

Large-Scale Production of the Immunomodulator c-di-GMP from GMP and ATP by an Enzymatic Cascade

Volker Spehr · Ralf Warrass · Klaus Höcherl · Thomas Ilg

Received: 12 January 2011 / Accepted: 24 May 2011 /
Published online: 28 June 2011
© Springer Science+Business Media, LLC 2011

Abstract (3′–5′)-Cyclic diguanylate (c-di-GMP) is a bacterial second messenger with immunomodulatory activities in mice suggesting potential applications as a vaccine adjuvant and as a therapeutic agent. Clinical studies in larger animals or humans will require larger doses that are difficult and expensive to generate by currently available chemical or enzymatic synthesis and purification methods. Here we report the production of c-di-GMP at the multi-gram scale from the economical precursors guanosine monophosphate (GMP) and adenosine triphosphate by a “one-pot” three enzyme cascade consisting of GMP kinase, nucleoside diphosphate kinase, and a mutated form of diguanylate cyclase engineered to lack product inhibition. The c-di-GMP was purified to apparent homogeneity by a combination of anion exchange chromatography and solvent precipitation and was characterized by reversed phase high performance liquid chromatography and mass spectrometry, nuclear magnetic resonance spectroscopy, and further compositional analyses. The immunomodulatory activity of the c-di-GMP preparation was confirmed by its potentiating effect on the lipopolysaccharide-induced interleukin 1 β , tumor necrosis factor α , and interleukin 6 messenger RNA expression in J774A.1 mouse macrophages.

Keywords C-di-GMP · Diguanylate cyclase · Enzymatic cascade · Immunomodulation · Biotechnological production

Introduction

(3′–5′)-Cyclic diguanylate (c-di-GMP) has been discovered in 1987 as an activator of cellulase synthase in *Gluconacetobacter xylinum* [1, 2]. Since that initial observation, it has become apparent that this cyclic dinucleotide is an important second messenger in many bacteria, which regulates a variety of cellular processes via binding to effector proteins and

V. Spehr · R. Warrass · T. Ilg (✉)
Intervet Innovation GmbH, Zur Propstei, 55270 Schwabenheim, Germany
e-mail: thomas.ilg@sp.intervet.com

K. Höcherl
Institut für Physiologie, Universität Regensburg, 93053 Regensburg, Germany

riboswitches [3–7]. The c-di-GMP-regulated bacterial processes encompass motility, biofilm formation, and cell cycle progression in many bacterial species, and diverse virulence mechanisms of pathogens [7–13].

c-di-GMP has drawn attention as an immunomodulator [14–16] with a novel mode of action [17]. The potential of c-di-GMP as a vaccine adjuvant has been demonstrated in mouse immunization experiments [18–23]. Earlier reports on the antiproliferative effect of c-di-GMP in cancer cells [24, 25] may be related to these activities. Furthermore, it has been reported that exogenously added c-di-GMP is able to inhibit biofilm formation in various pathogenic bacteria [26–28].

Further investigation of the biological roles of c-di-GMP and particularly the assessment of its clinical potential require a reliable and economical supply of this molecule. The predominant source of c-di-GMP has been chemical synthesis that, even after several improvements, has involved a complex and expensive multistep process using hazardous chemicals and is typically yielding milligram amounts [29–36]. An alternative biochemical production method became available after the identification of the genes encoding the c-di-GMP producing enzyme diguanylate cyclase (Dgc) in *G. xylinum* [37] and later in many other bacteria [38]. *Escherichia coli*-expressed recombinant Dgcs from various bacterial species were used to enzymatically generate partially purified c-di-GMP from guanosine triphosphate (GTP) or α - 32 P-GTP at an analytical scale [39–44]. Later studies expanded the enzymatic production of c-di-GMP from GTP by bacterial Dgcs to the 100-mg scale and reported purification schemes by reversed phase high performance liquid chromatography (HPLC) [45, 46].

In this study, we report a large-scale production method for c-di-GMP at the gram scale and at high purity from the, relative to GTP, more economical precursors guanosine monophosphate (GMP) and adenosine triphosphate (ATP) by an enzymatic cascade followed by low pressure anion exchange chromatography and solvent precipitation. Furthermore, we demonstrate the immunomodulatory activity of the c-di-GMP preparation on mouse macrophages.

Materials and Methods

Bacterial Strains, Plasmids, Chemicals, and Column Materials

E. coli strains JM109 (Promega), BL21(DE3)/pLysS (Invitrogen), TOP10 (Invitrogen), and M15 (Qiagen) were grown at 37 °C in Luria–Bertani (LB) medium modified with supplements as specified. *Caulobacter crescentus* strain CB2 (ATCC 15252) was grown at 30 °C in LB medium. The Ni $^{2+}$ -NTA agarose was from Qiagen. ATP and GMP were from AppliChem. Dowex 1-X2 (50–100 mesh, Cl $^{-}$) was from Serva.

Molecular Biology Techniques

Genomic DNA was isolated from bacterial pellets of *E. coli* JM109 and from *C. crescentus* by standard methods outlined in [47]. For the polymerase chain reaction (PCR) amplification of the genes for *E. coli* guanosine monophosphate kinase (GMPK, primer pair GGGATCCATGGCT-CAAGGCACGCTTATATTGTTTCTG and GAAGCTTCAGTCTGCCAACCAATTGCTG) and nucleoside diphosphate kinase (NDK, primer pair GGGATCCATGGCTATTGAACG-TACTTTTTCCATC and GAAGCTTAACGGGTGCGCGGGCACACTTC), reactions were performed using 4 ng/ml genomic DNA template and 0.5 μ M of each primer in standard PCRs (30 cycles, 30 s extension time, 57 °C annealing temperature). The expected DNA reaction products representing the GMPK and NDK encoding genes

were analyzed by 1% TAE agarose gel electrophoresis and isolated by silica adsorption (GENECLEAN, Qiogene). The PCR products were cloned into pCR2.1-TOPO (Invitrogen) and sequenced. Error-free inserts were subcloned via the primer-introduced restriction enzyme sites *Bam*HI and *Hind*III (underlined in the primer sequences) into pQE30 (Qiagen), and the resulting expression plasmids pQE30-Ecgmpr and pQE30-Ecndk were introduced into *E. coli* M15.

For the isolation and site-directed mutagenesis of the *C. crescentus* *Dgc* encoding *dgcA* gene, PCR was performed using 4 ng/ml genomic DNA of *C. crescentus* CB2 template and 1.0 μ M of primer Cacr-002 (CAGAAGCGTTGTCGTGCCCATGGTTG) and Cacr-004 (TTGCAGGCCAATGTGGT**CATGGGCGGCATCGTCGGCCGCATGGG**) in a standard PCR (35 cycles, 30 s extension time, 55 °C annealing temperature). The expected DNA band representing the 3'-end of the *dgcA* gene including the R153V-E154M-S155G-D155G mutation (bold in the primer sequence) was observed after 1% TBE agarose gel electrophoresis. The respective PCR band was excised, and the DNA fragment purified using the QIAquick PCR Purification Kit (Qiagen) and used as a megaprimer in the next PCR reaction in combination with 1.0 μ M primer Cacr-010 (GGTCTAGAAATCTCAGGCGCCCGACC) and 4 ng/ml genomic DNA of *C. crescentus* CB2 template. The expected DNA band representing the complete *dgcA* gene was excised after 1% TBE agarose gel electrophoresis, purified using the QIAquick PCR Purification Kit (Qiagen) and ligated into pCR-II TOPO (Invitrogen) to form pCacr-003b. Another PCR to introduce restriction enzyme sites *Bam*HI, *Nde*I, and *Xba*I was performed using 1 ng/ml pCacr-003b template and 1.0 μ M of primers Cacr-011 (CAGGATCCCGATCAAGCGCTCCTG) and Cacr-014 (GGTCTAGACATATGAAAATCTCAGGCGCCCGGA) in a standard PCR (35 cycles, 30 s extension time, 55 °C annealing temperature). The expected DNA band was observed after 1% TBE agarose gel electrophoresis, excised, purified by QIAquick PCR Purification Kit (Qiagen) and ligated into pCR-II TOPO to form pCacr-018a. For overexpression experiments, the open reading frame of *C. crescentus* *dgcA* from plasmid pCacr-18a was subcloned via the flanking *Bam*HI and *Nde*I sites into *Bam*HI/*Nde*I-cut pET-15b (Novagen) to form pCacr-20.

