Bacillus polymyxa*, Lipase from *Pseudomonase fluorescens*. Each chemical probes (1 μM) were incubated in the protein mixture of (5 mg/ml each) for 10 min and fluorescence signal was obtained from SDS-Gel image.

As a proof-of-concept study, we prepared 3 bacterial protein mixture containing proteases and lipase (protease from *Bacillus amyloliquefaciens* and *Bacillus polymyxa*; lipase from *Pseudomonas fluorescense*), and tested our hypothesis using 4 chemical probes. These proteins were chosen based on isoelectric points from 5 to 9 ($pI_{protease\_Bacillus.Amyloliquefaciens}$: 8.31, $pI_{protease\_Bacillus.polymyxa}$: 5.05, $pI_{lipase\_Pseudomonas.flujorescense}$: 5.12), which yield different charge state of protein at pH 7.4 conditions (20 mM, PBS). Protein solutions (5 mg/ml) were mixed with each chemical probe (10 μM) and incubated 10 min at room temperature followed by SDS PAGE (Fig S1). As we expected, reactivity profile exhibited unique fingerprints for individual protein and chemical probes (Fig 1, Fig S2). Generally, NHS probes (**3**, **4**) showed stronger fluorescence signal than benzyl halide probes (**1**, **2**) due to the high reactivity. However, detail reactivity trends differed not only depending on electrophiles, but also charge state and chemical structure of each probe. It is also noteworthy that fluorescent intensities of labeled bands were diminished in a dose dependent manner when pre-incubated with enzyme inhibitors (Fig. S3, S4). This observation indicated that electrophiles are targeted enzyme activity *in vitro*.

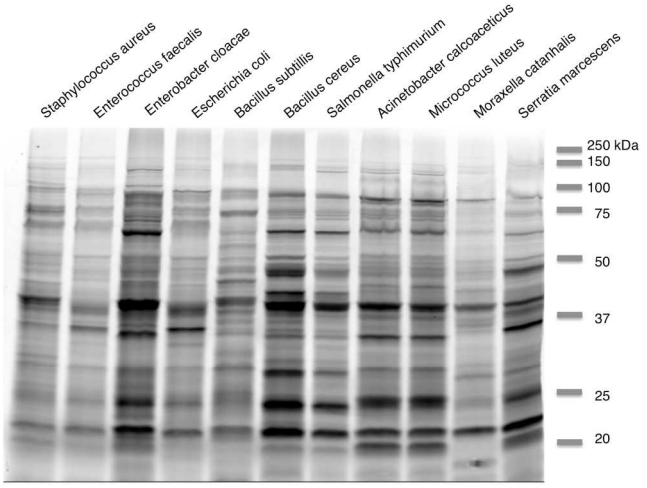


Fig 2. Bacterial proteome reactivity profiles of chemical probe **1**. 11 bacteria proteome exhibit divergent thiol reactivity. SDS PAGE was performed using 12 % gel. Fluorescence gel image was obtained using excitation laser 488 nm and emission filter 530 nm.

Encouraged by distinctive reactivity fingerprint of model protein mixture, we further examined the reactivity profile in pathogenic bacteria proteome. In point-of-care diagnostic field, a simple detection and discrimination methods for pathogenic bacteria are highly demanded. Most conventional methods for bacteria identification rely on bacterial 16S ribosomal RNA (rRNA) sequencing,[23, 24] antibody based ELISA,[25, 26] or bacteria specific enzyme activity measurement (e.g. Analytical Profile Index (API) test).[27, 28] These methods often require sophisticated facilities for PCR amplification and sequencing, or expensive reagents. Current standard procedures share the first sample culturing for 24 to 48 hours. Depending on the diagnostic techniques, following analysis procedures determine analysis speed and sensitivity. Culture-based methods, such as API test, takes at least 18 hours, and PCR method also needs more than 5 hours for DNA extraction and amplification.[29] Instead, reactivity profiling only requires 2 hours for chemical reaction and SDS-PAGE step. Therefore, reactivity profiling has merits in that sample preparation is straightforward, and profiling procedures are fast and cost-efficient.

