Label-Free Visualization of Carbapenemase Activity in Living Bacteria

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Abstract: Evaluating enzyme activity intracellularly on natural substrates is a significant experimental challenge in biomedical research. We report a label-free method for real-time monitor-ing of the catalytic behavior of class A, B, and D carbapene-mases in live bacteria based on measurement of heat changes. By this means, novel biphasic kinetics for class D OXA-48 with imipenem as substrate is revealed, providing a new approach to detect OXA-48-like producers. This in-cell calorimetry approach offers major advantages in the rapid screening (10 min) of carbapenemase-producing Enterobacteriaceae from 142 clinical bacterial isolates, with superior sensitivity (97 %) and excellent specificity (100 %) compared to conven-tional methods. As a general, label-free method for the study of living cells, this protocol has potential for application to a wider range and variety of cellular components and physiological processes.

Intracellular enzyme activity is exquisitely regulated by its physiological environment. Evaluating the progress of enzyme reactions in the context of their native cellular environment is therefore of utmost importance to gain valuable information on enzyme kinetics and for early disease detection.[1] Enterobacteriaceae (CPE) producing carbapene-mases are gram-negative bacteria, including E. coli, Serratia, Klebsiella, and Enterobacter, that efficiently hydrolyze a C N bond in carbapenems (e.g., imipenem, meropenem, and ertapenem), making these "last resort" antibiotics ineffective for treating infections caused by multi-drug resistant patho-gens.[2] Carbapenemases have been classified into A, B, and D groups by the Ambler system, according to amino acid homologies.[3] Classes A and D are composed of serine b-lactamases, which cleave the b-lactam with the formation of an acyl–enzyme intermediate. The most prevalent ones in clinic are K. pneumoniae carbapenemase (KPC) in Class A and oxacillinase-48 (OXA-48) in Class D. Class B carbape-nemases are metallo-b-lactamases, which require zinc for catalysis, including New Delhi metallo-b-lactamase (NDM-1) and imipenem-hydrolyzing lactamase (IMP) (Figure 1 a). These carbapenemase genes are often plasmid-encoded and can readily be transmitted to other pathogenic species, provoking nosocomial outbreaks or epidemics. Given the high morbidity and mortality caused by it, CPE has become one of today s most serious public health threats worldwide.[4] As a consequence, kinetic investigations of carbapenemase reaction are being pursued intensively in biomedical fields.

Real-time characterization of carbapenemase activity in living bacteria is accepted as crucial for mechanistic studies and rapid CPE detection. Intracellular hydrolytic activity of

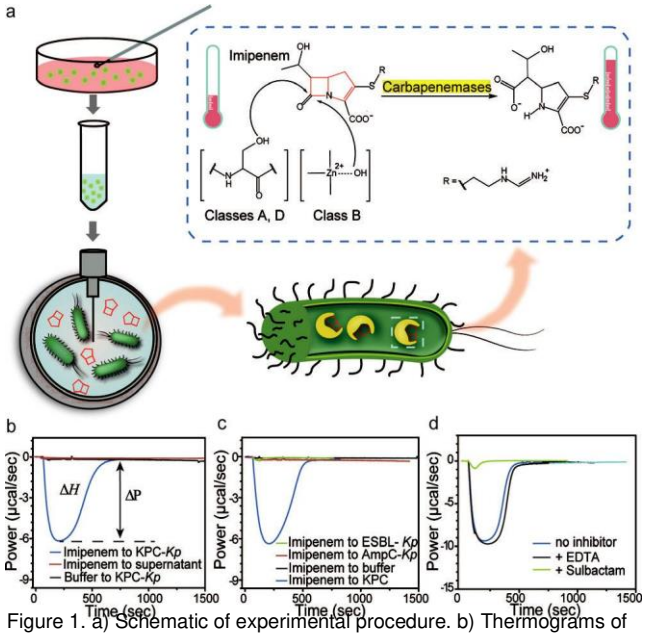

Figure 1. a) Schematic of experimental procedure. b) Thermograms of reaction, after injecting 28 mL of 5 mm imipenem, or buffer, into a calorimetric cell loaded with 210 mL of living KPC-Kp suspensions at OD_{600} =4. c) Thermograms of injection of imipenem into bacterial suspensions (ESBL-Kp and AmpC-Kp) and into 50 nm KPC protein. d) Thermograms of the hydrolysis of imipenem by KPC-Kp in the absence and presence of 500 mm EDTA or 1 mm sulbactam. All experiments were prepared in 50 mm Tris-HCl, pH 7.6, 0.1 mm $ZnCl_2$ at 25 8C.

