Cyclic mononucleotide- and Clr-dependent gene regulation in Sinorhizobium meliloti

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To identify physiological processes affected by cAMP in the plant-symbiotic nitrogen-fixing α-proteobacterium Sinorhizobium meliloti Rm2011, cAMP levels were artificially increased by overexpression of its cognate adenylate/guanylate cyclase gene cyaJ. This resulted in high accumulation of cAMP in the culture supernatant, decreased swimming motility and increased production of succinoglycan, an exopolysaccharide involved in host invasion. Weaker, similar phenotypic changes were induced by overexpression of cyaB and cyaG1. Effects on swimming motility and succinoglycan production were partially dependent on clr encoding a cyclic AMP receptor-like protein. Transcriptome profiling of an cyaJ-overexpressing strain identified 72 upregulated and 82 downregulated genes. A considerable number of upregulated genes are related to polysaccharide biosynthesis and osmotic stress response. These included succinoglycan biosynthesis genes, genes of the putative polysaccharide synthesis nodP2-exoF3 cluster and feuN, the first gene of the operon encoding the FeuNPQ regulatory system. Downregulated genes were mostly related to respiration, central metabolism and swimming motility. Promoter-probe studies in the presence of externally added cAMP revealed 18 novel Clr-cAMP-regulated genes. Moreover, the addition of cGMP into the growth medium also promoted clr-dependent gene regulation. In vitro binding of Clr-cAMP and Clr-cGMP to the promoter regions of Smc02178, Smb20906, Smc04190, Smc00925, Smc01136 and cyaF2 required the DNA motif (A/C/T)GT(T/C)(T/C/A)C (N4) G(G/A)(T/A)ACA. Furthermore, Smc02178, Smb20906, Smc04190 and Smc00653 promoters were activated by Clr-cAMP/cGMP in Escherichia coli as heterologous host. These findings suggest direct activation of these 7 genes by Clr-cAMP/cGMP.

INTRODUCTION

Second messengers are small molecules that are synthesized by the cell in response to a particular signal and perceived by cognate effectors, which regulate downstream processes. Bacteria use nucleotide signalling molecules, such as guanosine tetraphosphate or pentaphosphate [collectively called (p)ppGpp] and cyclic mononucleotide (cNMP) or cyclic dinucleotide. During the last decade, regulation by c-di-GMP attracted much attention and was explored in great detail (Römling et al., 2013). c-di-GMP was particularly associated to the sessile-to-motile switch, virulence or cell cycle progression (Römling et al., 2013). On the contrary, comprehensive studies of cNMP signalling have been restricted so far to Escherichia coli as model and a few bacterial species, which are mostly human pathogens like Mycobacterium tuberculosis and Pseudomonas aeruginosa (reviewed in McDonough & Rodríguez, 2011). Initially, 3′,5′-cyclic AMP (further referred to as cAMP) has been assigned mostly to the regulation of carbon metabolism in E. coli (Zheng et al., 2004). However, it is also essential for swimming motility (Soutourina et al., 1999) and recent data suggest global regulation, involving up to 378

Abbreviations: AC, adenylate cyclase; CMP, cyclic mononucleotide; CRP, cyclic AMP receptor protein; EMSA, electrophoretic mobility shift assay; EPS, exopolysaccharide; GC, guanylate cyclase.

The complete transcriptome data obtained in this study are available in the ArrayExpress database (accession no. E-MTAB-4932).

Three supplementary figures and three supplementary tables are available with the online Supplementary Material.
target promoters (Shimada et al., 2011). In other bacterial species, cAMP signalling was found to be mostly related to virulence and quorum sensing (reviewed in McDonough & Rodriguez, 2011).

The best-known bacterial effectors of cAMP are cAMP receptor proteins (CRPs), which are transcription regulators with a cyclic nucleotide-binding domain. Binding of cAMP to the E. coli CRP dimer results in structural rearrangements enabling DNA binding (Won et al., 2002). 3',5'-Cyclic GMP (further referred to as cGMP) is mostly known as a common second messenger in eukaryotes, though recently, several bacterial guanylate cyclases and cGMP-related processes were identified. In *Synechocystis* sp., cGMP is involved in the regulation of photosystem II repair, whereas in *Xanthomonas campestris*, binding of cGMP promotes dimerization and enzymatic activity of the pathogenicity-relevant diguanylate cyclase Xc_0249 (Ochoa et al., 2010). The biosynthesis of cGMP is positively controlled by the FeuNPQ regulon (Marden et al., 2011; Roychowdhury et al., 2015).

Genomes of symbiotic nitrogen-fixing members of the α-proteobacterial *Rhizobiaceae* encode an exceptionally high number of putative adenylate/guanylate cyclases (ACs/GCs). Previous annotation and our computational analysis predicted 28 class III AC genes in *Sinorhizobium meliloti* (Galibert et al., 2001) (Fig. S1, available in the online Supplementary Material), a soil-dwelling α-proteobacterium able to establish a nitrogen-fixing root nodule symbiosis with its host plants (Jones et al., 2007). The tripartite genome of the *S. meliloti* type strain Rm1021 consists of a chromosome (3.7 Mbp) and the megaplasmids pSymA and pSymB with 1.4 and 1.7 Mbp each (Galibert et al., 2001). The symbiotic interaction begins with a molecular dialogue between the partners requiring the bacteria to perceive plant signals and to respond with appropriate behaviour. This includes production of nodule factors, attachment to root hairs, proliferation inside infection threads and, finally, infection of plant cells and differentiation to nitrogen-fixing bacteroids (reviewed in Jones et al., 2007). At the stage of infection thread progression, bacterial production of periplasmic cyclic glucans and one of the exopolysaccharides succinoglycan (EPS I) or galactoglucomannan (EPS II) is essential for a successful establishment of symbiosis (Dickstein et al., 1988; Glazebrook & Walker, 1989; Pellock et al., 2000). Opposing regulation of exopolysaccharide (EPS) production and swimming motility by different environmental and genetic factors has been reported previously (Schäper et al., 2016; Bahlawane et al., 2008; Charoenpanich et al., 2013; Yao et al., 2004; de Lucena et al., 2010). The biosynthesis of cyclic glucans is positively controlled by the FeuNPQ regulatory system consisting of the FeuQ two-component system and the auxiliary small periplasmic protein FeuN (Griffitts et al., 2008; Carlyon et al., 2010).

