A non-radioactive mass spectrometry based differential radial capillary action of ligand assay (DRaCALA) to assess ligand binding to proteins

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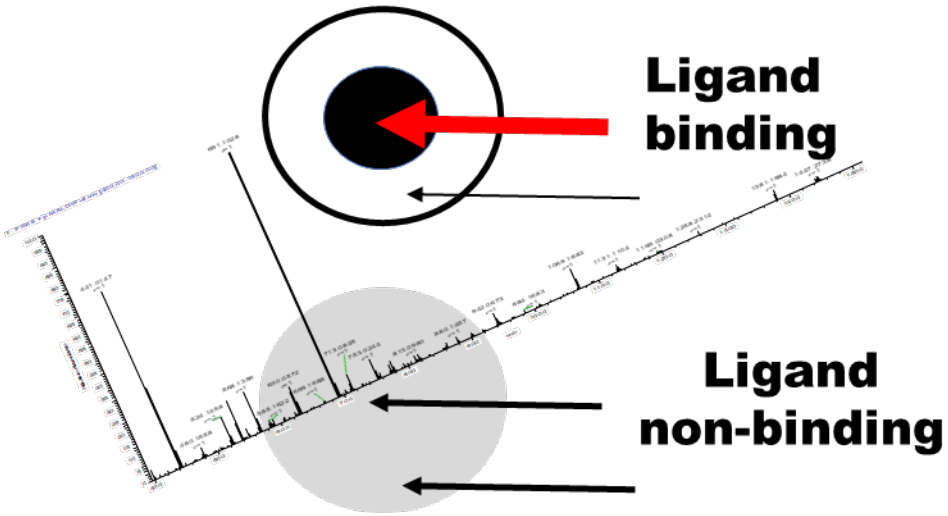
Abbreviations used: cyclic di-GMP, bis-(3′-5′)-cyclic dimeric guanosine monophosphate; cyclic di-AMP, bis-(3′-5′)-cyclic dimeric adenosine monophosphate; MALDI-TOF MS, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry; DRaCALA, differential radial capillary action of ligand assay; GGDEF motif, diguanylate cyclase motif; EAL motif, phosphodiesterase motif

Graphical abstract



Highlights

- Cyclic di-nucleotides are ubiquitous second messengers in Bacteria, however, few receptors have been identified.
- Previous screening of cell lysates by differential radial capillary action of ligand assay (DRaCALA) using radioactive ligand identified cyclic di-nucleotide binding proteins.
- A MALDI-TOF based DRaCALA was developed to detect cyclic di-nucleotide binding as a non-radioactive alternative.
- Known cyclic di-GMP binding proteins were verified and potential cyclic di-GMP binding proteins were identified.

Abstract
Binding of ligands to macromolecules changes their physicochemical characteristics. Cyclic di-GMP and other cyclic di-nucleotides are second messengers involved in motility/sessility and acute/chronic infection life style transition. Although the GGDEF domain encoding preferentially a diguanylate cyclase represents one of the most abundant bacterial domain superfamilies, the number of cyclic di-GMP receptors falls short. To facilitate screening for cyclic di-nucleotide binding proteins, we describe a non-radioactive, MALDI-TOF based modification of the widely applied differential radial capillary action of ligand assay (DRaCALA). The results of this assay suggest that $YciR_{Fec101}$, but not the $YciR_{TOB1}$ variant of the diguanylate cyclase/phosphodiesterase YciR binds cyclic di-GMP.

Introduction

Binding of small molecules to macromolecules such as proteins has a deep physiological impact as it alters the physico-chemical properties and the functionality of the protein including alteration of enzymatic parameters through allosteric regulation and change in protein-protein interactions due to substantial conformational changes upon small molecule binding [1].