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b-lactamases has been studied using UV spectroscopy,[5] MALDI-TOF MS,[6] and NMR spectroscopy.[7] These meth-ods are not widely applicable because of signal interference with turbid bacterial suspensions or with specific substrates (e.g., cephalosporins are incompatible with in-cell NMR spectroscopy), inoperability of continuous recording the course of enzymatic reactions, requirements for special sample preparation (e.g., deuterium labeling), or require-ments for expertise in data handling. Chromogenic and fluorogenic probes have been developed to overcome these impediments and facilitate analysis of b-lactamase activity in complex biological systems,[8] although, the changed specific-ity and kinetic properties of labeled substrates from those of natural ones remains a concern. Hence, there is a major need for a sensitive and uncomplicated approach for real-time monitoring of the activity of cellular carbapenemase activity using label-free, native substrates.

Heat changes are fundamental in all biological processes. Owing to the direct correlation between reaction rate and the rate of heat change from bond formation/breaking, reaction calorimetry has become a powerful tool for monitoring progress of enzyme reactions with natural substrates.[9] Although calorimetric real-time monitoring of the course of reaction yields accurate and significant data more rapidly than do classical kinetic techniques, the use of heat to study intracellular enzyme catalysis in living systems is still rare, largely because of concern over whether it can differentiate heat of reaction from background heat changes. We have shown that energies involved in breaking a b-lactam bond for metallo-b-lactamases are approximately 15–32 kcal mol ^{1}.[10] Because of the large enthalpy of b-lactam hydrolysis and the high efficiency of b-lactamases, the progress of micromolar b-lactam turnover (minutes) could easily be followed using a modern calorimetry instrument (signal noise < 0.01 mcal s ^{1}) at nanomolar concentration of enzyme. By contrast, meta-bolic heat derived from cell growth often takes place over a much longer period of time (hours to days),[11] and binding heat is known to be several orders of magnitude lower than reaction heat.[12] Given the substrate specificity of b-lacta-mases and significant heat changes in b-lactam catalysis, we anticipated that in situ b-lactamase activity in living bacteria could be probed accurately by tracking heat changes during turnover of an exogenous b-lactam under controlled condi-tions (Figure 1 a). In this work, we develop an in-cell calorimetry assay to investigate the real-time reaction prog-ress of Class A, B, and D CPE with imipenem, providing more direct and accurate data on the catalytic behavior of carbapenemases in live bacteria. We further explore its use in rapid identification of CPE in clinical samples.

We first tested in situ thermal monitoring of carbapene-mase activity in a calorimetry experiment using a KPC-producing K. pneumoniae strain (KPC-Kp; ATCC-1705) as reference. Titrating imipenem solution (588 mm in 50 mm Tris-HCl, pH 7.6, 0.1 mm $ZnCl_2$) into living KPC-Kp suspensions (OD_{600} = 4.0 in the same buffer; corresponding to approx-imately 57 nm KPC protein based on SDS-PAGE analysis of bacterial lysate) results in an instant negative change in heat-flow, showing the reaction is exothermic (Figure 1 b, blue curve). To make sure that the heat detected is from reaction inside the bacteria, KPC-Kp suspensions were spun down and the supernatant was used in a repeat experiment. The data show only negligible heat change with the supernatant solution (Figure 1 b, orange curve). Additional control experi-ments by injecting buffer into living KPC-Kp suspensions (Figure 1 b, black curve), injecting imipenem into two carba-penemase-negative bacterial suspensions (ESBL-Kp, Fig-ure 1 c green curve; AmpC-Kp, Figure 1 c orange curve) and injecting imipenem into buffer (Figure 1 c, black curve) show no heat contributions from metabolic heat, nonspecific bind-ing to cellular components, or dilution heat. Under the experimental conditions employed, the amplitude of heat change (DP) and the apparent enthalpy change (DH_{app}) for imipenem with KPC-Kp is at least 12-fold and 40-fold higher than background signals. To further confirm that the observed heat changes with KPC-Kp primarily result from enzyme catalysis, the calorimetry experiment was performed by titrating imipenem into a 50 nm purified KPC protein solution (Figure 1 c, blue curve). As expected, the features of the calorimetric trace with KPC-Kp resemble those of KPC protein, both indicating a constant phase of the thermal power over several minutes (150 to 275 s), representing initial velocities at saturating conditions followed by a decay curve (275 to 600 s) showing gradual depletion of imipenem, reaching complete consumption at 700 s. The values of DH_{app} are also comparable for bacterial suspensions (DH_{app} = 25 0.6 kcal mol ^{1}) and protein (DH_{app} = 23 1.2 kcal mol ^{1}), showing that bacteria cause no observable heat changes under the conditions tested.