Expression and Purification of *E. coli* GMPK and NDK and *C. crescentus* *Dgc*

Overnight cultures of *E. coli* M15 containing pQE30-Ecgmpr or pQE30-Ecndk in LB medium with 100 μ g/ml ampicillin and 25 μ g/ml kanamycin were diluted 1+9 in fresh medium, grown for 2 h at 37 °C, supplemented with 1 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) and then grown for further 4.5 h at 37 °C. The harvested cell pellets of 1-l culture were thoroughly resuspended in 40 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0/NaOH) supplemented with 0.5% Triton X-100, 1 mg/ml lysozyme (Sigma), and 25 U/ml benzonuclease (Merck). After incubation on ice for 1 h, the lysates were centrifuged for 1 h at 2,500 \times g. The pellet was washed once with 10 ml lysis buffer with supplements and centrifuged. The combined centrifugation supernatants were then applied onto a 4-ml Ni²⁺-NTA agarose (Qiagen) column that was equilibrated with lysis buffer without supplements. The column was washed with 5 column volumes of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0/NaOH). Elution was performed with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0/NaOH), and 5 ml fractions were collected. All fractions of the lysate processing and the Ni²⁺-NTA agarose column eluate were analyzed by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. GMPK- or NDK-containing fractions were pooled, diluted with an equal volume of 87% (v/v)

glycerol and stored at -20°C . The protein concentration was determined by the Coomassie Blue dye binding method using bovine serum albumin as a standard [48].

For production of DgcA^{VMGG}, *E. coli* BL21(DE3)/pLysS cells carrying the expression plasmid pCacr-20 were grown in LB medium with 100 $\mu\text{g}/\text{ml}$ ampicillin and 20 $\mu\text{g}/\text{ml}$ chloramphenicol at 37°C , and expression was induced by adding IPTG at A_{600} 0.4 to a final concentration of 1 mM. After induction, cells were grown for an additional 4 h at 37°C . After harvesting by centrifugation, cells were resuspended in buffer containing 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 5% glycerol (10 ml/g cell pellet), and Lysonase (20 $\mu\text{l}/\text{g}$ cell pellet, Novagen); incubated for 15 min at room temperature; and lysed by passage through a French pressure cell. 3-(1-Pyridino)-1-propane sulfonate (NDSB-201, Fluka) was added to a final concentration of 125 mM, and the mixture was incubated for another 15 min at room temperature. Soluble and insoluble protein fractions were separated by centrifugation for 15 min at $8,000\times g$. The pellet containing the inclusion bodies was washed once with wash buffer (10 ml/g cell pellet) containing 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 5% glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP, Fluka), and 125 mM NDSB-201 and centrifuged for 15 min at $8,000\times g$. Afterwards, the inclusion bodies were washed twice with resuspension buffer (10 ml/g cell pellet) containing 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 5% glycerol, and 1 mM TCEP and centrifuged for 15 min at $8,000\times g$. The purified inclusion bodies were stored at -80°C or resuspended in buffer containing 50 mM Tris–HCl, pH 8.0, 200 mM NaCl, 2 mM EDTA, 7 M guanidine hydrochloride, and 10 mM TCEP by stirring for 60 min at room temperature. Soluble and insoluble protein fractions were separated by centrifugation for 15 min at $25,000\times g$ and 4°C . The supernatant containing the denatured DgcA^{VMGG} was sterilized by filtration through a 0.45- μm filter and stored at -80°C until use. For refolding, denatured DgcA^{VMGG} (5 mg/ml) was added to 25 vol. buffer containing 500 mM L-arginine (Sigma), 50 mM HEPES (Sigma), pH 7.5 and incubated with stirring at 4°C for 18 h.

Analytical Procedures for c-di-GMP Detection, Structure Confirmation, and Determination of Purity

^1H -, ^{13}C -, and ^{31}P -nuclear magnetic resonance (NMR) spectra were recorded either on a Bruker DRX 400 spectrometer (400.13 MHz, 100.61 MHz, respectively) or on a Bruker DPX300 spectrometer (300.13, 75.47, and 121.50 MHz, respectively) and were internally referenced to the solvent (H_2O). Data for ^1H -NMR were reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet). HPLC with mass spectrometry and UV detection (HPLC-UV/MS) data were collected using an Agilent HPLC/MSD 1100 system that included a mass detector G1946D SL with ESI-source and an evaporating light detector Sedex 75. Samples were loaded onto an Atlantis C18-HPLC-column (4.6 \times 50 mm, dC18, 3 μm) and eluted at a flow rate of 1 ml/min by an acetonitrile gradient formed in 0.1% formic acid in H_2O (2 min 0% acetonitrile, 0–10% acetonitrile in 5 min, 1 min at 10% acetonitrile). Ion chromatography was performed using 761 Compact IC (Metrohm) using a Metrosep C2-150 column (150 mm \times 4 mm, flow rate 1 ml/min) with 2 mM HNO_3 as eluent for cations and a Metrosep A Supp 5-150 column (150 mm \times 4 mm, flow rate 0.7 ml/min) with 1 mM $\text{NaHCO}_3/3.2$ mM Na_2CO_3 as eluent for anions according to the manufacturer's (Metrohm) instructions. The endotoxin content was analyzed by L+S AG (Mangsfels, Germany) by the Limulus test (LAL) according to European Pharmacopeia 6th edition (method B).

Enzyme Assays of *E. coli* GMPK and NDK and *C. crescentus* Dgc

Both the GMPK and the NDK assays were performed in 50 mM Tris–HCl pH 7.5, 20 mM MgCl₂, 1 mM ATP, 2 mM phosphoenol pyruvate, 2 mM DTT, 0.2 mM NADH, 10 units (U)/ml pyruvate kinase (rabbit muscle, Sigma), and 10 U/ml lactate dehydrogenase (rabbit muscle, Sigma) at room temperature. After addition of the enzyme samples, the reaction was started by the addition of 2 mM GMP (GMPK assay) or 2 mM dTDP (NDK assay), and the optical density at 340 nm was monitored. A molar absorption coefficient ϵ_{340} of $6,220 \text{ M}^{-1} \text{ cm}^{-1}$ was used in calculations for NADH consumption. One unit of enzyme activity is defined as consumption of 1 μMol NADH/min.