An optimal number of symbiotic infections per plant is subject to regulatory fine-tuning. Recently, an important role in cAMP signalling was assigned to the CRP-like protein Clr during the symbiotic interaction between *S. meliloti* and *Medicago sativa* (Tian et al., 2012; Mathieu-Demazière et al., 2013). Here, Clr–cAMP was found to activate SMc02178 encoding a protein of unknown function, which is crucial for negative regulation of the number of infection initiation events. The binding of Clr–cAMP within the promoter region of SMc02178 was demonstrated, and the sequence motif TGTK(N4)AACAA has been suggested as cognate-binding site (Mathieu-Demazière et al., 2013).

The high number of putative AC/GCs in *S. meliloti*, the majority of which contain additional domains with putative regulatory function, suggests a high diversity in control of cAMP/cGMP biosynthesis and target processes of these cNMP second messengers. In this study, we explored genome-wide regulatory targets of cAMP during free-living growth of *S. meliloti*. We show that elevated levels of cAMP affect expression of more than 150 genes and report new targets of direct and indirect regulation by Clr. Furthermore, we redefine the Clr-binding site consensus and show that not only Clr–cAMP but also Clr–cGMP is able to bind to promoter regions of target genes and activate their expression.

**METHODS**

**Strains, plasmids and growth conditions.** Strains and plasmids used in this study are listed in Table S1. For cloning and conjugation procedures, *E. coli* was cultured at 37 °C on LB (tryptone, 10 g l⁻¹; yeast extract, 5 g l⁻¹; NaCl, 5 g l⁻¹). When required, kanamycin was added at 50 mg l⁻¹, gentamicin was added at 8 mg l⁻¹ and tetracycline was added at 10 mg l⁻¹. *S. meliloti* was cultured at 30 °C on TY (tryptone, 5 g l⁻¹; yeast extract, 3 g l⁻¹; CaCl₂·2H₂O, 0.4 g l⁻¹) or modified Vincent minimal medium with glucose as carbon source, further referred to as VMM (glucose, 2 g l⁻¹; NH₄Cl, 1 g l⁻¹; K₂HPO₄, 14.7 mM; KH₂PO₄, 11.5 mM; MgSO₄·10H₂O, 0.5 mM; CaCl₂, 0.05 mM; FeCl₃, 0.037 mM; biotin, 4.1 µM; H₂BO₃, 3 mg l⁻¹; MnSO₄·4H₂O, 2.23 mg l⁻¹; ZnSO₄·7H₂O, 0.287 mg l⁻¹; CuSO₄·5H₂O, 0.125 mg l⁻¹; CoCl₂·6H₂O, 0.065 mg l⁻¹; NaMoO₄·2H₂O, 0.12 mg l⁻¹). When required, streptomycin was added at 600 mg l⁻¹, kanamycin was added at 200 mg l⁻¹, gentamicin was added at 30 mg l⁻¹ and tetracycline was added at 10 mg l⁻¹. For liquid cultures, the antibiotic concentration was halved.

The detection of β-galactosidase activity in *E. coli* BTH101 carrying overexpression constructs to produce *S. meliloti* ACs was performed as follows. The cultures were grown overnight at 30 °C in 96-well polystyrene flat-bottom plates (Greiner) in 100 µl with shaking at 1200 r.p.m., diluted 1 : 10, and 5 µl was spotted onto LB agar containing 40 mg l⁻¹ X-Gal and IPTG as previously indicated. The phenotype was documented after approximately 24 h of growth at 30 °C.

For the detection of succinoglycan production, calcofluor-white dye was added to VMM agar to a final concentration of 200 mg l⁻¹. Five microlitres of bacterial suspension at an OD₆₀₀ of 0.2 was spotted onto the agar. Plates were documented after approximately 72 h of growth. Motility assays were performed on soft VMM agar (VMM medium diluted with water 1 : 4, 0.3 % agar). Stationary TY cultures of 2 µl were spotted onto the soft agar plate and the phenotype was documented after approximately 84 h.

For quantification of cyclic nucleotides in culture supernatants, the cultures were grown in duplicates in 25 ml of YMM in 100 ml flasks up to approximately 84 h. The detection of succinoglycan production, calcofluor-white dye was added to VMM agar to a final concentration of 200 mg l⁻¹. Five microlitres of bacterial suspension at an OD₆₀₀ of 0.2 was spotted onto the agar. Plates were documented after approximately 72 h of growth. Motility assays were performed on soft VMM agar (VMM medium diluted with water 1 : 4, 0.3 % agar). Stationary TY cultures of 2 µl were spotted onto the soft agar plate and the phenotype was documented after approximately 84 h.

For quantification of cyclic nucleotides in culture supernatants, the cultures were grown in duplicates in 25 ml of YMM in 100 ml flasks up to approximately 84 h. The detection of succinoglycan production, calcofluor-white dye was added to VMM agar to a final concentration of 200 mg l⁻¹. Five microlitres of bacterial suspension at an OD₆₀₀ of 0.2 was spotted onto the agar. Plates were documented after approximately 72 h of growth. Motility assays were performed on soft VMM agar (VMM medium diluted with water 1 : 4, 0.3 % agar). Stationary TY cultures of 2 µl were spotted onto the soft agar plate and the phenotype was documented after approximately 84 h.

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The OD<sub>600</sub> at harvest was 1.2 for Rm2011 with pWBT, 1.1 for Rm2011 with pWBT-cyaB and 0.9 for Rm2011 with pWBT-cyaS.

For microarray experiment, the cultures of Rm2011 carrying either empty vector pWBT or the cya<sup>−</sup> overexpression construct pWBT-cyaS were grown in 25 ml of TY in 100 ml flasks up to an OD<sub>600</sub> of 0.17 to 0.23, induced with 500 µM IPTG and grown further for 8 h. The OD<sub>600</sub> at harvest was 1.3 to 1.5 for Rm2011 pWBT and 1.1 for the cya<sup>−</sup>-overexpressing cultures.

