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Functional characterisation of Lp_2714, an EAL-domain protein from *Lactobacillus plantarum*

Richard Brown, Julian R. Marchesi, Andrew P. Morby *

School of Biosciences, Cardiff University, Cardiff CF10 3US, United Kingdom

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ABSTRACT

Bioinformatic analysis of *lp_2714* from *Lactobacillus plantarum* WCFS1 demonstrates that it encodes an EAL-domain protein associated with a membrane targeting signal-sequence. Comparison of the predicted primary amino-acid sequence of Lp_2714 shows that it lacks critical catalytic residues and heterologous expression has determined that it does not encode a functional phosphodiesterase. We designate Lp_2714 as a class-3 EAL domain protein probably involved in regulating polysaccharide synthesis on the cell surface the cell.

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1. Introduction

Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is synthesised by diguanylate cyclases (DGCs, containing a GGDEF domain) and degraded by phosphodiesterases (PDEs, containing an EAL domain). Proteins containing GGDEF or EAL domains appears to be limited to prokaryotes wherein their abundance varies widely, with *Vibrio vulnificus* possessing 66 GGDEF and 33 EAL proteins while *Escherichia coli* (*E. coli*) has 19 and 17 and *Bacillus subtilis* 4 and 3, respectively [1].

c-di-GMP was first described as a second messenger in *Gluconacetobacter xylinum* (formerly *Acetobacter xylinum*) in which it is a regulator of cellulose synthesis [2]. This compound has since been linked to signalling in prokaryotes with involvement in a range of pathways as diverse as cell cycle progression [3] and antibiotic synthesis [4] but also impacts on virulence and environmental colonisation, however, the primary function ascribed to c-di-GMP is as a mediator of the switch between sessility and motility (reviewed in [5,6]).

The focus of this work was to characterise the activity and derived phenotype of a putative c-di-GMP PDE from the commercially important organism, *Lactobacillus plantarum* WCFS1 (*L. plantarum*). The gene *lp_2714* encodes an EAL-domain associated with a signal-peptide motif consistent with membrane association.

2. Materials and methods

2.1. Bacterial strains and culture media

L. plantarum was NCIMB 8826 strain (WCFS1) cultured in MRS medium. Genomic DNA was extracted by "bead-beating" plus ammonium acetate and chloroform extraction. Sub-cloning grade competent DH5 alpha *E. coli* for transformation were obtained from Invitrogen. Sub cloning grade competent XL-1 blue *E. coli* was obtained from Stratagene. Plasmid transformed *E. coli* were cultured on LB broth or agar supplemented with ampicillin 100 μg/ml.

2.2. Chemicals, reagents and laboratory consumables

General laboratory chemicals were obtained from Sigma–Aldrich or Fisher Scientific UK and were of analytical grade or higher. Other reagent and consumable suppliers are listed in the text where appropriate.

2.3. Manipulation of DNA molecules

DNA manipulations were carried out in accordance with Sambrook et al. [7] unless otherwise specified.

2.4. BioInformatics

Gene identities for annotated cyclic-di-GMP metabolising enzymes of *L. plantarum* were obtained from the MiST2 microbial signal transduction database [8,9] and SMART [10] databases.

* Corresponding author. Address: School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3US, United Kingdom.
E-mail address: morby@cf.ac.uk (A.P. Morby).

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Operon analysis of *lp_2714* associated genes used NCBI genome. Comparative EAL domain alignment was performed using DIALIGN [11]. *lp_2714* upstream promoter sequence was analysed using HMMER [12].

2.5. Isolation and expression of lp_2714

lp_2714 (NC_004567.1 *L plantarum* WCFS1, 241939–241573) is an 824 bp gene [8].

