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ORIGINAL RESEARCH PAPER

Enhanced intracellular Ca^{2+} concentrations in *Escherichia coli* and *Bacillus subtilis* after addition of oligosaccharide elicitors

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Abstract Elicitation can lead to overproduction of secondary metabolites in plants and microbes. Potential changes in cytosolic Ca^{2+} levels in bacteria were studied in response to elicitation. We report, for the first time, the effect of oligosaccharide elicitors on intracellular Ca^{2+} levels. The apoaequorin gene was cloned into *Escherichia coli* DH5α and *Bacillus subtilis* 1604 cultures. Addition of elicitors, oligoguluronate and mannan oligosaccharides, to the cultures caused up to 11-fold increase in cytosolic Ca^{2+} in *E. coli* and tenfold increase in *B. subtilis*. These increases in Ca^{2+} levels could therefore contribute to the enhancement of secondary metabolite levels.

Keywords Oligosaccharide elicitors · Intracellular calcium · Apoaequorin · Secondary metabolites

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Introduction

Elicitation using carbohydrates stimulates morphological and physiological responses in fungi and bacteria. The overproduction of a range of antibiotics and enzymes has been achieved by adding small traces of oligosaccharide elicitors to cultures (Petruccioli et al. 1999; Nair et al. 2008; Murphy et al. 2007a). The effect of elicitation on fungal morphology and penicillin G production has also been studied: *Penicillium chrysogenum* supplemented with oligosaccharide elicitors had higher hyphal tip numbers compared to control cultures. Enhancement of penicillin G in elicited cultures may be related to the morphological changes caused by the elicitation (Radman et al. 2004). Elicitors also effect the transcription of genes for antibiotic biosynthesis (Murphy et al. 2007b; Nair et al. 2009). However, a possible mechanism by which elicitors cause these changes has yet to be defined.

Ca^{2+} is widely recognised as a secondary messenger that transmits external information into the eukaryotic cell (Campbell 1983). Eukaryotes maintain a very low cytoplasmic Ca^{2+} concentration and Ca^{2+} homeostasis has been reported for many years (Knight et al. 1991a). The role of Ca^{2+} in bacteria is less clear; however, it may be involved in prokaryotic cell division, chemotaxis, motility, competence, sporulation, cell defence, synthesis of specific proteins, gene expression and stress signals (Norris et al. 1996; Jones et al. 2002; Dominguez 2004). Cytosolic Ca^{2+} concentration in *Escherichia coli* is similar to

that in eukaryotic systems suggesting that bacteria regulate Ca^{2+} homeostasis and that the Ca^{2+} gradient could be used for transmitting information inside the cell (Jones et al. 2002). Due to the variety of responses observed after elicitation, we have investigated the effect of oligosaccharide elicitors on bacterial Ca^{2+} homeostasis; this adds to the knowledge base for the mechanism of elicitation contributing to a more robust system for enhanced secondary metabolite production via elicitation.

Methods

All materials were purchased from Sigma, unless stated otherwise. *E. coli* DH5α and *Bacillus subtilis* 1604 were used as models for Gram-negative and Gram-positive bacterial cultures.

Preparation of elicitors

Mannan oligosaccharide (MO) was prepared by enzymatic hydrolysis of locust bean gum; oligoguluronate (OG) and oligomannuronate (OM) were prepared by partial acid hydrolysis of sodium alginate (Asilonu et al. 2000). Elicitors were stored at 4°C.

Plasmids

To measure the intracellular Ca^{2+} in bacteria and to investigate the effect of oligosaccharide elicitors on calcium fluxes, aequorin technology was used. The apoaequorin protein from the jelly fish, *Aequorea victoria*, forms a complex with coelenterazine (the prosthetic group) and, upon Ca-binding, the aequorin complex emits bioluminescence allowing the measurement of intracellular Ca^{2+}. The pMMB66EH (Amp^r) containing the apoaequorin cDNA was a kind gift from Prof. Anthony Campbell (Cardiff University, UK). The apoaequorin gene was inserted between the restriction sites *Sal*I and *Pst*I at the multiple cloning site of the pMMB66EH. To express the apoaequorin gene in *E. coli* and *B. subtilis,* an *E. coli–B. subtilis* shuttle vector pHCMC05 (*Bacillus* Genetic Stock Center, USA) was used. This vector contains an IPTG-inducible *Spac* promoter, which is located upstream of the multiple cloning site and also harbours chloramphenicol- and ampicillin-resistance markers.