There are a variety of directed and unbiased experimental approaches to determine binding of small molecules to proteins and to assess their binding affinity [2]. Traditional biochemical approaches require purified protein and are not high-throughput. One of the most successful experimental approaches suitable for screening of a large number of candidate receptors for binding with candidate small ligand is the differential radial capillary action of ligand assay (DRaCALA), which does not require purification of the expressed protein, but uses whole bacterial cell lysates containing plasmid-expressed candidate gene products [3-6]. Thereby, small ligand binding to a protein is assessed by the spot intensity and diameter upon spotting the protein extract containing ligand onto a protein-binding nitrocellulose membrane. Binding of the radioactive ligand to the macromolecule creates a small intensive spot reflecting the protein location, while, upon non-binding, the ligand signal is more extended, but overall equally intensive reflecting the free ligand distribution over a larger spot area created by capillary forces.

Cyclic di-nucleotides are ubiquitous second messengers in Bacteria, however, the abundance of cyclic di-GMP turnover proteins is still not reflected by the number of identified receptors [7]. DRaCALA has been frequently used to identify receptors for cyclic di-nucleotides, in particular cyclic di-GMP and cyclic di-AMP, which are ubiquitous second messengers in Gram-negative and Gram-positive Bacteria [8, 9]. Thereby lysates of bacterial libraries containing all plasmid-expressed gene products of an organism have been screened for cyclic di-nucleotide binding proteins. With this experimental approach, receptors involved in, for example, regulation of cellulose biosynthesis such as BcsE and maintenance of osmohomeostasis and potassium transport have been identified [4, 10].

A radioactive ligand, as in the originally described approach, needs to be prepared and labeling is not necessarily readily applicable to every small molecular compound. However, MALDI-TOF MS or imaging mass spectrometry can be used to detect binding of a ligand to a protein receptor on the nitrocellulose membrane. Thereby, assessment of binding can be achieved by measuring the ligand concentration at the application spot and at the edge of the larger spot area that the liquid reached by capillary action (Fig. 1).

To perform this assay, a single colony of the *E. coli* strains (in case of the control with the vector containing a cloned cyclic di-GMP binding protein (YcgR or BcsE) and its relevant non-binding mutant) were inoculated 1:100 from an overnight culture in LB medium with the relevant antibiotic. After induction with 0.1% L-arabinose at 30°C for four hours, cells were harvested by resuspension in binding buffer (10 mM Tris pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, in Chromasolv H_2O; [11] with 100 µl lysozyme (500 µg/ml) per OD_{600}=3 ([4, 12]). Cells were disrupted by two freeze-thaw cycles of 1h at room temperature and at -80°C. 18 µl cell lysate and 2 µl 10 µM c-di-GMP were mixed, incubated 10 min at room temperature and 2 µl were spotted on a nitrocellulose membrane (Schleicher & Schuell Protan^{TM}, BA85). After the membrane had completely dryed, equally sized membrane pieces at the application spot and the edge of the diffused liquid were cut out with a scalpel. 2 µl 5 mg/ml alpha-cyano-4-hydroxy-cinnamic acid matrix in 50% acetonitrile and 0.05%

triflouroacetic acid were applied on a MALDI MassTech 96 spot sample plate, followed by the membrane piece and again 2 µl matrix. The matrix was left to dry at room temperature, the membrane removed and the sample measured in a QExactive, Thermo Scientific, with a MassTech AP MALDI PDF Ion source. Settings of the MALDI source were sheath gas: 0; aux gas: 0; sweep gas: 0; spray voltage: 3,75kV; spray current [µA]; capillary temperature: 280; S-lens RF level: 100.0; 5 min acquisition time, 2 microscans; lock masses OFF; $1e^5$ AGC target; maximal inject time 1.000. Signals for cyclic di-GMP were observed as expected at around 691.1 $[M+H]^+$and 713.1 $[M+Na]^+$.

In order to validate the procedure, we first expressed two previously characterized cyclic di-GMP binding proteins and their respective binding mutants, namely the cellulose biosynthesis enhancer BcsE and its non-binding mutant $BcsE_{R415D}$ and the flagella motor 'backstop-break' protein YcgR and its nonbinding mutant $YcgR_{R118D}$ in *E. coli* Top10 [4, 13]. Measurement of the cyclic di-GMP counts from cell lysates expressing these receptors after incubation with cyclic di-GMP showed higher counts derived from the membrane taken from the application spot than from the periphery. In contrast, counts were similar when membranes from cell lysates expressing the non-binding protein variants were investigated after incubation with cyclic di-GMP (Fig. 2 a).