For PCR, *lp_2714* insert variants all used the same reverse primer 5′-TTGCCGATTAACATCATCAG-3′ corresponding to location 2415791 slightly downstream (28 nt) of the stop codon. Respective forward primers were: insert plus complete upstream promoter (*lp_2714*) 5′-GTTCATAAGACAGGACTG-3′ (2414761 yielding a 1031 bp amplicon); insert with coding sequence alone (*lp_2714 ATG*) 5′-ATGAGTCTAGCACAATTAGAAATAGG-3′ (2414939 yielding an 852 bp amplicon).

lp_2714 amplicon variants were introduced into pGEM-T using a conventional AT cloning protocol (Promega). Plasmid inserts were generated by PCR from the extracted *L. plantarum* genomic DNA template employing the respective upstream forward primer plus the generic *lp_2714* reverse primer using Promega GoTaQ Flexi (2 mM Magnesium), dNTPs 250 nM, primers 150 nM. Cycling conditions were 95 °C 5 min, then 30 cycles of 95 °C 30 s, 45 °C 30 s, 72 °C 60 s followed by a final extension at 72 °C for 10 min. PCR products were purified by band excision from preparative 1% agarose gel electrophoresis followed by extraction from the gel band (QIAquick, Qiagen).

PCR products were ligated into pGEM-T (Promega) according to the protocol using overnight incubation at 4 °C. Ligated plasmid was used to transform competent DH5alpha *E. coli* by heat shock. Transformants were plated out on LB/ampicillin agar. Plasmid insert integrity was confirmed by Sanger sequencing.

2.6. Determination of Lp_2714 transcription by RTPCR

One millilitre of overnight confluent cultures of Lp_2714(P) and no insert control were added to one volume of RNA later (Ambion) and then centrifuged at 5000*g*. Cell pellets were stored at −80 °C prior to processing by lysozyme digestion followed by RNA extraction (RNeasy Qiagen) employing on column DNase treatment. cDNA was generated from extracted RNA using Quantitect (Qiagen) employing random hexamer primers. Presence of complementary Lp_2714 DNA was then detected in 1/50 dilution of the cDNA extract by PCR using a forward primer 5′-GAAGCCCGCGGTTACGGCAT-3′ and a reverse primer 5′-GGTTCAGGTCCAGCCAGACGG-3′ designed to amplify positions 487–646 (160 bp) of the transcribed Lp_2714 gene. Generated amplicon was identified by 2% agarose gel electrophoresis (data not shown).

2.7. Site directed mutagenesis of the Lp_2714 ECL motif

Lp-2714 contains an EAL-domain with ECL as the conserved motif. The ECL motif was mutated to ACL by a single base substitution at residue 65 by codon alteration from GAA to GCA. Primers were the complementary oligonucleotides 5′-CGCACTACCGGCTATGCATGT CTCCTGCG-3′ and 5′-CGCAGGAGACATGCATAGCCGGTAGTGCG-3′. Purified pGEM-T plasmid containing the *lp_2714* insert, complete with the upstream promoter sequence, was used as template in conjunction with the QuikChange system (Stratagene).

2.8. Non-specific phosphodiesterase (PDE) activity assay

Non-specific PDE activity was assessed by hydrolysis of bis (*p*-nitrophenyl) phosphate BNPP (Sigma). A 2 ml sample of broth culture (18 h at 37 °C, agitation 200 rpm) of DH5-alpha carrying a range of pGEM-T derivatives was harvested by centrifugation. The cell pellet was resuspended in 1 ml of assay buffer (50 mM Tris pH 8.5). The cell suspension was then added to 0.5 g of 0.1 mm glass beads and subjected to "bead beating" at 6.5 m/s for 30 s followed by centrifugation at 20,000*g* for 10 min. Duplicate volumes of the supernatant were transferred to the wells of a 96 well polystyrene micro-titre plate. BNPP substrate (10 mM) was dissolved in assay buffer containing 2 mM manganese chloride. The assay was incubated at room temperature for 1 h and the absorbance at 405nM measured on a plate reading spectrophotometer. Individual sample absorbance was normalised against the protein concentration (Bradford Reagent, Sigma) of the cell extract.

2.9. Confocal microscopy

Overnight confluent cultures were stained by acridine-orange [13]. A Leica TCS SP2 AOBS confocal microscope was used for imaging.

3. Results

3.1. Bioinformatic analysis of Lp_2714

Functional domain analysis compared Lp_2714 to canonical EAL proteins (Fig. 1). The predicted primary sequence of Lp_2714 appears to be partially degenerate in that it is missing 4/10 of these conserved residues.