Small-scale production of c-di-GMP from GTP was done with 250 μl freshly refolded DgcA^{VMGG} (0.025 mg/ml), 20 μl GTP (2 mM), and 730 μl reaction buffer A containing 50 mM Tris–HCl (AppliChem), pH 7.5, 10 mM MgCl₂, 0.5 mM EDTA, and 50 mM NaCl. Small-scale production of c-di-GMP from GMP was done with 250 μl freshly refolded DgcA^{VMGG} (0.025 mg/ml), 1 μl NDK (643 mU/ μl in 50% glycerol), 1 μl GMPK (103.5 mU/ μl in 50% glycerol), 10 μl GMP (2 mM final concentration), 50 μl ATP (5 mM final concentration), and 688 μl reaction buffer A. Both reactions were mixed and incubated, with slight shaking at 80 rpm, at 30 °C for 16 h. The production of c-di-GMP was monitored by HPLC-UV/MS analysis.

Large-Scale Production of c-di-GMP by an Enzymatic Cascade and Purification to Homogeneity

One thousand two hundred fifty milliliters of freshly refolded DgcA^{VMGG}, 1,148 U NDK (643 mU/ μl in 50% glycerol), 144 U GMPK (103.5 mU/ μl in 50% glycerol), GMP (4 g, 11 mmol), ATP (13.75 g, 22.8 mmol), and 3,750 ml reaction buffer A were mixed and incubated at 30 °C for 16 h, with slight shaking at 80 rpm. A 70-cm-long column with an inner diameter of 2.5 cm was filled with Dowex 1 \times 2 (100–200 mesh, Serva), washed extensively with 0.5% (v/v) acetic acid, and subsequently equilibrated with H₂O at a flow rate of 10 ml/min. The crude reaction mixture of the enzymatic cascade (up to 5 l) that was previously cleared from insoluble components by passage through cellulose filters (Schleicher and Schuell) was loaded onto the column at a flow rate of 10 ml/min. After loading, the column was washed with 2,000 ml of H₂O followed by 2,000 ml of 2 M NH₄-acetate, 1,500 ml H₂O, and 1,000 ml 10 mM HCl/50 mM LiCl. Elution of c-di-GMP was initiated by washing the column with 2,000 ml 10 mM HCl/500 mM LiCl. The elution of nucleotide-containing fractions was monitored by measuring samples for their optical density at 253 nm and by HPLC-UV/MS. The 10 mM HCl/500 mM LiCl elution fractions with optical density at 253 nm were pooled (~800 ml), the acid content neutralized by the addition of 1.5 ml aqueous NH₃ (32% w/w), and the solvent evaporated under reduced pressure until a highly viscous solution was obtained (~20–50 ml). Addition of 470 ml ethanol/acetone (1:1, v/v) and 1 M HCl (30 ml) led to the formation of a precipitate that was separated by filtration (glass filter; pore size 3) and dried under reduced pressure at 42 °C. In order to prepare the diammonium salt, the compound was dissolved in 20 ml 1% aqueous NH₃, filtered (PET filter, 0.45 mm pore size), and lyophilized overnight. The final product of the c-di-GMP synthesis and purification procedure was characterized by HPLC-UV/MS, ¹H-, ¹³C-, and ³¹P-NMR spectroscopy; by ion chromatography; and by the LAL test.

Immunomodulatory Activity of Enzymatically Produced c-di-GMP

The mouse macrophage cell line J774A.1 (ATTC) was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C and 5% (v/v) CO₂. For test purposes, the growth medium of J774A.1 cells with 80–90% confluency was replaced by DMEM, 0.5% FCS for 24 h before use. Thereafter, cells were incubated with or without the addition of 50 µM c-di-GMP or tylosin, followed by the addition of 1 µg/ml *E. coli* 0111:B4 lipopolysaccharide (LPS, Sigma) after 1 h. Controls consisted of cells exposed to neither LPS nor c-di-GMP. Quadruplicates were used for each data point. The cells were incubated for 18 h at 37 °C and 5% (v/v) CO₂. Thereafter, the J774A.1 cells were washed twice with phosphate-buffered saline, and total RNA was isolated using the total EZNA RNA Mini kit (Omega Biotek) according to the manufacturer's instructions. Complementary DNA was synthesized by reverse transcription by standard protocols [49]. Real-time PCR was performed using a LightCycler 480 (Roche), the LightCycler 480 SYBR Green I Master kit (Roche), and the primer pairs TCTCGCAGCAGCACATCA and CACACACCAGCAGGTTAT (mouse IL-1β gene), CTCAGCCTCTTCTCATTC and GGTGGTTTGCTACGACGT (mouse TNF-α gene), CCGGAGAGGAGACTTCAC and CTGCAAGTGCATCATCGT (mouse IL-6 gene), and TTCATTGACCTCAACTACAT and GAGGGGCCATCCACAGTCTT (mouse GAPDH gene). Amplification was performed after an initial denaturation step for 10 min at 95 °C by 40 cycles of 15 s denaturation at 95 °C, 15 s annealing at 58 °C, and 20 s elongation at 72 °C. Quantification was performed using standard curves generated from six dilutions of LPS-incubated RNA samples and using the method "Abs Quant/2nd Derivative Max" within the LightCycler 480 software 1.5.0. (Roche). The concentrations of IL-1β, TNF-α and IL-6 messenger RNAs (mRNAs) relative to that of the housekeeping gene encoding GAPDH were determined. The average value for the LPS-treated group was set at 100%. The standard error of the quadruplicates was assessed by two-sided Student's *t* test. *p* values smaller than 0.05 were considered statistically significant.

Results

Gene Cloning and Overexpression of *E. coli* GMPK and NDK

Enzymatic synthesis of c-di-GMP from GTP has been described [39–46], but GTP is an expensive chemical and of limited availability. Therefore, a method for the *in situ* synthesis of GTP from more economical precursors was sought. An enzymatic synthesis method starting from GMP and ATP with GMPK and NDK as catalysts was proposed (Fig. 1). To this end, based on the GenBank database DNA sequences of *E. coli* GMPK (M84400) and *E. coli* NDK (X57555), primers were designed for PCR amplification of the respective genes. The DNA sequences of *E. coli* JM109 *gmpk* and *ndk* were deposited at GenBank under the accession numbers JF776372 and JF776373, respectively. After subcloning into a pQE30 expression vector, *E. coli* M15 was transformed with pQE30-Ec*gmpk* and pQE30-Ec*ndk*, large-scale expressions (1-l cultures) were performed, and overexpressed GMPK and NDK were purified by Ni²⁺-NTA agarose chromatography. The yield of GMPK was extraordinarily high with 189 mg/l culture, and the enzyme displayed a specific activity of 16.4 U/mg. NDK was obtained with a yield of 86.7 mg/l culture and a specific activity of 74.2 U/mg. Based on the appearance of Coomassie Blue-stained SDS polyacrylamide gels (Fig. 2a), both products are estimated to be >95% pure.