YciR, an enzyme with opposing diguanylate cyclase and phosphodiesterase activities, has been initially characterized to downregulate expression of the agar-grown rdar biofilm morphotype in *Escherichia coli* and *Salmonella typhimurium* by a mechanism independent of its catalytic activities involving cyclic di-GMP sensing and protein-protein interactions [14-17]. Natural YciR protein variants encoded by semi-constitutive rdar biofilm strains, though, failed to efficiently downregulate this morphotype, even when overexpressed. Sensing and binding of cyclic di-GMP might be a determinative factor for the different variant behavior. To assess whether two YciR protein variants differentially bind cyclic di-GMP and to assess their binding capacity, we choose to express the two variants of the opposing activity enzyme YciR, $YciR_{Fec101}$, that has been previously shown to downregulate the rdar morphotype, and $YciR_{TOB1}$ that is nearly invariant to rdar morphotype expression upon plasmid-based expression [13]. Upon expression of YciR from the commensal *E. coli* strain Fec101, the MALDI-TOF MS -based DRaCALA binding assay showed higher counts for the membrane derived from the application spot compared to the membrane from the periphery suggesting binding of cyclic di-GMP to $YciR_{Fec101}$. In contrast, membranes from the application spot and the periphery of the membrane for the $YciR_{Fec101\text{-}K581A}$ mutant and a YciR double mutant in the GGDEF and EAL motif, $YciR_{Fec101\text{-}\ D316A,E317A/E440A}$, abolishing the two catalytic activities of the combined diguanylate cyclase and phosphodiesterase, showed similar counts suggesting those protein variants are either non-binding or not being expressed under the respective assay conditions. Equally, the counts from the application spot and the membrane periphery for $YciR_{TOB1}$ were even higher for the periphery suggesting that $YciR_{TOB1}$ does not bind cyclic di-GMP (Fig. 2b).

In conclusion, a MALDI-TOF MS-based DRaCALA assay to identify cyclic di-nucleotide binding proteins was successfully implemented which should save experiment time, as production of radioactive cyclic di-GMP is not necessary. Future miniaturization of the experiment will save material and optimization of the choice of the protein-binding matrix with optimal capillary forces for small compound diffusion will optimize the detection process. Furthermore, the approach can be transferred to

measure binding of other small ligands and molecules where methodological restrictions do not allow read radioactive labelling.

Author contribution

UR had the idea; ACA, RZ, AC and UR conceptualized the study; ACA and AC performed the experiments; ACA, RZ, AC and UR analyzed the data; UR and ACA wrote the manuscript with the input of all authors.

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Figures

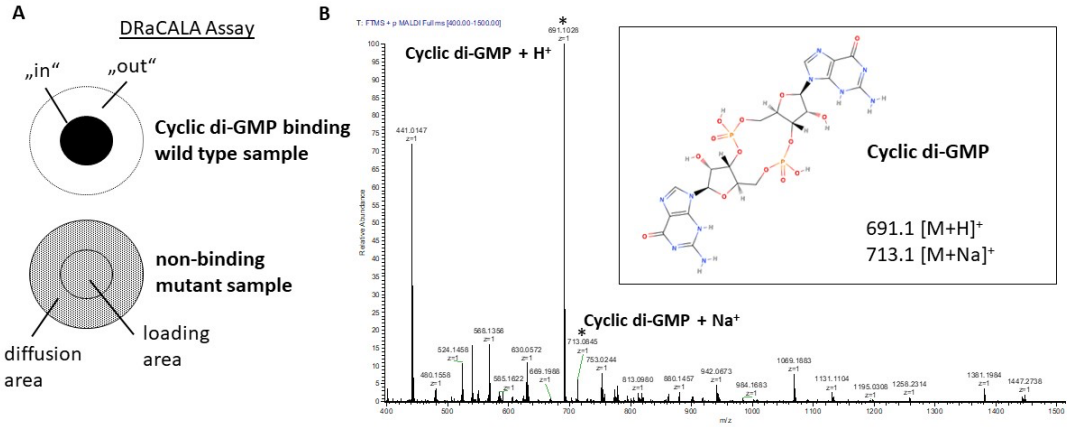

Figure 1: Basic principles of the non-radioactive, MALDI-TOF MS based DRaCALA assay

A Schematic drawing of the read out of the DRaCALA assay. If ligand is bound to the protein sample, the sample remains in the loading area while unbound ligand will diffuse [2,4]. Resulting ligand concentrations in the inner part of the loading spot („in“) and peripheral location („out“) can subsequently be determined by quantitative mass spectrometry.