3.2. Effect of insert on culture characteristics and cellular plasmid load

All of the analysed clones contained the insert orientated in the opposite sense to the pGEM-T P_{lac} promoter. Transformations also yielded cells that contained no detectable insert, these were assumed to contain recircularised plasmid and clonal replicates were used as "no insert controls". RTPCR indicated the presence of specific *lp_2714* transcript in cells transformed with the *lp_2714* insert but not in the controls (data not shown). This indicated that the *lp_2714* insert upstream promoter sequence appeared to be functional and capable of being recognised by host transcriptional apparatus. The optical density of 18 h broth cultures of replicate clones of transformants containing either control (no insert) and plasmid plus full length insert (*lp_2714*) or ACL mutated insert (*lp_2714 MUT*) were compared (Fig. 2 A,B). When assessed by conventional measurement of absorbance at 600 nm cells carrying w.t. or mutated *lp_2714* gave a higher value compared to the control. However, when viable cell density was measured [14] the CFU/ml were slightly higher in the control cells than for either strain carrying *lp_2714*.

lp_2714 forward PCR primer ATG was used to generate a truncated derivative lacking the associated promoter region alone and the Shine–Dalgarno motif.

Transformants containing plasmids with full length inserts (*lp_2714* and *lp_2714 MUT*) yielded minimal quantities compared to those containing truncated insert and no insert control (data not shown). Thus all strains in which the complete upstream promoter sequence was present consistently yielded plasmid at much lower levels than where this promoter sequence was absent.