Cloning of apoaequorin gene

On the multiple cloning site in the shuttle vector pHCMC05, *Xma*I and *Xba*I were the restriction sites chosen for cloning of the apoaequorin gene. *Xma*I and *Xba*I sites were engineered onto the 5′- and 3′- ends of the aequorin sequence during PCR reaction. The primers used were (5′- to 3′-): GGATCCTCTAGAAT GACCAGCGAACAATAC and CTGCAGCCCGGG TTAGGGGACAGCTCCACC. The PCR products were visualised on 1% (w/v) agarose gel and were purified using Qiagen Gel Extraction kit (Qiagen, UK) following the manufacturer's instructions. For the restriction and ligation reactions, all the enzymes were purchased from New England Biolabs, UK. The pHCMC05 vector and PCR products were digested with *Xba*I and *Xma*I at 37°C for 2 h. The digested PCR product and the plasmid were gel-purified using Qiagen Gel Extraction kit. The digested plasmid, PCR product, T4 DNA ligase buffer and T4 DNA ligase were mixed and incubated at 16°C for 16 h. Ligation reactions were used to transform *E. coli* DH5α and *B. subtilis 1604* using the electrophoretic method by Dower et al. (1988) and Xue et al. (1999), respectively.

Plasmid preparation was carried out using QIAprep spin miniprep kit (Qiagen), following the manufacturer's instructions. Sequencing of the constructed clone was carried out by GATC (Germany). The following primer sequence was designed (5′- to 3′-): CATTTGTTCCAGGTAAGG, with a melting temperature of 65°C and a GC content of 47%.

Growth of *E. coli* and *B. subtilis* expressing apoaequorin

Five milli liter of bacterial cultures were grown in LB medium containing 100 μg ampicillin ml^{-1} or 5 μg chloramphenicol ml^{-1} for *E. coli* DH5α and *B. subtilis* 1604, respectively. Incubation was carried out at 30°C at 250 rpm.

Expression of apoaequorin in bacterial cultures

0.5 ml of an overnight culture was inoculated into 10 ml LB medium containing the selective antibiotic. When the OD_{600} reached 0.45, the culture was induced with 0.4 mM IPTG for 2.5 h according to Nguyen et al. (2005).

Reconstitution of aequorin in vivo with coelenterazine H

To reconstitute aequorin, cells were centrifuged at 3000 g for 5 min and re-suspended in Buffer A (25 mM HEPES, 125 mM NaCl, 1 mM $MgCl_2$, pH 7.5) containing 2.5 μM coelenterazine H (Invitrogen) followed by incubation at room temperature in dark for 1 h.

Measurement of intracellular Ca^{2+} levels in bacterial system

Cells expressing functional aequorin were centrifuged and re-suspended in either of LB medium (in the case of *E. coli* DH5α) or Buffer A (in the case of *B. subtilis* 1604) in the presence or absence of 0.5 mM ethylene glycol tetraacetic acid (EGTA). Cells (100 μl) were challenged with $CaCl_2$ and elicitors. Luminescence readings were measured with a microplate reader and the light emitted was measured as relative luminescence units (RLU). Buffer A and LB were used to set the luminescence background. White Cornwell 96 well-plates were used for this assay.