For promoter-egfp studies, S. meliloti strains were grown in TY and E. coli strains were grown in LB using 100 µl medium in 96-well polystyrene flat bottom plates (Greiner) at 30 °C and shaking at 1200 r.p.m. For assays involving cya<sup>−</sup> overexpression in S. meliloti, stationary pre-cultures were used to inoculate the medium (dilution 1:100). These cultures were grown for 17 to 18 h, induced with 250 µM IPTG and grown further for 24 h. EGFP fluorescence was determined at the time point of IPTG addition (exponential growth phase), 8 h after IPTG addition (late exponential growth phase) and 24 h after IPTG addition (stationary growth phase). For assays including added nucleotides, stationary pre-cultures were used to inoculate the medium (dilution 1:100) either lacking added nucleotides or containing 400 µM 3′,5′-cAMP, 3′,5′-cGMP, GMP or 2′,3′-cAMP, followed by incubation for 24 h. EGFP fluorescence was determined for 24 h after IPTG addition (stationary growth phase). To study Clr-mediated gene regulation in E. coli, stationary pre-cultures were used to inoculate the medium (dilution 1:100) containing 100 µM IPTG and grown for 16 h.

Cloning and genetic manipulations. The constructs used in this work were generated using standard genetic techniques. All constructs were verified by sequencing. Plasmids were transferred to S. meliloti by conjugation with E. coli S17-1 as previously described (Krol & Becker, 2014). The primers used are listed in Table S2. The deletion of cya was generated using pCry-rel carrying DNA fragments upstream and downstream of cya and the sucrose selection marker sacB. Double recombinants carrying a gene deletion were selected on LB agar with 10 % sucrose as previously described (Schäfer et al., 1994) and verified by PCR.

Promoter-egfp fusions were generated by insertion of a S. meliloti promoter region of 300 to 500 bp, including the translation start site and up to the first 10 codons of the downstream gene, into the replicative medium-copy plasmid pPHU231-EGFP or replicative medium-copy plasmid pSRKkm-EGFP. Transcription from the inserted promoter resulted in fusion of cAMP to the first 10 codons of the downstream gene, into the replicative low-copy plasmid pPHU231-EGFP or replicative medium-copy plasmid pSRKkm-EGFP. The OD<sub>600</sub> was 0.23, induced with 500 µM IPTG and grown further for 8 h. The OD<sub>600</sub> at harvest was 1.3 to 1.5 for Rm2011 pWBT and 1.1 for the cya<sup>−</sup>-overexpressing cultures.

The OD<sub>600</sub> at harvest was 1.2 for Rm2011 with pWBT, 1.1 for Rm2011 with pWBT-cyaB and 0.9 for Rm2011 with pWBT-cyaS.

EGFP fluorescence measurements. EGFP fluorescence (excitation, 488 nm; emission, 522 nm; gain, 82) and optical density (absorbance at 600 nm) were measured using an Infinite 200 PRO multimode reader (Tecan) and calculated as relative fluorescence units (RFU) representing fluorescence values divided by optical density. In the experiments shown in Fig. 2b, the background, which ranged between 450 and 650 RFU, is included in the presented values. Otherwise, the background fluorescence of control strains carrying the corresponding empty promoter-probe vector was subtracted from the values. Three to four independent transconjugants or transformants of each strain containing the promoter-egfp fusion were used as biological replicates.

RNA isolation and labelling. RNA was isolated from 3 ml of culture, and collected as two 1.5 ml culture pellets, using the RNase Mini kit (Qiagen). The pellets were resuspended in 200 µl ice-cold 10 mM Tris/HCl (pH 8), mixed with 700 µl of RLT buffer (RNeasy Mini kit) in a lysing matrix tube (M.P. Biomedicals), crushed in a FastPrep-24 instrument (M.P. Biomedicals; power 6.5, 30 s), cooled on ice for 3 min and spun down for 3 min at 4 °C and 20,000 g. The lysates were transferred into a clean tube and spun down again with the same settings. Further steps were performed following the RNase Mini kit manufacturer’s instructions. Remaining DNA was digested using the RNase-free DNAse kit (Qiagen).

To generate the probes for microarray hybridization, 20 µg total RNA was reverse transcribed using Tetro reverse transcriptase (Bioline), terminally amino-allyl-modified random hexamers as primers and 0.5 mM of dATP, dCTP and dGTP, 0.1 mM dTTP and 0.4 mM amino-allyl dUTP (Thermo Fisher). Generated cDNA was purified with the QIAquick Nucleotide Removal kit (Qiagen), using 80 % ethanol to wash the column and 0.1 M NaHCO<sub>3</sub> (60 µl) as elution buffer. Cyan and Cy5 dyes (Amersham) were dissolved in 10 µl of DMSO; 1.5 µl was added to the cDNA followed by 1 h of incubation in the dark. The reactions were quenched by addition of 4.5 µl of 4 M hydroxylamine followed by 15 min incubation in the dark and finally purified with the QIAquick Nucleotide Removal kit (Qiagen), following the manufacturer’s instructions.