3.3. Determination of phosphodiesterase (PDE) activity

The PDE activity assay acts as a proxy indicator of specific c-di-GMP PDE activity and since Mn(II)-specificity for EAL domain PDE activity appears to be a consistent feature [15], the assays were performed in Mn(II)-supplemented conditions (Fig. 3). Ca(II) supplementation resulted in a significant reduction in the observed

```
                                                                                *
lp_2714         LENDDFNLRY FIQKQVDFRG rtTGYECLLR QQNTDGSWSL PPQLDSLplq
PdeA            IGRGEITPYF QPIVRLSTGA L.SGFEALAR WIHPRRGMLP PDEFLPLIEE
Yaha            LENHEFKPWI QPVFCAQTGV L.TGCEVLVR WEHPQTGIIP PDQFIPLAES
RocR            LDNGEFEAYY QPKVALDGGG L.IGAEVLAR WNHPHLGVLP PSHFLYVMET
A2389           VVRDDVVVYA QPICQARSHI V.ASYECLVR .IEDEGEIIL PGNFLPIITD
Catalytic                                E   r                    e

lp_2714         rvifl..... .LEDTFKALP D.....EA.. .ITLSINLEY EQIISPEFRY
PdeA            MGLMSELGAH MMHAAAQQLS TWRAAHPamg nLTVSVNLST GEIDRPGLVA
Yaha            SGLIVIMTRQ LMKQTADILM PVKHLLPdnf ..HIGINVSA GCFLAAGFEK
RocR            YNLVDKLFWQ LFSQGLATRR klaqlgQP.. .INLAFNVHP SQLGSRALAE
A2389           THLYTRLSRQ MITHTFNMMR H.....RP.. .EAFSINLSP QDLMSERTLQ
Catalytic                                              N
                                         *
lp_2714         FVRWAIANI. EPMHLA.IEY TPQYQPRRIN KRLfrRRIRE ARGYGMQFGI
PdeA            DVAETLRVNR LPRGALKLEV TESDIMRDPE RAAVI..LKT LRDAGAGLAL
Yaha            ECLNLVNKLG NDKIKLVLEL TErnpipvtp earaifdslh qh..NITFAL
RocR            NISALLTEFH LPPSSVMFEI TETGLIsapa ssl..ENLVR LRIMGCGLAM
A2389           HLEAAIKSVA DPARVG.LEV LESEQIKDYG RMIEV..CNH FRTLGATIIV
Catalytic                                E

                 *                       *
lp_2714         DNVGASLANL KNIQWLLKDI DTLKCSMRSF RKEDpsvwld lnlqfwnQLS
PdeA            DDFGTGFSSL SYLTRLPFDT LKIDRYFVRT MGNNAGSAKI VRSVV..KLG
Yaha            DDFGTGYATY RYLQAFPVDF IKIDKSFVQM ASVDeisghI VDNIV..ELA
RocR            DDFGAGYSSL DRLCEFPFSQ IKLDRTFVQK MktqprscaV ISSVV..ALA
A2389           DDFGSGYSNI DEIVKLEPQV IKLDGSLIRN IDQDVKQRRI AEQLV..KLC
Catalytic       DD                       k

                         *
lp_2714         KENNIDLILM GIENEADEQL AEQLQISIRQ GYLFGHPINP QQSttkenhd
PdeA            QDLDLEVVAE GVENAEMAHA LQSLGCDYGQ GFGYAPALSP QEAevyl...
Yaha            RKPGLSIVAE GVETQEQADL MIGKGVHFLQ GYLYSPPVPG NKFisewvmk
RocR            QALGISLVVE GVESDEQRVR LIELGCSIAQ GYLFARPMPE QHFldycsgs
A2389           QVLNAKTVAE FVHNQTVCRI SEDMGVDYLQ GYFLGRPsrl g........
Catalytic                E                       Q
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Fig. 1. Alignment of the predicted primary amino-acid sequence of Lp_2714 and representative EAL domains from *E. coli*, *V. cholerae*, *C. crescentus* and *P. aeruginosa*. Residues known to be conserved and catalytically important are highlighted, those that are crucial to the function of the phosphodiesterase are capitalised below the alignment. The sites at which Lp_2714 differs from these essential residues are denoted (*) above the alignment.