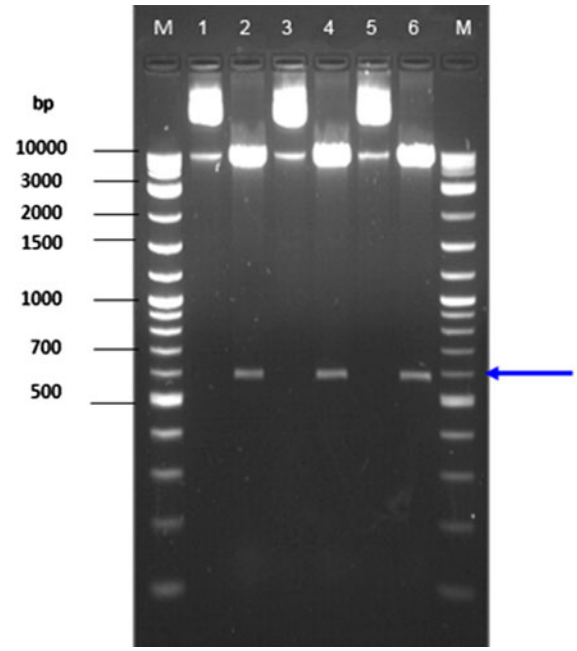

Fig. 1 Screening of positive colonies. Restriction digest of pHCMC05-Aq from *E. coli* DH5α. *Lane M*: 2-log DNA ladder (New England Biolabs). *Arrows* show the position of the released insert. Undigested pHCMC05-Aq: *lanes 1, 3, 5*. Double digest of pHCMC05-Aq with *Xma*I–*Xba*I: *lanes 2, 4, 6*

Results

Elicitation effects on intracellular Ca^{2+} levels in bacterial cultures

To investigate whether the addition of oligosaccharide elicitors changes the intracellular Ca^{2+} in bacteria, the apoaequorin gene was cloned into a Gram-negative/Gram-positive shuttle vector pHCMC05. The pHCMC05-Aq vector was digested with *Xma*I–*Xba*I. The released insert was 597 bp which was the expected size (Fig. 1). The apoaequorin gene in the donor and the expression vectors (PMMB66EH and pHCM05-Aq) were sequenced thereby confirming that no mutations had occurred during PCR and that the insert was correctly inserted in the expression shuttle vector (data not shown).

Expression of apoaequorin using 0.4 mM IPTG and its reconstitution with coelenterazine H was conducted as described in Methods. *E. coli* DH5α without the pHCMC05-Aq plasmid was tested for luminescence in the presence or absence of Ca^{2+}, elicitors, water and the luminescence exhibited by the culture was zero in all cases (data not shown). *E. coli* DH5α pHCM05-Aq cultures expressing aequorin were challenged with 100 mM $CaCl_2$ followed by elicitors. The elicitors were added at concentrations previously chosen to enhance secondary metabolites in bacteria (Murphy et al. 2007a). Supplementation of *E. coli* DH5α pHCM05-Aq with oligosaccharide elicitors (MO at 300 mg l^{-1}; OM at 200 mg l^{-1} or OG at 100 mg l^{-1}), after the first 100 mM $CaCl_2$ addition, increased Ca^{2+} levels (Fig. 2) by 11-fold with OG and by seven and fivefold with MO and OM, respectively.

We then investigated the effect of Ca^{2+} and elicitors in *B. subtilis* 1604 pHCM05-Aq using identical conditions to those used for *E. coli* DH5α pHCM05-Aq experiments. As no luminescence signal was detected under those conditions, lower $CaCl_2$ concentrations were tested without success (data not shown). When *B. subtilis* 1604 pHCM05-Aq cells were re-suspended in Buffer A (+EGTA) rather than