To further verify that the heat change observed with KPC-Kp arises specifically from KPC activity, experiments were repeated in the presence of sulbactam, an effective inhibitor of Class A carbapenemases, or EDTA, a strong metal-chelator and inhibitor for Class B enzymes.[13] As expected, the observed heat with imipenem (Figure 1 d, blue curve) is abolished in the presence of 1 mm sulbactam (Figure 1 d, green curve), whereas 500 mm EDTA (Figure 1 d, black curve) shows negligible effect on the thermograms of KPC-Kp with imipenem. Further inhibitory results using a clinical E. coli isolate encoding Class B NDM-1 (NDM-1-E. coli), a clinical isolate harboring both KPC and IMP enzymes (KPC/IMP-Kp), and a reference K. pneumoniae strain expressing Class D OXA-48 (OXA-48-Kp, NCTC-13442) also agree well with the inhibitory profiles of these enzymes, confirming that the heat change curves specifically reflect the progress of enzymatic hydrolysis of imipenem in live bacteria (Supporting Informa-tion, Figure S1).

A distinct biphasic calorimetric progress curve is observed with OXA-48-Kp (Figure 2 a, black curve). In this case, a short fast exothermic peak lasting circa 100 s accounts for most of the reaction, then changes to a much slower steady-state curve that lasts for 500 s at a steady rate until all substrate is consumed. Carbamylation of a conserved lysine in some OXAs, formed by the reaction of carbon dioxide and the non-protonated e-amino group of this lysine, is crucial for hydrolysis of some specific b-lactams.[14] While several crystal structures of OXA-48 confirmed the presence of an N-carbamylated lysine,[15] the effect of lysine carbamylation on carbapenem hydrolysis remains unresolved. To investigate

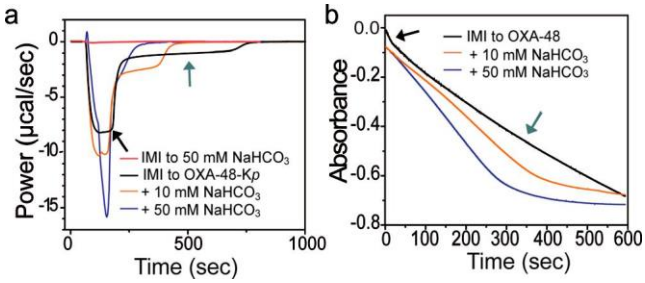


Figure 2. The biphasic kinetics of OXA-48 with imipenem (IMI). a) Effect of added CO_2 derived from sodium bicarbonate on the catalytic activity of OXA-48 with imipenem in a calorimetry assay using living OXA-48-Kp suspensions and b) a UV-spectrophotometric assay using OXA-48-Kp lysates.

whether the slow phase in the calorimetry assay with OXA-48-Kp suspension is a result of enzyme deactivation, experi-ments were performed with added bicarbonate as a source of carbon dioxide to facilitate lysine carbamylation. Increasing bicarbonate from 10 to 50 mm causes the slow secondary phase to be progressively subsumed into the initial fast phase (Figure 2 a, orange and blue curves). The UV-spectroscopic assay also confirms the effect of bicarbonate on the hydrolytic activity of OXA-48 with imipenem (Figure 2 b; supernatant of OXA-48-Kp lysate is used to avoid the interference of bacterial suspensions in UV readings). It is notable that the biphasic feature observed in the calorimetry assay in the absence of bicarbonate is barely visible in the UV-spectro-scopic assay, which highlights the much greater sensitivity of the calorimetry approach. This distinction is likely due to the calorimetry method using a differential rate method as opposed to the integrated rate analysis of the UV assay, hence the change in reaction rate is more clearly identified.

Next, we evaluated the dose-dependent effect of carba-penemases on reaction thermograms, by recording heat-flow on injecting imipenem (final concentration, 588 mm) into 2-fold serial dilutions of the above-mentioned reference bacterial suspensions (Figures 3 a and Figure S2a,d). The amplitude of thermal power (DP) is considered as enzyme activity and is in a linear relation to bacterial density, in the