B Mass spectrum for cyclic di-GMP (Biolog), m/z 400-1500, main peak corresponds to positively charged protonated cyclic di-GMP (m/z 691.1). The inserted box depicts the structure of cyclic di-GMP and shows the molecular mass of the positively charged cyclic di-GMP molecules ($[M+H]^+$, $[M+Na]^+$).

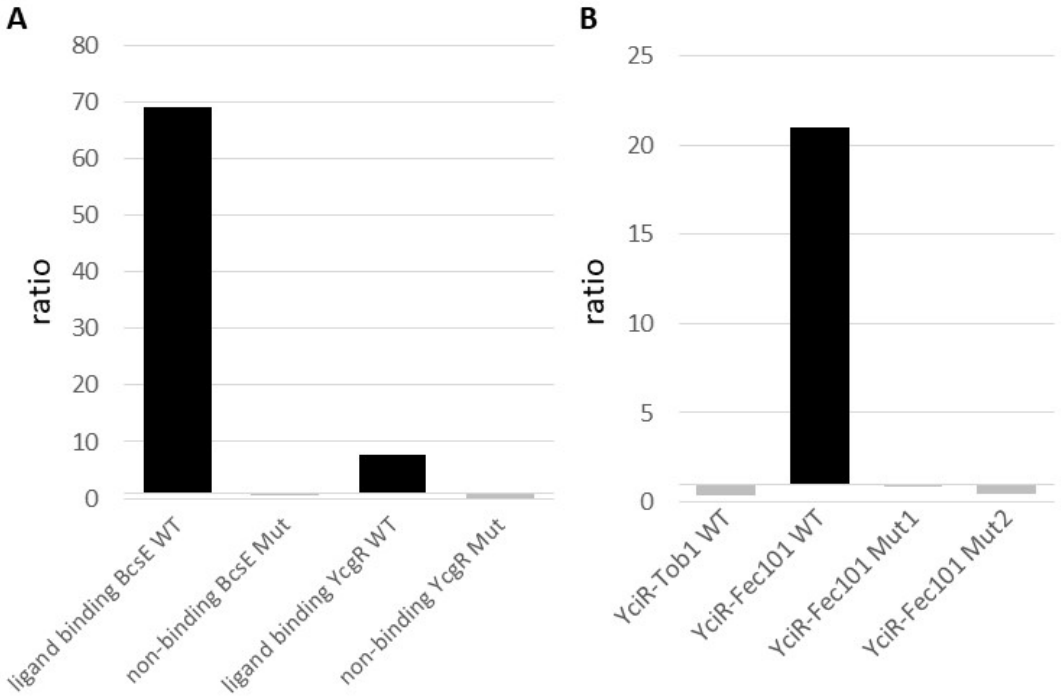

Figure 2: Determination of cyclic di-GMP binding capability of cyclic di-GMP receptors and candidate receptor proteins.

Ratio of counts derived from representative absolute values measured by FTMS MALDI-TOF on the membrane at the application spot and the peripheral membrane (range m/z 691.0950 – 691.1050). A value >1 is indicative for cyclic di-GMP binding to the expressed protein (black bars), a value <1 indicates no binding (gray bars). **A** Proof of principle shown with the known cyclic di-GMP receptors BcsE and YcgR and their respective non-binding catalytic mutants [3,12]. **B** Cyclic di-GMP binding capabilities of the cyclic di-GMP metabolizing protein YciR from the commensal *E. coli* strains TOB1 and Fec101. While $YciR_{Fec101}$ binds c-di-GMP, the respective catalytic mutants and $YciR_{TOB1}$ do not.

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