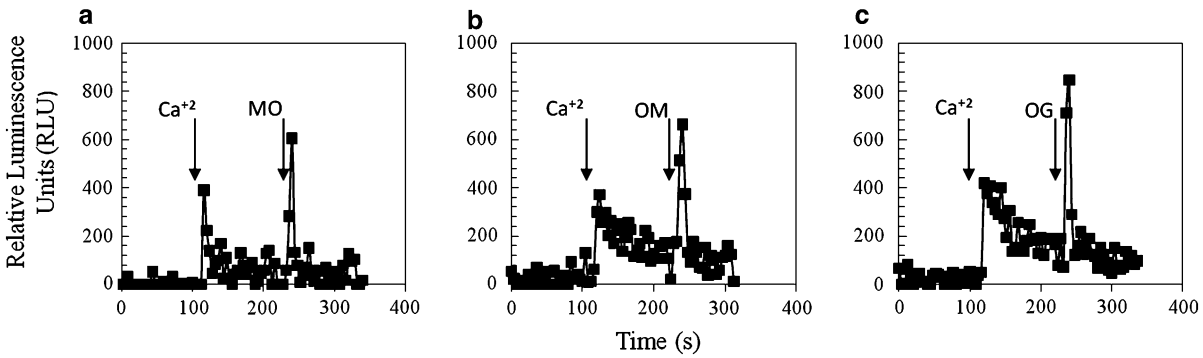

Fig. 2 Effect of 100 mM $CaCl_2$ followed by elicitor additions on *E. coli* DH5α pHCMC05-Aq: **a** mannan oligosaccharide, MO at 300 mg l^{-1}; **b** oligomanuronate, OM at 200 mg l^{-1}; **c** oligoguluronate, OG at 100 mg l^{-1}. Cells were re-suspended in LB broth (in the absence of EGTA)

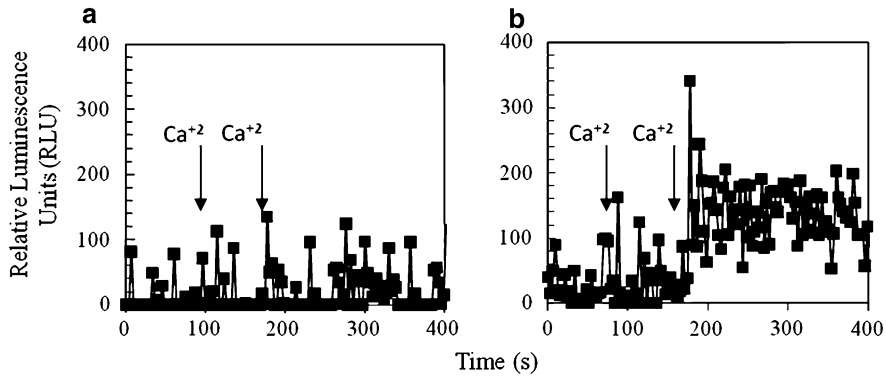

Fig. 3 Effect of double addition of 0.1 mM $CaCl_2$ on *B. subtilis* 1604 pHCMC05-Aq. Cells were re-suspended in either LB broth (**a**) or buffer A (**b**) in the presence of EGTA. *Arrows* represent the addition of 0.1 mM $CaCl_2$ to the culture

LB broth (Fig. 3a), addition of 0.1 mM $CaCl_2$ increased the intracellular Ca^{2+} levels (Fig. 3b).

Stimulation of *B. subtilis* 1604 pHCM05-Aq cells with OG and MO, after $CaCl_2$ addition, increased Ca^{2+} levels significantly ($P < 0.05$) by ten and threefold, respectively. Neither water nor OM, changed the Ca^{2+} levels as shown in Fig. 4.

We therefore interrogated the influence of elicitors in the absence of extracellular Ca^{2+} in *B. subtilis* 1604 pHCM05-Aq. Individual addition of OG or MO increased Ca^{2+} levels by 168- and 33-fold, respectively. Addition of water caused an increase of fourfold. When cells were challenged with OG followed by MO, 170- and 39-fold increases in cytosolic Ca^{2+} levels were observed (Fig. 5).

Discussion

Calcium signalling has recently been recognised to be present in bacterial cultures. The role of Ca^{2+} has been harder to demonstrate due to the difficulties in measuring intracellular Ca^{2+} levels in this type of cells. This is due to the size of the cells, the presence of cell wall, dye loading into the bacteria, dye toxicity and background fluorescence (Norris et al. 1996). Aequorin technology however, can measure intracellular Ca^{2+} levels in living cells without any of the above mentioned drawbacks (Norris et al. 1996).

Calcium homeostasis, calcium channels, calcium-binding proteins and a calcium-dependent adenylate cyclise (Knight et al. 1991b) have been reported in *E. coli* and *B. subtilis* (Herbaud et al. 1998; Jones et al. 2002; Dominguez 2004). Alterations in intracellular Ca^{2+} levels trigger cell activation through protein phosphorylation (Campbell 1983; Norris et al. 1996).