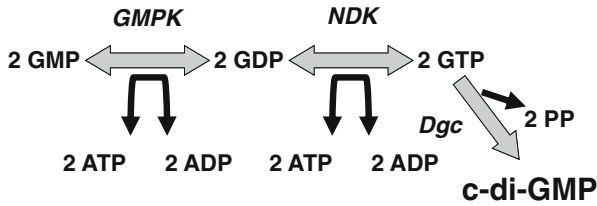
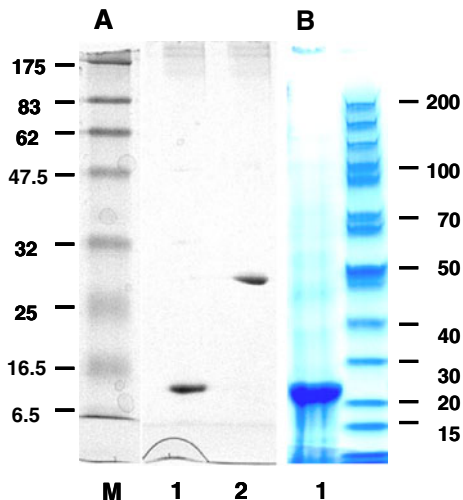


Fig. 1 Enzymatic cascade leading to the synthesis of (3'-5')-cyclic diguanylate (c-di-GMP). *GMP* guanosine monophosphate, *GDP* guanosine diphosphate, *GTP* guanosine triphosphate, *ATP* adenosine triphosphate, *ADP* adenosine diphosphate, *PP* pyrophosphate, *GMPK* guanosine monophosphate kinase, *NDK* nucleoside diphosphate kinase, *Dgc* diguanylate cyclase

Gene Cloning, Site-Directed Mutagenesis, and Overexpression and Refolding of *C. crescentus* Dgc

Potent product inhibition of diguanylate cyclase (Dgc) by c-di-GMP is a well-studied phenomenon ([50] and references therein), which limits the yields of enzymatic c-di-GMP synthesis. For the *C. crescentus* Dgc, it has been shown that a R153V-E154M-S155G-D155G mutation abolishes c-di-GMP feedback inhibition [50]. Based on GenBank database DNA sequences of *C. crescentus* DgcA (AE005673), primers were designed for PCR amplification of the gene. Subsequently, site-directed mutagenesis was performed by the “megaprimer” strategy [51–53]. The DNA sequence of *C. crescentus* CB2 *dgca*^{VMGG} was deposited at GenBank under the accession number JF776374. *E. coli* expression of this enzyme in an active soluble form (e.g., as maltose binding protein (MBP) fusion construct in pMalc2x) led to poor yields in the low milligram per liter culture range (not shown). Therefore, the *dgca*^{VMGG} open reading frame was subcloned into the pET-15b expression vector, *E. coli* BL21(DE3)/pLysS was transformed, and large-scale expression (2-l cultures) was performed. Overexpressed DgcA^{VMGG} was obtained as inclusion bodies at >>10× higher amounts than in the expression experiments aiming at soluble,

Fig. 2 SDS-PAGE of purified enzymes of the enzymatic cascade. *M* marker proteins, the molecular masses in kilodalton are indicated. **a** Lane 1: *E. coli* NDK 2 μg; lane 2: *E. coli* GMPK 2 μg. **b** Lane 1: *C. crescentus* DgcA^{VMGG} >10 μg



enzymatically active MBP–DgcA fusion. The purified inclusion bodies (0.5 g wet weight) were dissolved in guanidine chloride solution and subsequently refolded, yielding 325 mg DgcA^{VMGG}. Based on the appearance of Coomassie Blue-stained SDS polyacrylamide gels (Fig. 2b), the enzyme is estimated to be >95% pure. The enzymatic activity of the preparation was verified by small-scale production of c-di-GMP from GTP (compare Fig. 3 IIA).

Large-Scale Production of c-di-GMP by a “One-Pot” Enzymatic Cascade and Setup of a Purification Procedure

One thousand two hundred fifty milliliters of freshly refolded DgcA^{VMGG}, 1,148 U NDK, 144 U GMPK, 4 g GMP (11 mMol), 13.75 g ATP (22.8 mMol), and 3,750 ml reaction buffer A were mixed and incubated, with slight shaking at 80 rpm, at 30 °C for 16 h. Precipitates formed during the incubation period were subsequently removed by filtration and ~4.8 l of the mixture that contained as major nucleotide derivatives GTP, ATP, ADP, AMP, and c-di-GMP (Fig. 3 IIA) was loaded onto a Dowex 1×2 anion exchange column (Fig. 3 I). No c-di-GMP could be found in the flowthrough suggesting quantitative binding of the molecule. Nonbinding components were removed by washing the column with H₂O. For the subsequent elution steps, a focus was set on potential eluents that could be removed from c-di-GMP by lyophilization or solvent extraction. Numerous salts and solvents, and

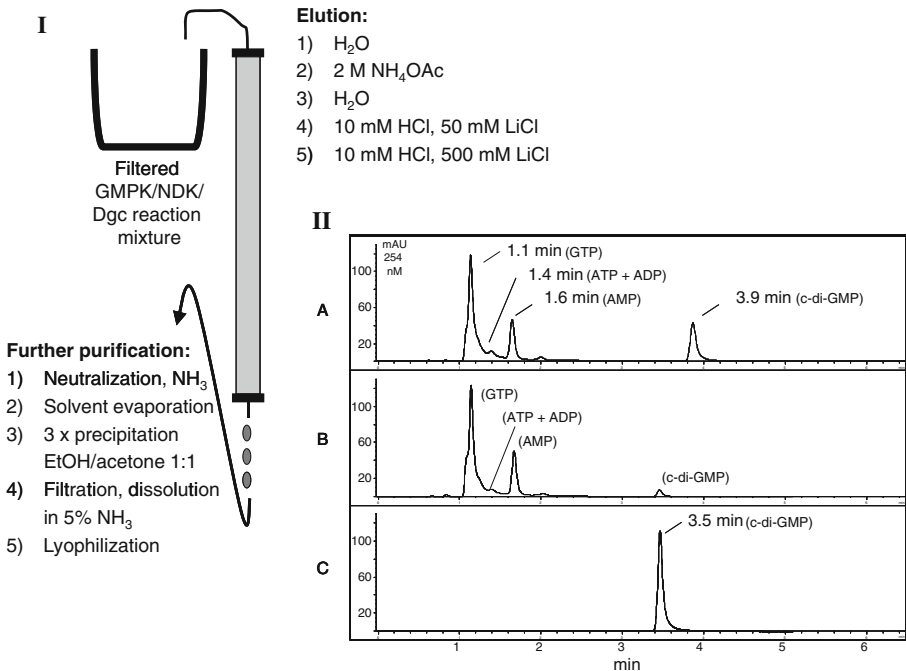


Fig. 3 Purification of c-di-GMP from the enzymatic cascade reaction mixture. *I* Purification scheme of Dowex 1×2 column chromatography and solvent precipitations. *II* Reversed phase HPLC analysis of aliquots from the reaction mixture after incubation (*A*), the 2-M NH₄OAc eluate (*B*), and the 10-mM HCl/500 mM LiCl eluate (*C*). Elution positions of nucleotide derivatives are indicated