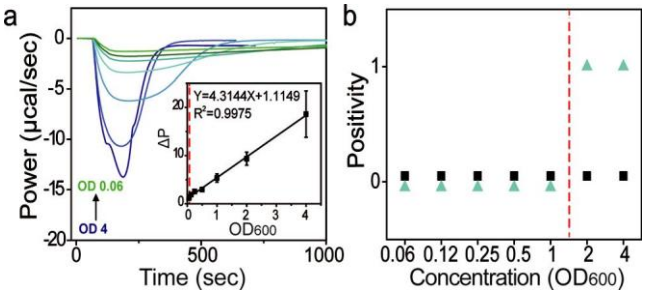
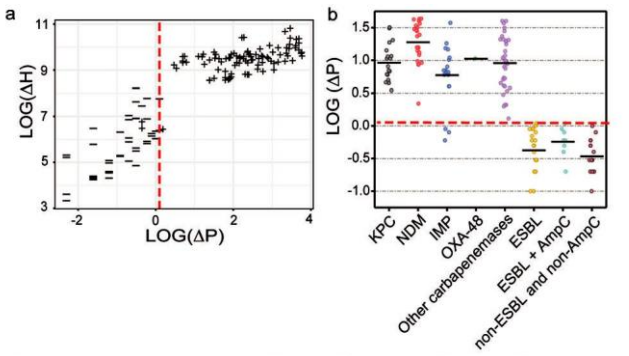


Figure 3. Comparison of in-cell calorimetry with Carba-NP test for sensitivity in detecting carbapenemase activity using live bacteria of KPC-K. pneumonia at given concentrations. a) Raw calorimetric data using 588 mm imipenem as substrate, and linear dependence of heat change rate (DP) on bacterial density. b) Dependence of results on bacterial density in Carba-NP test, using both 588 mm (black squares) and 20 mm imipenem (cyan triangles).

range of OD_{600} of 0.06–4.0 for KPC-Kp (Figures 3 a), 0.03–4.0 for NDM-1-E. coli (Figures S2b) and 2–8 for OXA-48-Kp (Figures S2 e). Similar experiments were repeated using pure recombinant carbapenemases, from which the detection limit was estimated to be lower than 3 nm for KPC/NDM-1 and 19 nm for OXA-48 (Figure S3). These results indicate that the DP values in the calorimetry assay quantitatively reflect intracellular carbapenemase with high sensitivity and have potential to be used for rapid CPE detection. Currently, the Carba-NP test is the only carbapenemase activity-based colorimetric assay suggested by Clinical and Laboratory Standards Institute (U.S.) for CPE detection.[16] To demon-strate the advantages of the in-cell calorimetry assay, an updated Carba-NP assay was used for direct comparison. However, neither of the three strains show an apparent change of color at 588 mm imipenem (Figure S4 a). With a 30-fold larger substrate concentration, positive results for this updated Carba-NP assay are obtained with bacterial suspen-

sions with OD_{600} values as low as 2 for KPC-Kp (Figure 3 b and Figure S4 b), 0.12 for NDM-1-E. coli (Figures S2 c and S4 c), while OXA-48-Kp is still not detected at OD_{600} of 16 after 120 min (Figures S2 f and S4 d). This is not surprising since Carba-NP assay has been reported to have difficulties with OXA-48 producers.[17] Considering the difference in substrate concentration between the two assays, the in-cell calorimetry assay is at least two orders of magnitude more sensitive than the updated colorimetric assay, with the advantages of a significantly shorter testing time (10 min) and ability to provide fingerprint thermograms for OXA-48-producers.

Timely detection of CPE is regarded as one of the key approaches to prevent rapid spread of resistant strains and to guide clinical treatment.[4] Having demonstrated the specific-ity, sensitivity, and rapidity of the in-cell calorimetry approach in detecting carbapenemase in live bacteria, we sought to apply it to the screening of clinical CPE and to compare its performance with current phenotypic tests. For this purpose, a collection of 142 clinical Enterobacteriaceae strains were included in the study. Of these, 97 are identified as CPE and 45 are non-CPE (Tables S1 and S2).[3a, 18] In-cell calorimetry tests were performed on these isolates as described above, and results were evaluated by extracting DP and DH values from calorimetric data for analysis using logistic regression models. The regression coefficient of DP in the model is statistically significant (P = 0.029), suggesting that intracellu-lar carbapenemase activity is well reflected by DP values. The cut-off value of DP determined by the decision-tree model is 1.1. Using this cut-off value, the calorimetry assay successfully detects 94 of 97 CPE within 10 min of each test, while all the non-carbapenemase producers are correctly determined (Fig-ure 4 a,b). The results give a sensitivity of 97 % and a specific-ity of 100 % for the calorimetry test (Figure 4 c). The three samples that fail the in-cell calorimetry test are IMP-producers (2 strains of IMP-4 and 1 strain with an unknown subtype of IMP; Figure 4 b) and have a low level of resistance to imipenem (MICs in the range 0.25–8 mg L^{-1}). It is possible that the enzyme expression level/activity in these strains may be too low for ready detection.