enzyme activity (data not shown). PDE activity was normalised to sample protein. PDE activity was higher in transformants containing *lp_2714* or *lp_2714 MUT* inserts than controls with either no insert, or *lp_2714* lacking a promoter region and the Shine–Dalgarno motif. However no difference in activity was observed between the two full-length insert types indicating that the ACL mutant is phenotypically equivalent to w.t.

3.4. Cell morphology by light microscopy

Fluorescent microscopy of acridine orange stained transformants (Fig. 4) demonstrated that cells containing plasmid with full length inserts (*lp_2714* and *lp_2714 MUT*, (Fig. 4B and D) displayed a filamentous phenotype characteristic of impairment of septal cell wall development. Control cells containing plasmids with either no insert, or *lp_2714* lacking a promoter region and ATG-start codon, displayed normal morphology (Fig. 4A and C).

4. Discussion

Genome sequencing and analysis of *L. plantarum* WCFS1 shows that the genome contains 8 genes encoding proteins that are involved in turnover of c-di-GMP (MiST2, [9]). Of these, 3 contain a GGDEF domain characteristic of GMP di-guanylate cyclase and 5 contain an EAL domain characteristic of c-di-GMP phosphodiesterase (PDE) activity. We chose to focus on *lp_2714* as, unlike the other four genes encoding EAL domain proteins identified within the *L. plantarum* genome, it also encodes a hydrophobic leader sequence consistent with cell membrane attachment (SMART [10,16]). Tchingvintsev et al. [17] analysed 13 EAL domain proteins, both active and inactive with respect to the degradation of c-di-GMP and the comparison of activity/structure with primary sequence identified 5 absolutely conserved catalytically important residues and 10 highly conserved residues also involved in catalysis. Whilst the predicted primary sequence of Lp_2714 aligns well with EAL-domain proteins, it is missing 3/5 absolutely conserved residues and this demonstrates that, although it has similarity to known c-di-GMP degradative phosphodiesterases, critical catalytic residues are absent. This suggests that Lp_2714 may bind and perhaps signal the presence of c-di-GMP rather than degrade it. Rao et al. [18] and Römling [19] identified three classes of EAL domain proteins based on comparison of structural characteristics and functional catalytic activity. On this basis, we classify Lp_2714 as a class 3 EAL domain protein lacking crucial residues and deemed to be catalytically inactive.

In order to investigate the function of Lp_2714, we chose to express the gene from its own promoter within *E. coli*. This analysis generated cells with a significantly altered phenotype to that of the control cells. It is clear that the product of *lp_2714* is detrimental to the cell, this is demonstrated by the presence of a filamentous morphology, altered cell size and the minimal plasmid loading found in cells expressing *lp_2714*. The low plasmid levels in such cells may be viewed as a compromise between the minimal-level of plasmid required to confer effective antibiotic resistance and constitutive expression of a functionally active gene product that was harmful to the host. It is unclear if the phenotype displayed is due to protein over-expression, a consequence of Lp_2714 interacting with the c-di-GMP network within the

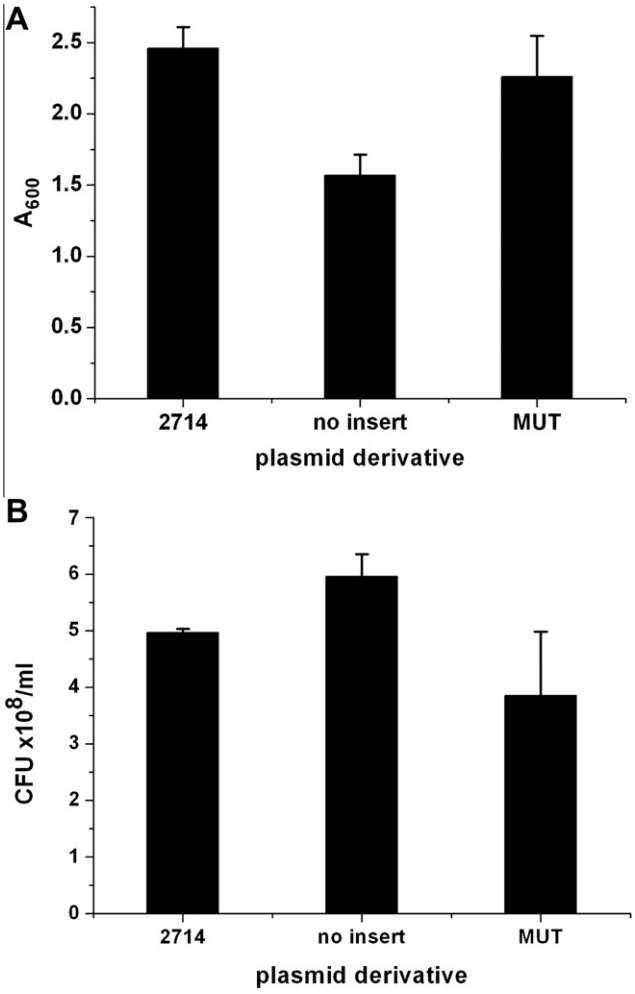