combinations thereof, were tested, and the most straightforward solution is presented here (Fig. 3 I): consecutive step elutions (Fig. 3 I) were monitored by HPLC-UV/MS (Fig. 3 IIA–C). The application of 2 M NH_4 -acetate led to the elution of the bulk of GTP (1.1 min; $[\text{M}+1]^+=523.5$ amu), ATP (1.4 min; $[\text{M}+1]^+=507.8$ amu), ADP (1.4 min; $[\text{M}+1]^+=427.9$ amu), and AMP (1.6 min; $[\text{M}+1]^+=348.0$ amu) (Fig. 3 IIB), as well as traces of GDP (2.0 min; $[\text{M}+1]^+=443.9$ amu) and GMP (2.5 min; $[\text{M}+1]^+=364.0$ amu), while c-di-GMP (3.9 min; $[\text{M}+1]^+=691.4$ amu) was largely retained on the column (Fig. 3 IIB). A further wash with 10 mM HCl/50 mM LiCl led to the elution of further traces of nucleoside mono-, di-, and tri-phosphates, whereas c-di-GMP remained bound to the Dowex 1 \times 2 resin (not shown). Selective elution of c-di-GMP was achieved by a further wash with 10 mM HCl/500 mM LiCl (Fig. 3 IIC). No other peaks were apparent, neither by UV detection at $\lambda=254$ nm nor in mass detection (3.5 min; $[\text{M}+1]^+=691.4$ amu). Neutralization, concentration, and removal of salts by precipitation in ethanol/acetone led to colorless and pure c-di-GMP. The generation of the diammonium salt of c-di-GMP was achieved by the addition of ammonia and lyophilization; 1.75 g of the diammonium salt of c-di-GMP (724 g/mol; 2.4 mMol) was obtained as a white solid, which corresponds to an overall yield of ~44%, based on the GMP input into the reaction.

Structure Confirmation of c-di-GMP and Purity Assessment

The final product had the appearance of a white powder and was characterized by LCMS (Fig. 3 IIC), ^1H -NMR spectroscopy (Fig. 4), as well as ^{13}C - and ^{31}P -NMR (not shown). The NMR spectroscopy and HPLC-UV/MS signals of enzymatically produced c-di-GMP were compared to c-di-GMP samples synthesized by a modified published procedure [31, 32] and were found to be largely identical (Fig. 4a, c and data not shown). Estimation of purity by HPLC-UV/MS based on the absorption at $\lambda=210$ nm and 254 nm was 100% (see Fig. 3 IIC and data not shown), while quantitative NMR analysis using maleic acid as internal standard (Fig. 4b) revealed a c-di-GMP (free acid) content of 82% (w/w). Karl–Fischer analysis revealed a water content of approximately 10%, while ion chromatography determined the presence of sodium (0.4%), ammonium (5.3%; expected from the diammonium salt formation—5.0%), and lithium (0.04%) cations and small amounts of phosphate (0.15%) and chlorine (0.7%) anions in the final product. The lipopolysaccharide contamination of the purified product was determined by the LAL assay to be in between 0.192 and 0.384 endotoxin units (EU)/mg c-di-GMP. Typically, *E. coli* LPS possesses an activity of 1–10 EU/ng, which suggests that the endotoxin contamination of the c-di-GMP preparation is below 0.00005% (w/w).

Immunomodulatory Activity of c-di-GMP in the Mouse Macrophage Cell Line J774A.1

Earlier studies using chemically synthesized c-di-GMP have established this molecule as an immunostimulator [16]. To determine whether enzymatically synthesized c-di-GMP is also able to modulate the activity of immune cells, we investigated the effect of c-di-GMP on the LPS-induced production of IL-1 β , TNF- α , and IL-6 mRNA of the mouse macrophage cell line J774A.1 by a real-time PCR approach. Incubation of J774A.1 cells with 1 $\mu\text{g}/\text{ml}$ LPS leads to a massive induction of IL-1 β , TNF- α , and IL-6 mRNA (Fig. 5a–c). Coapplication of 50 μM tylosin, a macrolide antibiotic used in veterinary practice [54], showed no effect, while combination of LPS with 50 μM (~35 $\mu\text{g}/\text{ml}$) c-di-GMP increases the amount of IL-1 β and TNF- α mRNA in J774A.1 cells by ~80% and 160%, respectively, indicating (p values < 0.1) a stimulatory effect (Fig. 5a, b), while

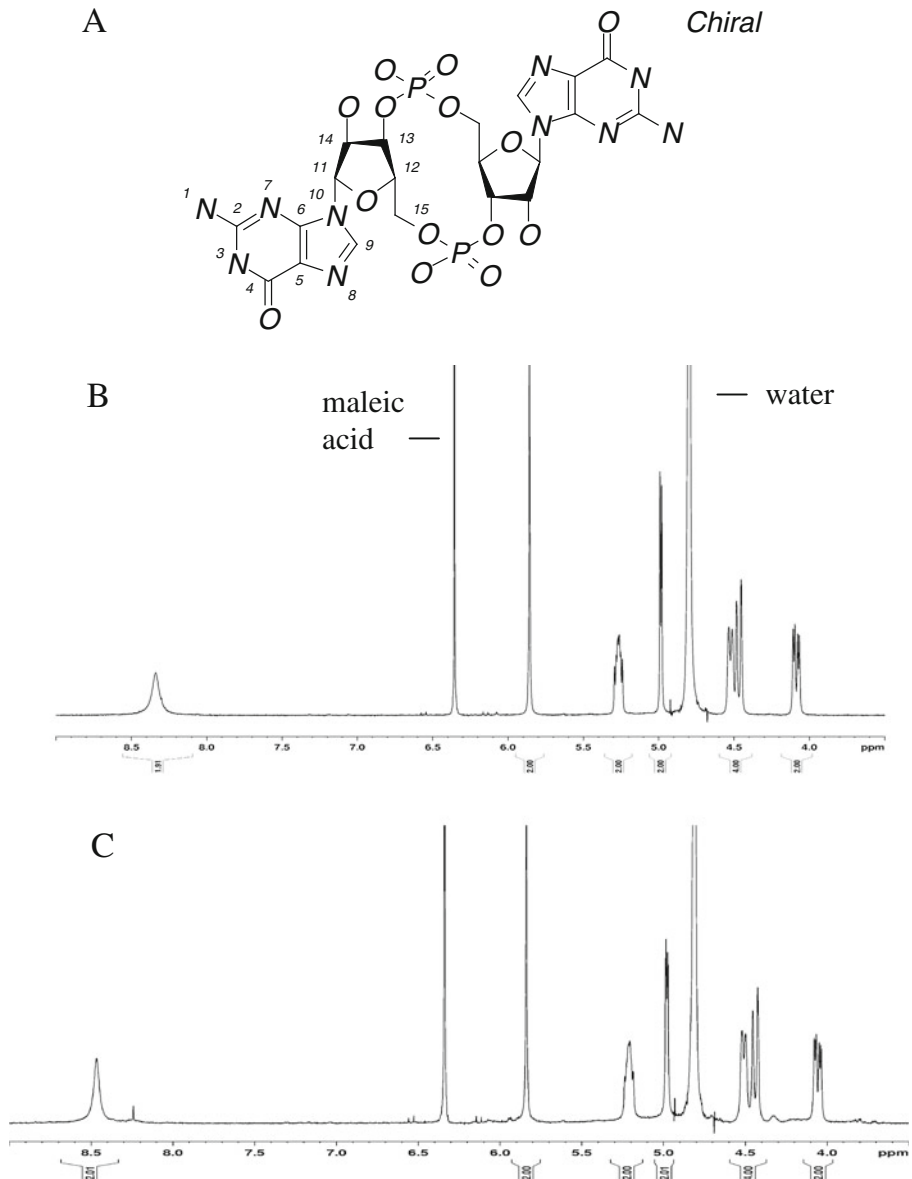
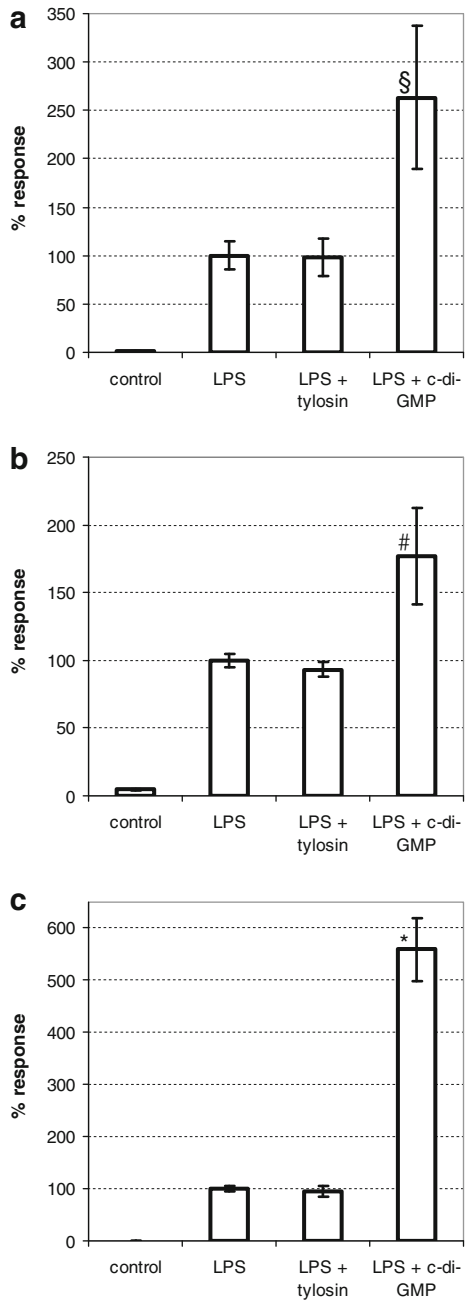