Transcriptome profiling experiments. Microarray hybridizations were performed using labelled cDNA synthesized from RNA isolated from four independent bacterial cultures. Hybridization, image acquisition and data analysis were performed as previously described (Krol & Becker, 2004; Serrania et al., 2008). Spot detection, image segmentation and signal quantification were performed using the ImaGene 8.0 software (Biodiscovery). Spots were flagged as ‘low signal spots’ if R<sub>x</sub> < 2.0 in both channels, where R<sub>x</sub> = signal mean – background mean)/background SD. The log<sub>2</sub> value of the ratio of intensities was calculated for each remaining spot using the formula M<sub>x</sub> = log<sub>2</sub>(R<sub>G</sub>/R<sub>B</sub>), where R<sub>G</sub> = I<sub>bG</sub> - B<sub>G</sub> and G<sub>x</sub> = I<sub>bG</sub> - B<sub>G</sub> (where I<sub>bG</sub> and I<sub>bG</sub> are the intensities of spots in channel 1 and channel 2, respectively, and B<sub>G</sub> and B<sub>G</sub> are the background intensities of spots in channel 1 and channel 2, respectively). The mean intensity (A<sub>x</sub>) was calculated for each spot as follows: A<sub>x</sub> = log<sub>2</sub>(R<sub>G</sub>/R<sub>B</sub>)<sup>2/3</sup>. A normalization method based on local regression that accounts for intensity and spatial dependence in dye biases was used (Yang et al., 2002). Normalization and t-statistics were carried out using the EMMA2.8.2 microarray data analysis software developed at the Bioinformatics Resource Facility, Center for Biotechnology, Bielefeld University (http://www.cebitec.uni-bielefeld.de/groups/bio/software/emma_info/) (Dondrup et al., 2009). Genes were classified as differentially expressed with P<0.05 and M<1 or M<−1 (at least a twofold difference). The complete transcriptome data are available in the ArrayExpress database (accession no. E-MTAB-4932).
**Protein purification.** E. coli BL21-Gold (DE3) cells were transformed with pET36b/CLR and grown in 41 LB medium containing 35 µg ml⁻¹ kanamycin at 30 °C. For induction, IPTG was added at an OD₆₀₀ of 0.6 to a final concentration of 0.1 mM. The cells were harvested after 7 h by centrifugation at 1800 g for 10 min at 4 °C, resuspended in 40 ml lysis buffer [50 mM NaH₂PO₄ (pH 8.0), 1 M NaCl and 10 mM imidazole] and immediately frozen in liquid nitrogen. After thawing, the cell suspension was treated for 15 min with 0.2 mM PMSF and 50 µg ml⁻¹ lysozyme and disrupted by a French Press cell (Aminco). The crude lysate was cleared by centrifugation for 60 min at 22 600 g and 4 °C and subsequently filtered through a 0.45 µm filter. The clear supernatant containing the C-terminally His₆-tagged Clr protein was applied onto a Ni-NTA column (5 ml; Macherey-Nagel). After washing with lysis buffer until a stable baseline at 280 nm was achieved, Clr-His₆ was eluted with a linear gradient from 0 to 250 mM imidazole in 18 column volumes. The Clr-His₆-containing fractions were checked by SDS-PAGE, pooled and concentrated with a concentrator (molecular weight cutoff: 10 000 Da, Millipore) at 3220 g and 4 °C up to 4 ml. The protein was further purified by size-exclusion chromatography using a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) in gel-filtration buffer [10 mM Tris/HCl (pH 8.0) and 100 mM NaCl]. The fractions of the peak corresponding to the Clr-His₆ monomer (27 kDa) were collected. The purity of the protein was assessed by SDS-PAGE.

**Electrophoretic mobility shift assay.** An electrophoretic mobility shift assay (EMSA) reaction mixture contained 10 mM Tris/HCl (pH 8.5), 50 mM KCl, 850 ng sonicated salmon sperm DNA (GE Healthcare), 1 µg of BSA (Sigma) and 20 ng of Cy3-labelled DNA in a final volume of 10 µl.

Native Cy3-labelled DNA fragments were obtained by PCR with primer Cy3-egfp-28-rev, uni-48 or rev-67 (Table S2) using the corresponding pSRKKm-promoter-EGFP or pPHU-promoter-EGFP constructs as template. The protein was added at 5.4 µg per reaction and 3',5'-cAMP, 3',5'-cGMP or 2',3'-cAMP at 1 mM, when indicated.

Synthetic constructs carrying Clr-binding sequences were obtained as follows. The oligonucleotides containing the native or mutated Clr-binding palindromes, four adjacent nucleotides and sticky end overhangs for XbaI (ctag) and HindIII (agct) (Table S2) were phosphorylated with polynucleotide kinase, hybridized and cloned into the XbaI/HindIII digested plasmid pSRKKm-EGFP. Resulting plasmids were used as template for the amplification of the DNA fragments for EMSA using the primers uni-48 and Cy3-egfp-28-rev. The protein was added at 500 ng per reaction and 3',5'-cAMP at 1 mM.

The reaction mixtures were incubated at room temperature for 15–30 min. We added 1 µl of 90% glycerol and 1.5 µl of 5× TBE buffer and loaded the reaction onto a 10% polyacrylamide gel in TBE. Following electrophoresis at 9 V cm⁻¹ at room temperature for 4 h for native Clr-binding sequences and 2.5 h for synthetic constructs with inserted Clr-binding site, gel images were scanned using a Typhoon 8600 variable-mode imager (Amersham Bioscience).

**RESULTS**

**Enhanced levels of cAMP stimulate succinoglycan production and repress swimming motility**

To identify processes influenced by cAMP in S. meliloti, the cellular CAMP content was increased by induced AC overproduction. S. meliloti genes encoding active ACs were identified by heterologous complementation of the AC-deficient E. coli strain BTH101. This strain is defective in production of the β-galactosidase enzyme due to the lack of activation of the lac operon by Crp-cAMP. Complementation by CAMP synthesized by ectopically produced AC enzymes was detected in a β-galactosidase assay. To this end, 24 expression constructs were generated under the control of the IPTG-inducible and CRP-cAMP-independent T5 promoter in the pWBT vector and introduced into E. coli strain BTH101. These constructs contain the coding sequences of the predicted S. meliloti Rm2011 AC genes and cyaI₁-642 encoding a truncated Cya protein missing the C-terminal predicted transmembrane region (Fig. S1). Reconstitution of AC activity in E. coli strain BTH101 was detected upon IPTG-induced expression of 14 of these plasmid-borne AC genes (Fig. S2). While induction with 10 µM IPTG was sufficient for the detection of AC activity mediated by seven of these AC gene expression plasmids, for the other seven, AC activity was observed upon induction with 250 µM IPTG (Fig. S2). To artificially increase the level of cAMP in S. meliloti, we have selected those nine ACs as candidates for overproduction, which include the putative catalytic domain but lack additional domains that are likely to serve a regulatory function (Fig. S1). Fig. 1a shows the complementation results from the expression of these nine AC genes induced with 10 µM and 250 µM IPTG in the AC-deficient E. coli strain. Expression of six of these AC genes reconstituted AC activity in this strain. While induction of cyaB, cyaE1, cyaG1 and cyaJ expression with 10 µM IPTG resulted in significant β-galactosidase activities, functional complementation by the expression of cyaG2 and cyaH required much higher induction levels. Induced production of a C-terminally truncated variant of Cya lacking the transmembrane part resulted in lower β-galactosidase activities of the complemented E. coli strain, suggesting a lower enzymatic activity or stability of the truncated protein. Moreover, overexpression of cyaJ induced with 250 µM IPTG inhibited growth of E. coli BTH101, which might be attributed to accumulation of very high levels of cAMP.