We collected 11 bacteria strains that cause various infectious respiratory diseases or food poisoning from ATCC (details are

available in SI).[30-34] It has been reported that bacteria detection efficiency differs depending on Gram-positive and negative strains due to the efficiency of cell wall disruptions during sample preparation.[29] Therefore, we choose 5 Gram-positive and 6 Gram-negative species to evaluate general applicability of our method. Proteome reactivity profiles of individual chemical probes were obtained from fluorescence images of SDS PAGE (Fig 2, S6-S8), and quantitative intensity values were analyzed using *ImageJ* software (detail protocols are available in SI). Depending on electrophiles, reactivity profiles were distinguishable and also different pattern compared with protein abundance (Fig S5). Notably, benzyl halide probes **1** and **2** showed remarkably different reactive profiles even though they share identical electrophile (Fig 2, S6), and NHS probe **3** and **4** also exhibited significantly distinct profile (Fig S7, S8). This observation supported that diversity in electrostatic state play roles for diversifying engaged target proteins.

For reactivity pattern analysis and predictive capability for bacteria discrimination, we used one of non-supervised discriminant method, principal component analysis (PCA).[35] Non-supervised discriminant analysis is most suitable method to evaluate classification of original profile data without artificial interpretation. Ten independent labeling experiments were performed for the statistical data process (Fig S9), and series of combinations of chemical probes were tested for the best visual discrimination (Fig S10). In particular, the reactivity profile of 10 replicate data appeared in perfectly separated groups using benzyl halide probes **1** and **2** (Fig 3). Depending on the probe set used for PCA, the most distinct bacteria varied. In case of NHS probe set, *Enterobacter cloacae* was the most significantly distinguishing bacteria due to the unique strong reactivity of protein near 23 kDa profiled using probe **4** (Fig S8, S10-a). On the other hands, *Escherichia coli* was most distinctive in benzyl halide probe set, because of strong reactivity of protein near 37 kDa, which is visualized by positively charged probe **2** (Fig 3, S6).

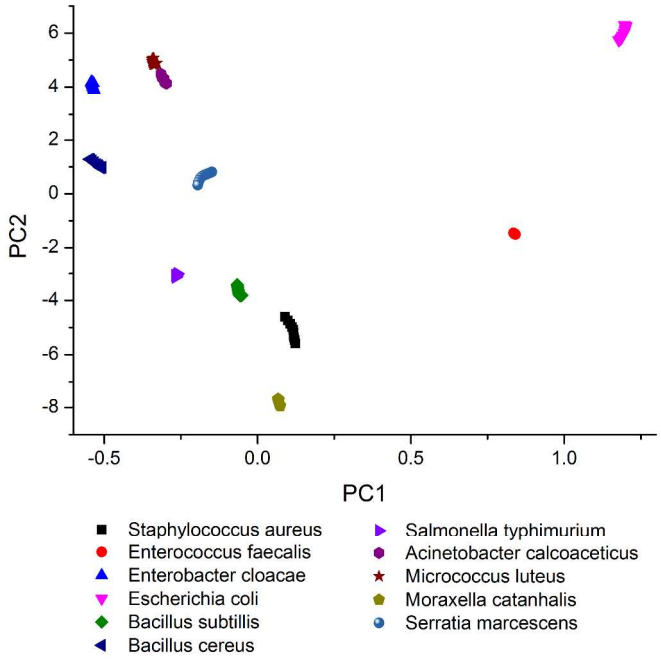


Fig 3. Principal component analysis (PCA) plot of 11 bacteria using benzyl halide probes (**1**, **2**). PC1 variance 42.4 %; PC2 variance 29.3 %.

In conclusion, all 11 bacteria proteome was successfully clustered and discriminated each other using proteome reactivity profiling. We demonstrated the analytical power of proteome reactivity profiling using differentially charged electrophiles. Though majority of efforts have pursued to design new ABPP probes using electrophiles at the moments, its application for multi-analyte or pathological status sensing is still in an early stage. Our observation suggests proteome reactivity profiling has potential for a simple point-of-care diagnostic method as well as a primary screening method to discover molecular markers. Based on this initial study of bacteria discrimination, the practical application in various disease states of human is under study.

This work was supported by intramural funding from KIST (2E24860-2Z04070), Eco Innovation Technology Development Program of the Korea Ministry of Environment, and Converging Research Center Program through the Ministry of Science, ICT and Future Planning (2013K000340).

Notes and references

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† Electronic Supplementary Information (ESI) available: For experimental procedures, fluorescence SDS-gel analysis, and discriminant analysis, see DOI: 10.1039/c000000x/

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