Fig. 4 $^1\text{H-NMR}$ spectroscopy of enzymatically and chemically synthesized c-di-GMP. **a** Chemical structure of c-di-GMP. **b**, **c** $^1\text{H-NMR}$ spectra of c-di-GMP preparations; s (8.32, 2H), s (5.85, 2H), m (5.26, 2H), m (5.00, 2H), s (4.79, H_2O), m (4.50, 4H), m (4.10, 2H); signal for maleic acid (s, 6.45) was used as internal reference for quantification. **b** Enzymatically synthesized c-di-GMP as described in this paper; **c** chemically synthesized according to a modified published procedure [31, 32]; synthesis modifications will be published elsewhere

elevation of IL-6 mRNA levels was highly significant by a factor of 4.5 (p value 0.0003) (Fig. 5c). These results demonstrate the immunomodulatory potential of enzymatically synthesized c-di-GMP.

Fig. 5 Effect of 50 μ M c-di-GMP or 50 μ M tylosin on LPS-induced cytokine mRNA expression in mouse J774A.1 macrophages. **a** Interleukin 1 β ; **b** tumor necrosis factor α ; **c** interleukin 6. The standard error of quadruplicate determinations is indicated; $\$p$ value 0.0743, $\#p$ value 0.0757, $*p$ value 0.0003



Discussion

Apart from its role as a bacterial second messenger, the cyclic dinucleotide c-di-GMP has attracted considerable interest as an immunomodulator and as a vaccine adjuvant, which holds promise for human medicine [7] and potentially for veterinary applications. However,

while *in vivo* proof-of-concept studies for these c-di-GMP activities have been exclusively performed in mice, further clinical studies in humans or farm and companion animals will require much larger quantities of c-di-GMP.

One strategy to provide c-di-GMP has been chemical synthesis, and various protocols have been published by different laboratories [21, 29, 31–34, 36, 55]. However, yields of the reported methods were in the low milligram scales; they required expensive, in part noxious precursors and reagents; and preparative reversed phase HPLC was used to give reasonable purity. Furthermore, while our own efforts to achieve upscaling of chemical c-di-GMP synthesis based on procedures published earlier [31, 32] resulted in gram amounts, it became obvious in the process that cost of goods, yields, and purification will remain challenges (R. Warrass et al., unpublished observations).

As an alternative strategy for the synthesis of c-di-GMP, enzymatic dimerization of GTP by the enzyme diguanylate cyclase (Dgc) has been employed up to the milligram scale [39–46], and in one study, the generation of 200 mg of c-di-GMP by pooling of several batches was reported. In view of further upscaling of enzymatic c-di-GMP production and purification, disadvantages of the published procedures include the use of the expensive precursor GTP and the need for reversed phase HPLC purification.

To replace the expensive GTP in the c-di-GMP production process, a synthesis cascade for GTP from the more economical precursors GMP and ATP was established using the *E. coli* enzymes GMPK and NDK that could be generated in large amounts by homologous overexpression. This led to a decrease of cost of goods for the precursors by a factor of more than 40. DgcA^{VMGG} from *C. crescentus* engineered by mutagenesis to be devoid of product feedback inhibition [50] was used as c-di-GMP synthesis enzyme, and expression as enzymatically inactive inclusion bodies proved to be crucial for high yields (>100 mg/l culture), while expression levels of the soluble, enzymatically active maltose binding protein fusion Dgc construct were poor (<<5 mg/l). It is conceivable that overexpression of enzymatically active Dgc, particularly if c-di-GMP feedback inhibition is abolished, is toxic for *E. coli*, which may explain the strong differences in expression levels. The solubilized and refolded *C. crescentus* DgcA inclusion bodies could be used in c-di-GMP synthesis without further purification and gave a specific yield of ~7 mg c-di-GMP/mg enzyme, which is higher than that observed for other mesophilic Dgcs [46], but somewhat lower than that seen recently for a thermophilic Dgc [45]. The conversion of GMP and ATP to c-di-GMP by a three enzyme cascade was performed in a “one-pot reaction” typically of 5-l volume, with c-di-GMP yields based on GMP input of about 50–70%. The product c-di-GMP had to be concentrated and separated from other substances in the reaction mixture such as HEPES, Tris, NaCl, EDTA, L-arginine, GTP, ATP, ADP, and AMP, the enzymes, as well as several minor contaminants. This was achieved by anion exchange chromatography on Dowex 1×2, where cationic and uncharged contaminants remained unbound, whereas the nucleotide derivatives were retained. Consecutive step elution with NH₄-acetate and LiCl/HCl resulted in a pure c-di-GMP fraction. It was unexpected that c-di-GMP remained bound to the Dowex 1×2 column in the 2 M NH₄-acetate (~pH 7) wash, while ATP, ADP, AMP, and GTP were eluted (Fig. 3 IIB). The net charges of ATP (and presumably GTP), ADP, and AMP at pH 7 are –4, –3, and –2, respectively [56], while that of c-di-GMP is expected to be –2. Perhaps differences in the geometry of negative charge distribution account for the counter-intuitive anion exchange column elution behavior. The c-di-GMP purification protocol using the low pressure resin Dowex 1×2 is more suitable to further upscaling and process development than the previously reported preparative reversed phase HPLC methods due to the ready availability of bulk column material, the ease of package of any column format, and the

avoidance of organic solvents during chromatography. The use of LiCl as a c-di-GMP eluent allowed near quantitative removal of excess salt by solvent precipitations.