Growth, swimming motility and production of calcofluor-white-binding EPS was monitored upon overexpression of the nine selected AC genes in S. meliloti Rm2011 (Fig. 1b–d). Overexpression of cyaB, cyaE1, cyaG1 and cyaJ resulted in reduced motility, while overexpression of cyaP had a slight effect. Overproduction of the remaining AC/GC had no effect (Fig. 1b). The negative effects of overproduction of CyA and CyaE1 on motility correlated with impaired growth (Fig. 1d). In the case of overexpression of cyaB, cyaG1 and cyaJ, the decrease in motility correlated with an increase in production of calcofluor-white-binding EPS (Fig. 1c). The strongest increase in calcofluor-mediated fluorescence was observed upon overproduction of CyaJ, whereas effects of CyaB, CyaG1 and CyaH overproduction were less prominent. In the AC overproduction strains, the increase in calcofluor-mediated fluorescence was abolished by knockout of exoY, encoding the galactosyltransferase initiating succinoglycan biosynthesis (Fig. 1c). This suggests that increased levels of cAMP induce succinoglycan biosynthesis.

Previously, Clr has been identified as a cAMP-dependent transcription regulator (Tian et al., 2012). Therefore, we...
Transcriptome profiling reveals genes differentially expressed at elevated levels of cAMP

To characterize the effect of elevated levels of cAMP on gene expression, we compared the transcriptome of the strain overexpressing cyaJ to the corresponding WT Rm2011 carrying the empty vector. Genes were considered as differentially expressed if upregulation or downregulation exceeded a twofold threshold. A total of 72 upregulated and 82 downregulated genes were identified in this study (Tables 1 and 2). Consistent with the phenotype of agar cultures observed upon cyaJ overexpression, transcripts of seven genes of the exo-exs gene cluster directing succinoglycan biosynthesis were more abundant and transcripts of 18 genes associated with swimming motility were less abundant in the strain overexpressing cyaJ. Interestingly, Smc00507, which is unrelated to the motility gene cluster and encodes a small PilZ domain protein involved in c-di-GMP-mediated repression of swimming motility (Schäper et al., 2016), was also found among the repressed genes.

Of the upregulated genes, 19 have been detected previously in a transcriptome study characterizing the response to an upshift in osmolarity (Domínguez-Ferreras et al., 2006), whereas 7 upregulated genes belong to the feuNPQ regulon, activated by low external osmolarity (Griffitts et al., 2008). The functions of osmolarity-regulated genes detected in our study are mostly unknown, except for the trehalose/maltose transport genes thuF and thuG, the gene SMB20842, which encodes a putative TonB-dependent receptor, and feuN, the first gene of the feuNPQ operon. A total of 11 cyaJ overexpression-activated genes were previously identified as regulatory targets of ChvI, the transcription regulator of the ExoS-ChvI two-component system (Chen et al., 2009). Among these, six are located in the exsl-exo gene cluster and the remaining five (SMB21440, SMB21491, Smc00404, Smc01580 and Smc01774) are unrelated genes of unknown function. Twelve and 11 upregulated genes were previously found to be induced by phosphate starvation in a phoB-independent or partially phoB-dependent manner and by a shift to acidic pH, respectively (Table 1). Enhanced levels of cAMP also increased transcription of nine genes from a putative exopolysaccharide biosynthesis gene cluster containing nodP2 and exoF3 (Table 1, Fig. S3). Moreover, we found increased transcript abundance of two further surface-related genes, Smc01003 encoding a diacylglycerol lipase (Sahonero-Canavesi et al., 2015) and mrcA2 encoding a putative penicillin-binding protein, which might be involved in peptidoglycan biosynthesis. Out of 72 upregulated genes, 43 are genes without known or predicted function, and 16 of these encode proteins comprising less than 100 amino acids. Repressed genes are related to respiration, e.g. the transcription regulator fixK1, as well as carbon and amino acid metabolism. Of the downregulated genes, 34 were also repressed upon an osmotic upshift (Domínguez-Ferreras et al., 2006). Although below the twofold threshold, we note a 1.85-fold repression of ctrA encoding a master regulator of cell cycle progression.

Clr-mediated regulation of gene expression is promoted by both cAMP and cGMP

To verify the results of our microarray-based transcriptome study, promoter activities of 14 genes detected as differentially expressed were analysed in the WT and the clr mutant with and without overexpression of cyaJ. For this analysis, promoter-egfp fusions were constructed based on the low-
Fig. 1. Phenotypes of E. coli and S. meliloti strains overproducing a subset of S. meliloti ACs. (a) Heterologous complementation of an E. coli BTH101 cya mutant strain. β-Galactosidase activity was detected on plates containing X-Gal. (b) Swimming motility is inhibited upon overexpression of cyaB, cyaE1, cyaG1, cyaJ, cyaP and clr. Upper panel, flaA promoter activity in cNMP signalling in S. meliloti ac.
copy vector pHU-EGFP, which is compatible with the cya expression plasmid. This assay confirmed cya overexpression and clr-dependent regulation of the promoter activities of all these genes, except for Smc03999 (Fig. 2a). Expression of genes related to swimming motility could have been mediated by visNR. In order to identify genes affected by exogenously added cAMP, we analysed promoter activities of 20 genes, identified as differentially expressed in our transcriptome study, in the WT and the clr mutant. For this analysis, promoter-egfp fusions were constructed based on pSRKKm-EGFP to improve detection of weakly expressed genes taking advantage of the medium-copy number of this vector. Since a number of members of the feuNPQ regulon were found to be activated upon cya overexpression, the previously identified feuNPQ targets ndvA and Smb20838 were included in this assay. In accordance with our transcriptome data, promoter activities of 16 genes were affected in a clr-dependent manner by addition of 400 µM cAMP, whereas the promoter activities of ndvP1, Smb20495, Smc00864 and Smc00888 were unaffected in this assay (Fig. 2b). Moreover, the promoter activities of ndvA and Smb20838 were found to be regulated in a clr- and cAMP-dependent manner. Thus, Clr-cAMP might be involved in positively regulating production of periplasmic cyclic glucans. Addition of 400 µM cGMP to the growth medium resulted in clr-dependent effects on promoter activities similar to the addition of cAMP. Altogether, this strongly implies that S. meliloti is able to take up cAMP and cGMP from the medium and uses both cNMPs for clr-mediated gene regulation.