Fig. 2. The impact of Lp_2714 and its derivatives on cell growth and viability. Data are the mean plus the standard deviation of four clonal replicates for each derivative: no insert control; gene plus full upstream promoter sequence as (2714), with ECL to ACL mutation (MUT); coding sequence only (ATG). (A) Shows absorbance (600 nm). (B) Shows colony forming units obtained on the same cultures.

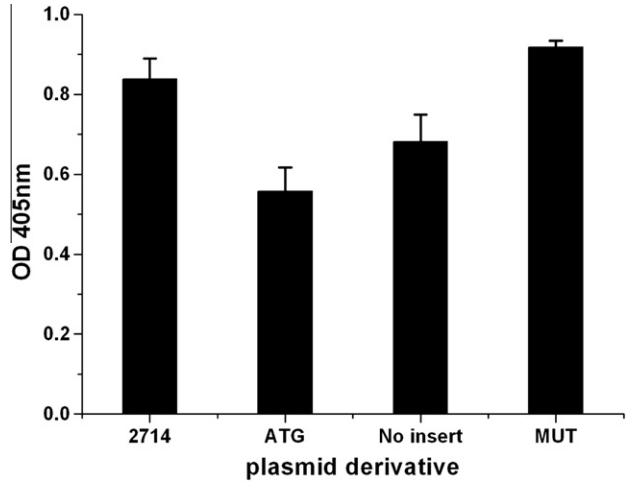

Fig. 3. Effect of Lp_2714 and its derivatives on non-specific phosphodiesterase (PDE) activity in cell extracts. PDE activity was measured in the presence of 1 mM Mn(II). The absorbance (420 nm), representing the metabolised substrate, is shown normalised for protein concentration. The data shown are mean plus the standard deviation of four clonal replicates for each insert.

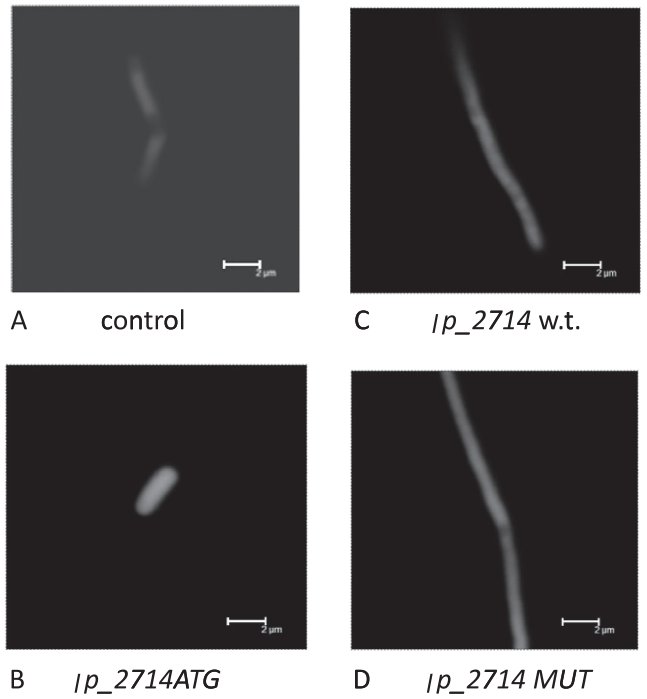

Fig. 4. Alterations in cellular morphology in response to Lp_2714 and its derivatives. Confocal fluorescent microscopy of acridine-orange stained transformed *E. coli* showing control cells containing pGEM-T alone (A) the control plasmid, w.t. Lp_2714 (B) and derivatives lacking an ATG start codon (C) or a mutated derivative (MUT) encoding an ECL to ACL alteration (D).

heterologous host or the creation of inhibitory sequences within the plasmid construct, however, one or both of the two former interpretations are thought more likely.

The primary characteristic of an EAL domain protein is the degradation of c-di-GMP, when tested, w.t. *lp_2714* conferred a slight increase in PDE activity in comparison with controls. However, PDE activity associated with cells containing the w.t. construct and the ACL derivative were equivalent, demonstrating that *lp_2714* does not encode an active PDE, this data is consistent with the bioinformatic categorisation of the protein product as a class 3 EAL domain protein deemed to be catalytically inactive. Similar experiments have been performed for other EAL domain proteins [15] and the results are supportive of our conclusions. Hence, we propose that any increase in PDE activity seen in cells carrying *lp_2714* is due to the interaction of its product with the cell system and not due to the presence of PDE activity associated with Lp_2714. It is unclear if the observed increase in cellular PDE activity is due to a specific interaction between Lp_2714 and the *E. coli* c-di-GMP network or non-specific effects engendered by its expression.

Analysis of the *L. plantarum* WCSF1 genome indicates that *lp_2714* appears to be part of a three-component operon along with *lp_2715* and *lp_2716*. Analysis of *lp_2715/2716* shows that both encode putative cytoplasmic membrane proteins, LP_2715 has no assigned function, however, Lp_2716 has similarity to glycosyl-transferases known to be involved in the synthesis of polysaccharides. This is of interest, as it is well known that c-di-GMP has an impact on polysaccharide synthesis in other organisms [2, reviewed in 19].

In conclusion, our data lead us to propose that Lp_2714 is a class 3 EAL-domain protein acting as a sensor for c-di-GMP and is involved in the regulation of a polysaccharide synthetic protein complex that directs the production of cellulose, or a similar derivative, on the cell surface.

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