Based on HPLC and NMR spectroscopy analyses, c-di-GMP was devoid of organic contaminants, and analysis of content by $^1\text{H-NMR}$ spectroscopy, water analysis, and ion chromatography showed a c-di-GMP content of 82% (w/w) for the final product, the balance largely accounted for by H_2O , Na^+ , NH_4^+ , Li^+ , PO_4^{3-} , and Cl^- . Despite the fact that two *E. coli*-expressed recombinant enzymes and crude refolded inclusion bodies were employed in the c-di-GMP synthesis, the endotoxin contamination of the final product was extremely low, making it suitable for immunological applications. We have confirmed the immunomodulatory activity of our c-di-GMP preparations in a mouse J774A.1 macrophage LPS stimulation model by detecting massive potentiation of LPS-induced IL-6 mRNA production, while, albeit statistically less significant, a similar effect was also seen for IL-1 β and TNF- α mRNA induction.

In summary, we have reported the synthesis of gram amounts of c-di-GMP from the economical precursors GMP and ATP by an enzymatic cascade using the enzymes *E. coli* GMPK, *E. coli* NDK, and *C. crescentus* DgcA^{VMGG}. C-di-GMP purification to homogeneity was achieved by an anion exchange chromatography and solvent precipitation protocol that is amenable to further upscaling and process development.

Acknowledgments We thank Nicole Engelmann, Karl-Heinz Grimm, Susanne Schmalz, and Heinz-Jörg Wennesheimer for expert technical assistance.

References

1. Ross, P., Aloni, Y., Weinhouse, C., Michaeli, D., Weinberger-Ohana, P., Meyer, R., et al. (1985). An unusual guanyl oligonucleotide regulates cellulose synthesis in *Acetobacter xylinum*. *FEBS Letters*, *186*, 191–196.
2. Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., et al. (1987). Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. *Nature*, *325*, 279–281.
3. Sudarsan, N., Lee, E. R., Weinberg, Z., Moy, R. H., Kim, J. N., Link, K. H., et al. (2008). Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science*, *321*, 411–413.
4. Smith, K. D., Lipchock, S. V., Ames, T. D., Wang, J., Breaker, R. R., & Strobel, S. A. (2009). Structural basis of ligand binding by a c-di-GMP riboswitch. *Nature Structural and Molecular Biology*, *16*, 1218–1223.
5. Kulshina, N., Baird, N. J., & Ferré-D'Amaré, A. R. (2009). Recognition of the bacterial second messenger cyclic diguanylate by its cognate riboswitch. *Nature Structural and Molecular Biology*, *16*, 1212–1217.
6. Chang, A. L., Tuckerman, J. R., Gonzalez, G., Mayer, R., Weinhouse, H., Volman, G., et al. (2001). Phosphodiesterase A1, a regulator of cellulose synthesis in *Acetobacter xylinum*, is a heme-based sensor. *Biochemistry*, *40*, 3420–3426.
7. Yan, H., & Chen, W. (2010). 3',5'-Cyclic diguanylic acid: A small nucleotide that makes big impacts. *Chemical Society Reviews*, *39*, 2914–2924.
8. Römling, U., & Amikam, D. (2006). Cyclic di-GMP as a second messenger. *Current Opinion in Microbiology*, *9*, 218–228.
9. Jenal, U., & Malone, J. (2006). Mechanisms of cyclic-di-GMP signaling in bacteria. *Annual Review of Genetics*, *40*, 385–407.
10. Cotter, P. A., & Stibitz, S. (2007). c-di-GMP-mediated regulation of virulence and biofilm formation. *Current Opinion in Microbiology*, *10*, 17–23.
11. Tamayo, R., Pratt, J. T., & Camilli, A. (2007). Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annual Review of Microbiology*, *61*, 131–148.
12. Wolfe, A. J., & Visick, K. L. (2008). Get the message out: Cyclic-Di-GMP regulates multiple levels of flagellum-based motility. *Journal of Bacteriology*, *190*, 463–475.

13. Hengge, R. (2009). Principles of c-di-GMP signalling in bacteria. *Nature Reviews Microbiology*, 7, 263–273.
14. Brouillette, E., Hyodo, M., Hayakawa, Y., Karaolis, D. K., & Malouin, F. (2005). 3',5'-Cyclic diguanylic acid reduces the virulence of biofilm-forming *Staphylococcus aureus* strains in a mouse model of mastitis infection. *Antimicrobial Agents and Chemotherapy*, 49, 3109–3113.
15. Karaolis, D. K., Newstead, M. W., Zeng, X., Hyodo, M., Hayakawa, Y., Bhan, U., et al. (2007). Cyclic di-GMP stimulates protective innate immunity in bacterial pneumonia. *Infection and Immunity*, 75, 4942–4950.
16. Karaolis, D. K., Means, T. K., Yang, D., Takahashi, M., Yoshimura, T., Muraille, E., et al. (2007). Bacterial c-di-GMP is an immunostimulatory molecule. *Journal of Immunology*, 178, 2171–2181.
17. McWhirter, S. M., Barbalat, R., Monroe, K. M., Fontana, M. F., Hyodo, M., Joncker, N. T., et al. (2009). A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. *The Journal of Experimental Medicine*, 206, 1899–1911.
18. Ebensen, T., Schulze, K., Riese, P., Link, C., Morr, M., & Guzmán, C. A. (2007). The bacterial second messenger cyclic diGMP exhibits potent adjuvant properties. *Vaccine*, 25, 1464–1469.
19. Ebensen, T., Schulze, K., Riese, P., Morr, M., & Guzmán, C. A. (2007). The bacterial second messenger cdiGMP exhibits promising activity as a mucosal adjuvant. *Clinical and Vaccine Immunology*, 14, 952–958.
20. Ogunniyi, A. D., Paton, J. C., Kirby, A. C., McCullers, J. A., Cook, J., Hyodo, M., et al. (2008). c-di-GMP is an effective immunomodulator and vaccine adjuvant against pneumococcal infection. *Vaccine*, 26, 4676–4685.
21. Yan, H., KuoLee, R., Tram, K., Qiu, H., Zhang, J., Patel, G. B., et al. (2009). 3',5'-Cyclic diguanylic acid elicits mucosal immunity against bacterial infection. *Biochemical and Biophysical Research Communications*, 387, 581–584.
22. Hu, D. L., Narita, K., Hyodo, M., Hayakawa, Y., Nakane, A., & Karaolis, D. K. (2009). c-di-GMP as a vaccine adjuvant enhances protection against systemic methicillin-resistant *Staphylococcus aureus* (MRSA) infection. *Vaccine*, 27, 4867–4873.
23. Chen, W., Kuolee, R., & Yan, H. (2010). The potential of 3',5'-cyclic diguanylic acid (c-di-GMP) as an effective vaccine adjuvant. *Vaccine*, 28, 3080–3085.
24. Steinberger, O., Lapidot, Z., Ben-Ishai, Z., & Amikam, D. (1999). Elevated expression of the CD4 receptor and cell cycle arrest are induced in Jurkat cells by treatment with the novel cyclic dinucleotide 3',5'-cyclic diguanylic acid. *FEBS Letters*, 444, 125–129.
25. Karaolis, D. K., Cheng, K., Lipsky, M., Elnabawi, A., Catalano, J., Hyodo, M., et al. (2005). 3',5'-Cyclic diguanylic acid (c-di-GMP) inhibits basal and growth factor-stimulated human colon cancer cell proliferation. *Biochemical and Biophysical Research Communications*, 329, 40–45.
26. Karaolis, D. K., Rashid, M. H., Chythanya, R., Luo, W., Hyodo, M., & Hayakawa, Y. (2005). c-di-GMP (3'-5'-cyclic diguanylic acid) inhibits *Staphylococcus aureus* cell–cell interactions and biofilm formation. *Antimicrobial Agents and Chemotherapy*, 49, 1029–1038.
27. Ishihara, Y., Hyodo, M., Hayakawa, Y., Kamegaya, T., Yamada, K., Okamoto, A., et al. (2009). Effect of cyclic bis(3'-5')diguanylic acid and its analogs on bacterial biofilm formation. *FEMS Microbiology Letters*, 301, 193–200.
28. Yan, W., Qu, T., Zhao, H., Su, L., Yu, Q., Gao, J., et al. (2010). The effect of c-di-GMP (3'-5'-cyclic diguanylic acid) on the biofilm formation and adherence of *Streptococcus mutans*. *Microbiology Research*, 165, 87–96.
29. Ross, P., Mayer, R., Weinhouse, H., Amikam, D., Huggirat, Y., Benziman, M., et al. (1990). The cyclic diguanylic acid regulatory system of cellulose synthesis in *Acetobacter xylinum*. Chemical synthesis and biological activity of cyclic nucleotide dimer, trimer, and phosphothioate derivatives. *Journal of Biological Chemistry*, 265, 18933–18943.
30. Kawai, R., Nagata, R., Hirata, A., & Hayakawa, Y. (2003). A new synthetic approach to cyclic bis (3'→5')diguanylic acid. *Nucleic Acids Research. Supplement (2001)*, 3, 103–104.
31. Hayakawa, Y., Nagata, R., Hirata, A., Hyodo, M., & Kawai, R. (2003). A facile synthesis of cyclic bis (3'-5')diguanylic acid. *Tetrahedron*, 59, 6465–6471.
32. Hyodo, M., & Hayakawa, Y. (2004). An improved method for synthesizing cyclic bis(3'-5')diguanylic acid (c-di-GMP). *Bulletin of the Chemical Society of Japan*, 77, 2089–2093.
33. Zhang, Z., Gaffney, B. L., & Jones, R. A. (2004). c-di-GMP displays a monovalent metal ion-dependent polymorphism. *Journal of the American Chemical Society*, 126, 16700–16701.
34. Yan, H., & Humes, E. (2006). Convenient synthesis of 3',5'-cyclic diguanylic acid (cdiGMP). *Nucleic Acids Symposium Series (Oxford)*, 50, 5–6.
35. Kiburu, I., Shurer, A., Yan, L., & Sintim, H. O. (2008). A simple solid-phase synthesis of the ubiquitous bacterial signaling molecule, c-di-GMP and analogues. *Molecular BioSystems*, 4, 518–520.
36. Gaffney, B. L., Veliath, E., Zhao, J., & Jones, R. A. (2010). One-flask syntheses of c-di-GMP and the [Rp, Rp] and [Rp, Sp] thiophosphate analogues. *Organic Letters*, 12, 3269–3271.