**Direct targets of regulation by Clr**

The Clr-binding site TGTT TCCCGCGG AACA was previously identified in the promoter region of Smc02178 and the Clr-binding sequence motif TGTT(N₈)AACA was suggested (Mathieu-Demazière et al., 2013). Sequences exactly matching this motif were only found in the upstream non-coding region of Smc04164 and not in the upstream of other Clr-cAMP/cGMP-regulated genes identified in our study. However, we identified slightly divergent motifs upstream of Smc00925, Smc01136, Smc04190 and Smb20906 (Fig. 3a). Mutation of (A/T)ACA to TTCA in the right half of the palindrome (Fig. 3a) abolished promoter activation by Clr and cNMPs (Fig. 2b), which indicates a role of these sequences in Clr-mediated transcription regulation. We noted that the N₈ spacer positions 2 and 7 were represented by C and G, respectively, in all the Clr-binding sites, whereas positions 1 and 4 of the palindrome varied (Fig. 3a). Based on these findings, we applied the sequence GT(T/C)(T/C/A)CNG-NNG(A/T)(A/T)AC to a genome-wide search using the PatScan online tool (D'souza et al., 1997). Among 279 hits, matching sequences were found upstream of Smc00653, Smc01003 and Smc02278, detected as upregulated in our transcriptome study (Table S3). In addition to the motif match (Table S3) at position −225 relative to the predicted transcription start site of Smc00653 (Schlüter et al., 2013), an additional sequence (CGTC ACCGCGGT AACT) resembling the consensus motif was found centred at position −40. Moreover, a putative Clr-binding site was found in the promoter region of the AC-encoding cyaF2 gene (Fig. 3a). Activation of the cyaF2 promoter by added cAMP and cGMP was clr-dependent and required an intact Clr-binding site (Fig. 2b). Based on this analysis, we suggest the new Clr-binding sequence consensus to be HGTYHC(N₈)GRWACA.

Activation of Smc02178, Smc04190, Smc00653 and Smb20906 promoters by Clr-cAMP/cGMP was also demonstrated in E. coli as heterologous host. In this assay setup, the promoter-reporter constructs were combined with expression constructs to produce the S. meliloti AC CyaG1 or the Synechocystis sp. GC Cya2, alone or in conjunction with Clr in the AC-deficient E. coli strain BTH101. Promoter activation was observed upon biosynthesis of cAMP or cGMP only when clr was co-expressed with the nucleotidyl cyclase gene. These results provide an additional proof for Clr being activated by cAMP and cGMP and suggest that Clr is able to interact with the E. coli RNA polymerase. Moreover, they show that endogenous E. coli Crp is not able to activate Clr targets, since promoter activation was not detected upon expression of nucleotidyl cyclase genes only. Finally, binding of purified Clr-His₈ in the promoter regions of genes identified as directly regulated by Clr was demonstrated in an EMSA. Recombinant Clr-His₈ only bound to the DNA in the presence of cAMP or cGMP and not if 2',3'-cAMP was provided (Fig. 3c). In all cases, addition of cAMP resulted in a larger electrophoretic mobility shift than addition of cGMP. The promoter DNA fragments
of exoY, exoH, exoL, flaA, visN, pilA, feuN and ndvA failed to bind Clr-His$_6$ in an EMSA (data not shown).

To further evaluate the role of the palindrome in Clr binding, native and mutated Clr-binding consensus sequences were inserted into the polylinker of the promoter-probe vector pSRKKm-EGFP and 167 bp composite DNA fragments carrying the binding motif and parts of the vector sequence were amplified. Whereas the synthetic fragments carrying the native binding sequences bound Clr in an EMSA, the changes in the right half of the palindrome to GRTTCA abolished binding (Fig. 3d). The latter result is consistent with missing activation of the promoters by Clr in vivo, when (A/T)ACA was exchanged for TTCA in the palindrome (Fig. 2b). These observations strongly support the imperfect palindromic sequence HGTYHCNNNNGR-WACA as the Clr-binding site.

Fig. 2. Studies of promoter-egfp activities to identify Clr-cAMP/cGMP-regulated genes. (a) EGFP fluorescence mediated by promoter-egfp constructs on the low-copy vector pPHU-EGFP upon overexpression of cyaJ in the WT and clr mutant. Fluorescence was measured 24 h after IPTG addition (250 µM). WT, Rm2011 carrying pWBT; WT cyaJ++, Rm2011 carrying pWBT-cyaJ; clr, clr mutant carrying pWBT; clr cyaJ++, clr mutant carrying pWBT-cyaJ. (b) EGFP fluorescence mediated by promoter-egfp constructs on the medium-copy vector pSRKKm-EGFP in the WT and clr mutant grown in TY with or without added cAMP or cGMP (400 µM). Fluorescence was measured 24 h post-inoculation. Asterisks mark constructs with the AACA motif in the putative Clr-binding palindrome mutated to TTCA. RFU, relative EGFP fluorescence units. Error bars represent the SD of three to four biological replicates.
Table 1. Genes induced upon overexpression of cyaJ

Genes analysed in promoter-egfp studies are given in boldface. M value, log2 differential ratio of transcript abundance; O, previously detected as induced upon osmotic upshift (Dominguez-Ferreras et al., 2006); EE, exs-exo gene cluster; NE, nodP2-exoF3 gene cluster; F, FeuNPQ regulon Griffitts et al.(2008); C, ChvI regulon (Chen et al., 2009); P, induced upon phosphate starvation in phoB-independent or partially phoB-dependent manner (Krol & Becker, 2004); A, genes upregulated following a shift to acidic pH (Chen et al., 2009); S, annotated protein product smaller than 100 aa.

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*Genes detected as regulated by clr in presence of added cAMP and cGMP and/or upon cyaJ overexpression.
†Genes detected as regulated dependent on clr in presence of added cAMP and cGMP and binding Clr in the promoter region.