In this work, the effect of exogenous calcium was tested in *E. coli* pHCM05-Aq and *B. subtilis* pHCM05-Aq cultures. Ca^{2+} transients were observed upon $CaCl_2$ addition with resting levels re-

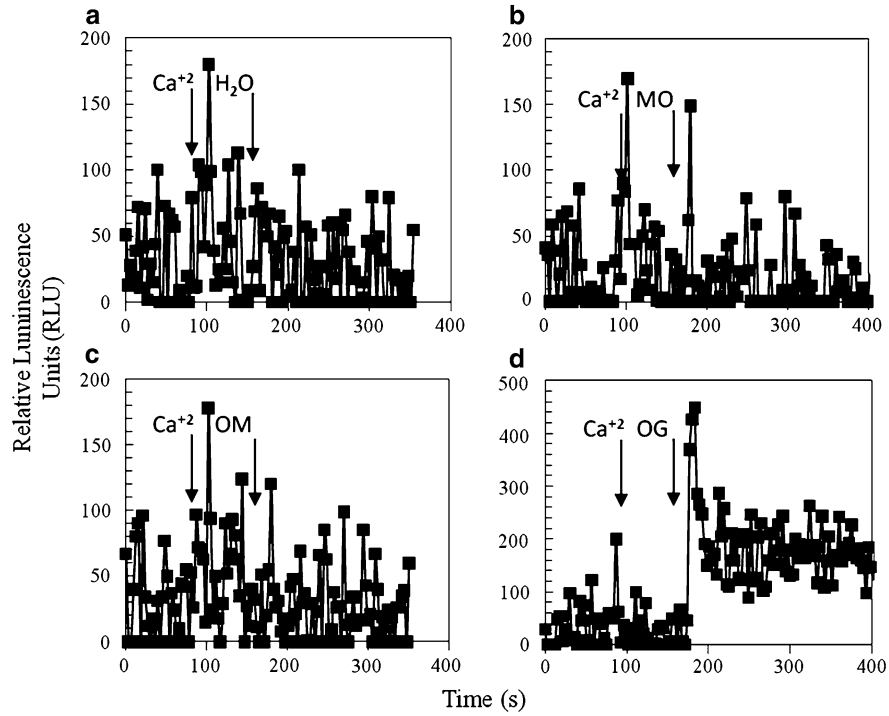

Fig. 4 Effect of 0.1 mM $CaCl_2$ followed by **a** water or elicitors; **b** mannan oligosaccharide, MO at 300 mg l^{-1}; **c** oligomanuronate, OM at 200 mg l^{-1}; **d** oligoguluronate, OG at 100 mg l^{-1} on *B. subtilis* 1604 pHCMC05-Aq. Cells were re-suspended in buffer A (in the presence of EGTA). *Arrows* represent additions to the culture