37. Tal, R., Wong, H. C., Calhoun, R., Gelfand, D., Fear, A. L., Volman, G., et al. (1998). Three *cdg* operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: Genetic organization and occurrence of conserved domains in isoenzymes. *Journal of Bacteriology*, *180*, 4416–4425.
38. Römmling, U., Gomelsky, M., & Galperin, M. Y. (2005). C-di-GMP: The dawning of a novel bacterial signalling system. *Molecular Microbiology*, *57*, 629–639.
39. Ryjenkov, D. A., Tarutina, M., Moskvina, O. V., & Gomelsky, M. (2005). Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: Insights into biochemistry of the GGDEF protein domain. *Journal of Bacteriology*, *187*, 1792–1798.
40. Christen, M., Christen, B., Folcher, M., Schauerte, A., & Jenal, U. (2005). Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. *Journal of Biological Chemistry*, *280*, 30829–30837.
41. Kazmierczak, B. I., Lebron, M. B., & Murray, T. S. (2006). Analysis of FimX, a phosphodiesterase that governs twitching motility in *Pseudomonas aeruginosa*. *Molecular Microbiology*, *60*, 1026–1043.
42. Tamayo, R., Tischler, A. D., & Camilli, A. (2005). The EAL domain protein *VieA* is a cyclic diguanylate phosphodiesterase. *Journal of Biological Chemistry*, *280*, 33324–33330.
43. Merighi, M., Lee, V. T., Hyodo, M., Hayakawa, Y., & Lory, S. (2007). The second messenger bis-(3′-5′)-cyclic-GMP and its PilZ domain-containing receptor Alg44 are required for alginate biosynthesis in *Pseudomonas aeruginosa*. *Molecular Microbiology*, *65*, 876–895.
44. Hickman, J. W., & Harwood, C. S. (2008). Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. *Molecular Microbiology*, *69*, 376–389.
45. Rao, F., Pasunooti, S., Ng, Y., Zhuo, W., Lim, L., Liu, A. W., et al. (2009). Enzymatic synthesis of c-di-GMP using a thermophilic diguanylate cyclase. *Analytical Biochemistry*, *389*, 138–142.
46. Zähringer, F., Massa, C., & Schirmer, T. (2011). Efficient enzymatic production of the bacterial second messenger c-di-GMP by the diguanylate cyclase YdeH from *E. coli*. *Applied Biochemistry and Biotechnology*, *163*(1), 71–79.
47. Sambrook, J., & Russell, D. W. (2001). *Molecular cloning. A laboratory manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
48. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.
49. Höcherl, K., Dreher, F., Vitzthum, H., Köhler, J., & Kurtz, A. (2002). Cyclosporine A suppresses cyclooxygenase-2 expression in the rat kidney. *Journal of the American Society of Nephrology*, *13*, 2427–2436.
50. Christen, B., Christen, M., Paul, R., Schmid, F., Folcher, M., Jenoe, P., et al. (2006). Allosteric control of cyclic di-GMP signaling. *Journal of Biological Chemistry*, *281*, 32015–32024.
51. Kammann, M., Laufs, J., Schell, J., & Gronenborn, B. (1989). Rapid insertional mutagenesis of DNA by polymerase chain reaction (PCR). *Nucleic Acids Research*, *17*, 5404.
52. Landt, O., Grunert, H. P., & Hahn, U. (1990). A general method for rapid site-directed mutagenesis using the polymerase chain reaction. *Gene*, *96*, 125–128.
53. Sarkar, G., & Sommer, S. S. (1992). Double-stranded DNA segments can efficiently prime the amplification of human genomic DNA. *Nucleic Acids Research*, *20*, 4937–4938.
54. Anadón, A., & Reeve-Johnson, L. (1999). Macrolide antibiotics, drug interactions and microsomal enzymes: Implications for veterinary medicine. *Research in Veterinary Science*, *66*, 197–203.
55. Amiot, N., Heintz, K., & Giese, B. (2006). New approach for the synthesis of c-di-GMP and its analogues. *Synthesis*, *24*, 4230–4236.
56. Alberty, R. A., Smith, R. M., & Bock, R. M. (1951). The apparent ionization constants of the adenosine phosphates and related compounds. *Journal of Biological Chemistry*, *193*, 425–434.