DISCUSSION

Physiological processes affected by cAMP in S. meliloti

In this study, we found that cAMP contributes to opposing regulation of succinoglycan production and swimming motility in S. meliloti. Our data suggest this regulation to be partially dependent on the Crp-like protein Clr. cAMP-mediated regulation of swimming motility seems to be a common feature in various bacteria. Similarly to S. meliloti, cAMP-mediated repression of swimming motility was also reported in P. aeruginosa. Here, the CRP-like regulator Vfr negatively regulates swimming motility by direct transcriptional repression of flaQ, which encodes the master regulator of flagella production (Dasgupta et al., 2002). In contrast, in E. coli, Serratia marcescens or Salmonella typhimurium, CRP-cAMP activates expression of flhDC encoding the master regulator of the flagellar cascade (Soutourina et al., 1999; Stella et al., 2008; Kutsukake, 1997). Although expression of flagellin gene flaA and the visNR operon encoding the master regulator of swimming motility (Sourjik et al., 2000) was altered in a cAMP-dependent and a partially Clr-dependent manner in S. meliloti, Clr-cAMP did not bind to the upstream regions of flaA and visN, suggesting that this transcriptional regulation is probably indirect.

Transcriptome changes upon cyaJ overexpression overlapped with the ChvI regulon, which points to a possible activation of ChvI under these conditions. Interestingly, S. meliloti phosphate starvation and acidic stress regulons include chvI itself and genes activated by ChvI, which were also detected in our transcriptome study (exo genes, SMb21440, SMb21491, SMc00404, SMc01580 and SMc01774) (Chen et al., 2009; Krol & Becker, 2004; Hellweg et al., 2009). Considering that activation of succinoglycan biosynthesis and the exoY promoter upon cyaJ overexpression was only partially dependent on clr and that the exoY promoter DNA fragment was not bound by
Table 2. Genes repressed upon overexpression of cyaJ

Genes analysed in promoter-egfp studies are given in boldface. M value, log2 differential ratio of transcript abundance; O, detected previously as repressed upon osmotic upshift (Dominguez-Ferreras et al., 2006).

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*Genes detected as regulated by clr in presence of added cAMP and cGMP and/or upon cyaJ overexpression.

Clr in an EMSA, ChvI is a potential candidate for a factor mediating the clr-independent regulation of exs-exo genes under these conditions.

Our data suggest a link between cAMP signalling and osmotic stress response. A considerable number of genes related to osmotic stress response showed cAMP-mediated differential expression. This includes induction of the feuNPQ operon and several target genes of the encoded regulatory system. Activation of this operon under low osmolarity conditions and a role in regulation of biosynthesis of osmoprotective cyclic glucans has been reported previously (Griffit et al., 2008). Regulatory connections between cAMP signalling and osmoregulation were also reported in Anabaena sp. and Myxococcus xanthus. In Anabaena sp., an osmotic upshift induces expression of genes related to heterocyst envelope formation, supposedly via a CyaC-cAMP signal transduction system (Imashimizu et al., 2005). In M. xanthus, the AC CyaA was suggested to function as an ionic osmosensor during spore germination and is required for osmotic tolerance during sporulation (Kimura et al., 2002). Membrane lipid remodelling, characteristic for cells growing under high osmolarity, was regulated by crpO in Rhodobacter sphaeroides (Tsuzuki et al., 2011). In our study, we detected Clr-cAMP/cGMP-dependent activation of SMc01003 expression. This gene was recently reported to encode a diacylglycerol lipase contributing to the release of free fatty acids in S. meliloti (Sahonero-Canavesi et al., 2015). It remains to be investigated how Clr- and cAMP-dependent signalling affects osmolarity-regulated physiological processes.
Besides this possible link to osmoregulation, we observed enrichment of cAMP-induced genes in the \textit{nodP2-exoF3} cluster and confirmed Clr-cAMP- and Clr-cGMP-mediated activation of the \textit{nodP2}, \textit{SMb21240} and \textit{exoF3} promoters, which are likely to drive transcription of 12 genes in this cluster (Fig. S3). Functional predictions for 14 genes in this

<table>
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<td>SMc02178</td>
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<tr>
<td>SMb20906</td>
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<td>cyaF2</td>
<td>GGGT CCGCAGA AACA</td>
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Fig. 3. Identification of direct regulatory targets of Clr. (a) Clr-binding site in promoter region of \textit{SMc02178} and homologous sequences in promoter regions of other Clr-regulated genes. Nucleotides conserved in all analysed binding sites are shaded. Adenines mutated to thymidines to obtain an exchange (T/A)ACA to TTCA are underlined. Ambigous codes: H: T, C or A; Y: T or C; R: G or A; W, A or T. (b) EGFP fluorescence mediated by promoter-\textit{egfp} constructs in the \textit{E. coli} BTH101 strain producing AC CyaG1 or GC Cya2Sy alone or in combination with Clr. Fluorescence was measured 16 h post-inoculation. RFU, relative EGFP fluorescence units. Error bars represent the SD of four biological replicates. (c) EMSA with purified Clr-His\textsubscript{6}. The sizes of the promoter DNA fragments were 570 bp for \textit{Smc02178}, 435 bp for \textit{SMb20906}, 465 bp for \textit{SMc00925}, 560 bp for \textit{SMc01136}, 458 bp for \textit{SMc04190}, 492 bp for \textit{cyaF2} and 475 bp for \textit{SMc00653}. (d) EMSA with purified Clr-His\textsubscript{6}. The short synthetic DNA fragments (167 bp) contained either the native Clr-binding site (WT) or the binding site with the exchange (A/T)ACA to TTCA in the right half of the palindrome (TTCA).
gene cluster suggest that it is possibly involved in biosynthesis of an unknown polysaccharide. Thus, elevated levels of cAMP not only induce succinoglycan biosynthesis but also may induce biosynthesis of another yet unknown polysaccharide. This gene cluster encodes ExoF3, which shows moderate homology (27% amino acid sequence identity) to ExoF1, an outer membrane polysaccharide export lipoprotein involved in terminal steps of succinoglycan biosynthesis (Cuthbertson et al., 2009). It also encodes NodP2, which is identical with NodP1, involved in Nod factor sulfurylation (Schwedock et al., 1994). Sequence identity of these two genes is restricted to the protein coding sequence, whereas the upstream non-coding regions differ. This provides an explanation for detection of cAMP- and clr-mediated upregulation of nodP1 and nodP2 in our transcriptome study, while the promoter-probe assay only confirmed this regulation for nodP2. Out of 82 genes identified as downregulated in our transcriptome study, 10 encode cytochromes, cytochrome oxidases or haem biosynthesis enzymes. Moreover, mdh and sucC, encoding two enzymes of the citrate cycle, were among the repressed genes. In several other bacterial species, the situation is opposite. In E. coli, CRP-cAMP positively regulates expression of TCA cycle enzymes (Nanchen et al., 2008), whereas in Shewanella oneidensis and Mycobacterium smegmatis, CRP genes were reported to directly activate genes encoding cytochromes (Kasai et al., 2015; Aung et al., 2014). In Corynebacterium glutamicum, the cAMP-responsive protein GlxR was found to bind upstream of genes related to both aerobic and anaerobic respiration and central carbon metabolism (Kohl et al., 2008). In contrast to glucose-mediated catabolite repression in E. coli, S. meliloti employs catabolite repression by succinate, which is mediated by the HPK kinase/phosphatase and the two-component regulatory system SMa0113-SMa0114 (Bringhurst et al., 2002; Pinedo et al., 2009; Garcia et al., 2010). A role of cAMP in this process has not been reported. Thus, Clr-cAMP-mediated regulation of central metabolism probably differs between E. coli and S. meliloti.