c. Performance of each test on CPE detection using 142 clinical isolates

	in cell-Calorimetry	Carba NP	mCIM	MHT	MIC
Sensitivity	97.0%	89.9%	95.0%	89.9%	88.6%
Specificity	100%	100%	95.4%	83.7%	68.9%
Testing Time	10 min	2 h	12 h	12 h	36 h

Figure 4. In-cell calorimetry assay results compared to older methods on the detection of CPE using 142 clinical isolates. a) Distribution of calorimetry results using log(DH) and log(DP) values. Carbapenemase-positive samples are labelled "+" and carbapenemase-negative samples are labelled " ". The cut-off value for log(DP) (red dash line) is determined by a decision tree model. b) Distribution of the log(DP) values based on enzyme type for all clinical isolates. c) Comparison of in-cell calorimetry assay with Carba-NP, mCIM, MHT, and MIC methods in sensitivity, specificity, and testing time for CPE screening.

The results of the in-cell calorimetry tests were then compared to those of an updated Carba-NP test, a modified carbapenem inactivation method (mCIM) test, a modified Hodge test (MHT) test, and a minimum inhibitory concen-tration (MIC) assay following reported procedures (Fig-ure 4 c).[16b, 19] The in-cell calorimetry test shows the best sensitivity (97 %) and specificity (100 %) with our collection of isolates, followed by Carba-NP (89.9 % and 100 %), mCIM (95 % and 95.4 %), MHT (89.9 % and 83.7 %) and MIC (88.6 % and 68.9 %). Regarding the experimental duration, from availability of bacterial colonies on agar plates (ca. 16– 18 h), the in-cell calorimetry test delivers results within 10 min of the reaction owing to the real-time nature of the method. This is a great reduction in testing time compared to visual interpretation of color change in Carba-NP (2 h) or assessment of bacterial growth in mCIM (12 h), MHT (12 h), and MIC (36 h). Other features that make the calorimetric method preferable include ease of sample preparation, potential for automatic operation (commercial calorimeters are capable of running 384 samples unattended), and interpretation of results.

Although we have used bacterial colonies in this screen-ing, the in-cell calorimetry approach is also applicable to bacteria directly recovered from blood culture bottles (Fig-ure S5). The presence of blood cells and debris does not interfere with reaction heat signals, avoiding the need to purify microorganisms from positive blood cultures. The above experiments show that the bacterial load required for the calorimetric approach is approximately 10^6 to 10^8 bacteria for the successful identification of CPE. There should be enough bacteria in positive blood culture samples identified by an automatic blood culture detection system, such as BACTEC FX (Becton Dickinson, USA). Hence, compared to the conventional susceptibility methods, the use of the in-cell calorimetry test directly on positive blood cultures has the potential to reduce the time for identification of CPE in blood stream infections by at least one day. Such a reduction in time is critical for survival of sepsis patients, as rapid initiation of effective antimicrobials in the first hours of sepsis is essential.[20] While this study only focuses on early identification of CPE, it is of note that other mechanisms may also contribute to carbapenem resistance in Enterobacteriaceae, such as defect in membrane permeability, efflux pump, and mutations on target proteins. When these mechanisms are present, integration of this in-cell calorimetry test with other tradi-tional tests (e.g., MIC assay) may offer a more robust strategy for more efficient prevention of nosocomial outbreaks and better guidance on antibiotic treatment.

In conclusion, we demonstrate in detail that heat changes can be exploited as a label-free approach to track intracellular carbapenemase activity in living organisms. This approach features high selectivity and sensitivity for all major carbapenemases. Since there is currently no specific inhibitor for Class D carbapenemases, the characteristic biphasic thermo-grams with OXA-48 may provide a new approach for detecting the OXA-48-like producers. We further demon-strate the applicability of this technique in very fast identifi-cation of clinical CPE with greatly improved accuracy as compared to conventional phenotypic methods. Although we have exemplified the advantages of utilizing heat changes to quantify enzyme activities in living pathogens, the concept of in-cell calorimetry is simple and should readily be applicable in other areas that need to characterize enzyme activity in complex biological systems (e.g., tumor cells, immune cells, and vesicles) and for a wider range of applications, such as monitoring abnormal enzyme activity in tumors, evaluating new inhibitors, and synthetic biology.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: calorimetry · bacterial detection · carbapenemase · Enterobacteriaceae · enzyme kinetics

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