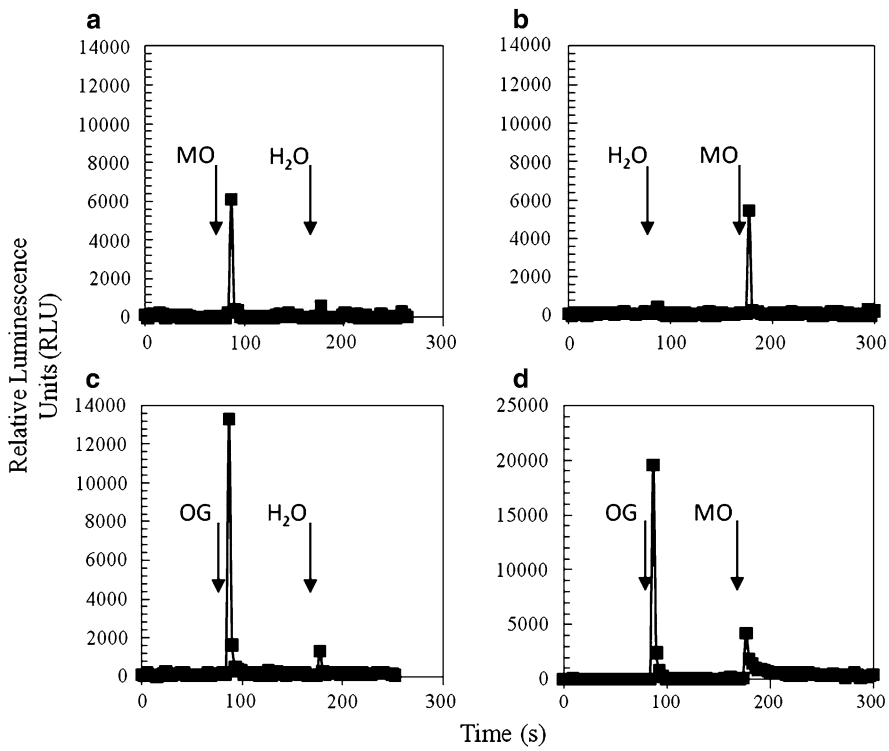

Fig. 5 Effect of water or elicitor addition on *B. subtilis* 1604 pHCMC05-Aq: **a** mannan oligosaccharide (MO at 300 mg l^{-1}) followed by water; **b** water followed by mannan oligosaccharide (MO at 300 mg l^{-1}); **c** oligoguluronate (OG at 100 mg l^{-1}) followed by water; **d** oligoguluronate (OG at 100 mg l^{-1}) followed by mannan oligosaccharide (MO at 300 mg l^{-1}). Cells were re-suspended in buffer A (in the presence of EGTA). *Arrows* represent the addition of either water or elicitors to the culture

establishing rapidly as reported by other investigators (Knight et al. 1991b; Jones et al. 1999; Watkins et al. 1995; Herbaud et al. 1998). Transient response in Ca^{2+} levels due to external stimulus should disappear fast for the cell to generate a rapid and efficient response to new situations (Norris et al. 1996).

As shown in Figs. 2 and 3, higher exogenous calcium concentrations were required to create a notable intracellular Ca^{2+} increase in *E. coli* compared to *B. subtilis* cultures. Similar observations were reported in bacterial cultures during the investigation of the role of Ca^{2+} in chemotaxis. Higher external calcium levels were required by *E. coli* (1 mM) compared to *B. subtilis* (100 nM) to promote Ca^{2+} transients that would result in the cells tumbling instead of swimming. The differences in exogenous calcium concentration required by both cultures to generate intracellular Ca^{2+} changes are thought to be due to the difference in the cell envelope/wall structures between Gram-positive and Gram-negative bacteria (Watkins et al. 1995).

Different Ca^{2+} signatures were observed upon addition of OG or MO to *E. coli* pHCM05-Aq and *B. subtilis* pHCM05-Aq, suggesting that different calcium messages might be transmitted probably due to the difference in the three-dimensional structure between MO and OG. This may explain why higher antibiotic levels are achieved when *P. chrysogenum* and *Bacillus licheniformis* are stimulated with multiple elicitor additions using different elicitors (OG and MO) in comparison to repeated addition of the same elicitor to the culture (Murphy et al. 2007b; Nair et al. 2009).

Addition of different elicitors to *Nicotiana plumbaginifolia* produced different Ca^{2+} signatures varying in intensity and duration. The increase in intracellular Ca^{2+} levels is a fast event in elicitor sensing mechanisms in plants and is dependent on the interaction and binding of elicitors to the specific receptors (Lecourieux et al. 2002). Similar results were obtained by (Knight et al. 1991a) studying the effect of yeast elicitors on *N. plumbaginifolia.* The authors suggested that signalling occurs through Ca^{2+} level changes and the signal transmitted depends on the Ca^{2+} signatures.

The results presented in this study confirm that *E. coli* and *B. subtilis* cultures are able to regulate intracellular Ca^{2+} levels and to sense external signals which are transmitted through Ca^{2+} transients. Here we report, for the first time, the effect of elicitors on intracellular Ca^{2+} in bacterial cultures. It is suggested that cytosolic Ca^{2+} levels increase due to addition of OG and MO elicitors and therefore the effects on secondary metabolite production and gene expression observed in many fungal and bacterial cultures upon addition of elicitors is, at least partly, due to the increase in Ca^{2+} levels which activates the signalling mechanisms in both systems. Further investigation into the elucidation of elicitation mechanism and its understanding could lead to the application of this novel, cheap and easy method for enhancement of secondary metabolites production at large-scale.

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