Considering that overexpression of the AC-encoding gene cyaJ slowed down growth and resulted in lower final cell densities of batch cultures, repression of ctrA under these conditions is noteworthy. Moreover, AC overexpression resulted in partially clr-dependent repression of three targets of direct regulation by CtrA, namely pilA1, flaA and ctrA itself (Pini et al., 2015). Depletion of CtrA leads to growth arrest of S. mellitondo (Pini et al., 2015). Thus, it is tempting to speculate that an increase in cAMP levels may influence stability or phosphorylation status of CtrA, which in turn may be responsible for the slow-down in growth and downregulation of flaA and pilA1.

**Clr-cAMP and Clr-cGMP bind to a weakly conserved palindromic sequence**

Using in vivo and in vitro assays, we identified SMb20906, SMc00925, SMc00653, SMc01136, SMc04190 and cyaF2 as targets of direct activation by Clr-cAMP/cGMP, and we confirmed the previously reported target SMc02178. For SMc04190, SMb20906 and cyaF2, the transcription start sites were previously determined (Schlüter et al., 2013). Clr-binding sites in these promoters are centred at positions −77, −41 and −43, respectively, relative to the transcription start site. These positions are in accordance with the classical mode of gene activation by a CRP, by binding at or upstream of the −35 promoter element (Busby & Ebright, 1999). We found that an imperfect palindromic with one mismatch either in first or in fourth position was still functional in Clr binding. Putative binding sites differing in three positions from the consensus motif TGTT(N8)AACA were found upstream of SMc00653, whose promoter was activated by Clr-cAMP/cGMP in E. coli and Clr bound to the corresponding DNA fragment. Although the role of these sequences in Clr-dependent regulation was not investigated, it implies that Clr may be able to bind sequences differing from the consensus in more than one position. Similar observations were made for the C. glutamicum CRP homologue GlxR and Haemophilus influenzae CRP, which bind sequences with mismatches in the palindromic part of the binding site (Kohl et al., 2008; Redfield et al., 2005). Moreover, in X. campestris, even sequences with more than two mismatches in the palindrome were bound by the CRP homologue Clp (Chen et al., 2010). We note that the 8 bp spacer of the S. meliloti Clr-binding site contains five or more C or G. Moreover, positions 2 and 7 of the spacer, occupied by C and G, appeared to be conserved. The CG-rich spacer was experimentally shown to be important for Clp binding (Chen et al., 2010); thus, this feature might be important for Clr binding as well. Our initial characterization suggests that the number of Clr-binding sites in the S. meliloti genome is much higher than 27, which is the number of hits detected using the PatScan online tool (Dsoouza et al., 1997) for a search with the previously suggested Clr-binding site TGTT(N8)AACA. Upstream regions of the genes Smc00653, Smc01003 and Smc02278 induced upon cyaJ overexpression were among the 279 hits for the alternative consensus GTYHC(N8)GRWAC. Moreover, a site upstream of cyaF2 was identified and cyaF2 could be confirmed as a direct target of Clr. In contrast to E. coli cyaJ, which is negatively regulated by CRP (Inada et al., 1996), cyaF2 transcription was activated by Clr-cAMP/cGMP. Based on these findings, we redefine the consensus sequence as HGTYHC(N8)GRWAC. However, we expect that identification of additional Clr-binding sites probably will lead to an even more relaxed consensus sequence.

Since cGMP was previously detected in S. meliloti cells (Tian et al., 2012) and, in our study, CyaB was found to be capable of cGMP synthesis, we asked if Clr could be activated by cGMP as well. Both cNMPs activated Clr for regulation of its gene targets, when added to the S. mellitondo growth medium and when ectopically produced in E. coli. In vitro, Clr-His6 bound the target sequences in the presence of either cAMP or cGMP. Binding required the presence of one of these cNMPs, and the shift in electrophoretic mobility appeared larger with cAMP than with
cGMP. This implies that binding of both cNMPs may cause different changes in protein conformation, which may result in either a different stoichiometry for the protein-DNA interaction or differences in bending of Clr-bound DNA. However, the magnitude of clr-dependent activation of the majority of analysed promoters did not dramatically differ between cAMP and cGMP added to the growth medium. Therefore, Clr-cAMP and Clr-cGMP complexes are likely to be structurally diverse but functionally similar. This is a clear difference from the CRPs of other bacteria studied so far. For example, E. coli and Pseudomonas putida CRPs are activated by cAMP, but not cGMP, whereas R. centenum CRP CgrA has a much higher affinity to cGMP than to cAMP (Seok et al., 2014; Arce-Rodriguez et al., 2012; Roychowdhury et al., 2015). Interestingly, just a few amino acid exchanges can alter the specificity of a CRP toward cAMP or cGMP. A triple mutant version of E. coli CRP (R123N, T127C and S128T) that was activated by both cNMPs was recently described (Ryu et al., 2015). The structural basis of cAMP- and cGMP-induced binding of target DNA by the S. melliloti Clr protein remains to be elucidated.

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REFERENCES


cNMP signalling in S. melloti


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