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Supplementary information to the article

Identification and Characterization of Four c-di-GMP-Metabolizing Enzymes from *Streptomyces ghanaensis* ATCC14672 Involved in the Regulation of Morphogenesis and Moenomycin A Biosynthesis

Desirèe Nuzzo, Roman Makitrynskyy*, Olga Tsypik and Andreas Bechthold*

Pharmaceutical Biology and Biotechnology, Institute of Pharmaceutical Sciences, Albert-Ludwigs University, Freiburg 79104, Germany

* Correspondence: R.M. (email: roman.makitrynskyy@pharmazie.uni-freiburg.de), A.B. (email: andreas.bechthold@pharmazie.uni-freiburg.de)

Supplementary Table S1. Strains and plasmids used in this work. .

Strains or plasmids	Description/Functions	Source
Strains		
S. ghanaensis ATCC14672	Wild-type (WT) moenomycin producer	ATCC
S. ghanaensis Δ$cdgE_{gh}$	WT derivative, Δ$cdgE_{gh}$ deletion	This work
S. ghanaensis Δ$cdgD_{gh}$	WT derivative, Δ$cdgD_{gh}$ deletion	This work
S. ghanaensis Δ$rmdA_{gh}$	WT derivative, Δ$rmdA_{gh}$ deletion	This work
S. ghanaensis Δ$cdgA_{gh}$	WT derivative, Δ$cdgA_{gh}$ deletion	This work
S. ghanaensis pIJcdgE	WT derivative, $cdgE_{gh}$ overexpression	This work
S. ghanaensis pIJcdgD	WT derivative, $cdgD_{gh}$ overexpression	This work
S. ghanaensis pIJrmdA$_{gh}$	WT derivative, $rmdA_{gh}$ overexpression	This work
S. ghanaensis pIJcdgA$_{gh}$	WT derivative, $cdgA_{gh}$ overexpression	This work
S. ghanaensis pIJrmdA^{AAL}	WT derivative, $rmdA_{gh}^{AAL}$ overexpression	This work
S. ghanaensis pIJcdgA^{AAL}	WT derivative, $cdgA_{gh}^{AAL}$ overexpression	This work
E. coli XL1Blue	Host strain for DNA cloning	Agilent
E. coli ET12567 (pUZ8002)	Host for *E. coli*-streptomycetes conjugation	[1]
E. coli BW25113	Host for REDIRECT technology with helper plasmid pIJ790	[2]
E. coli BL21 Star (DE3)	Host for protein production	Thermo Fisher Scientific
E. coli BL21 Star (DE3)/pLysS	Host for protein production	Thermo Fisher Scientific
Plasmids		
pBluesctiptIIKS+	Cloning vector, Ap^R	Addgene
pKGLP2	Suicide vector carrying *gusA*, Hyg^R	[3]
pSET152	φC31-based integrative vector, Am^R	[4]
pUWLCre	Vector carrying *cre* under *ermEp*, Tsr^R	[5]
pIJ10257	φBT1-based integrative vector carrying *ermEp*, Hyg^R	[6]
pLERECJ	Vector carrying apramycin resistance cassette with *loxP*-sites for gene replacement	Prof. Luzhetskyy, Saarland University
pET28a	Vector for protein production, Km^R	Novagen
pET32a	Vector for protein production, Ap^R	Novagen
pBluecdgE	pBluescriptIIKS+ carrying $cdgE_{gh}$	This work
pBluecdgD	pBluescriptIIKS+ carrying $cdgD_{gh}$	This work
pBluermdA	pBluescriptIIKS+ carrying $rmdA_{gh}$	This work
pBluecdgA	pBluescriptIIKS+ carrying $cdgA_{gh}$	This work
pBluecdgE::aac(3)IV	pBlue02707, Δ$cdgE_{gh}$ deletion	This work
pBluecdgD::aac(3)IV	pBlue02343, Δ$cdgD_{gh}$ deletion	This work

pBluermdA::aac(3)IV	pBluermdA, Δ*rmdA$_{gh}$* deletion	This work
pBluecdgA::aac(3)IV	pBluecdgA, Δ*cdgA$_{gh}$* deletion	This work
pSETcdgA	pSET152 carrying *cdgA$_{gh}$* along with its promoter	This work
pKGcdgE::aac(3)IV	pKGLP2 carrying *cdgE::aac(3)IV*	This work
pKGcdgD::aac(3)IV	pKGLP2 carrying *cdgD::aac(3)IV*	This work
pKGrmdA::aac(3)IV	pKGLP2 carrying *rmdA::aac(3)IV*	This work
pKGcdgA::aac(3)IV	pKGLP2 carrying *cdgA::aac(3)IV*	This work
pIJcdgE	pIJ10257 carrying *ermEp-cdgE$_{gh}$* fusion	This work
pIJcdgD	pIJ10257 carrying *ermEp-cdgD$_{gh}$* fusion	This work
pIJrmdA	pIJ10257 carrying *ermEp-rmdA$_{gh}$* fusion	This work
pIJcdgA	pIJ10257 carrying *ermEp-cdgA$_{gh}$* fusion	This work
pIJrmdA^{AAL}	pIJ10257 carrying a mutated version of *rmdA$_{gh}$*	This work
pIJcdgA^{AAL}	pIJ10257 carrying a mutated version of *cdgA$_{gh}$*	This work
pETcdgE	pET28b carrying His-tagged CdgE$_{gh}$	This work
pETcdgE^{AADEF}	pET28b carrying a mutated version of CdgE$_{gh}$	This work
pETrmdA	pET28b carrying His-tagged RmdA$_{gh}$	This work
pETcdgA	pET28b carrying His-tagged CdgA$_{gh}$	This work
pETrmdA^{GGDEF}	pET28b carrying solely the His-tagged GGDEF domain of RmdA$_{gh}$	This work
pETrmdA^{AAL}	pET28b carrying a mutated version of His-tagged RmdA$_{gh}$	This work
pETcdgA^{AAL}	pET28b carrying a mutated version of His-tagged CdgA$_{gh}$	This work
pET32acdgA^{AAL}	pET32a carrying a mutated version of Trx-His-tagged CdgA$_{gh}$	This work

Supplementary Table S2. Primers used in this work.

Primers	Sequence
02707_del_for	AAATCTAGAAACGACGAGACGATGCCG
02707_del_rev	AAATCTAGATACGGGTGGAGGCGCTCG
02707_kn_for	AATGTACCCGTTCGCCCGGAATCTCCCTAGCCTGGAGGGATGGATATCTCTAGATACCG
02707_kn_rev	GCGCGTCACACCCGCCGGAGCCGCGGCGGGGCGTGCGGTCAAACAAAAGCTGGAGCTC
02343_del_for	AAATCTAGAAGTCTGTAGGTGCATCGAGC
02343_del_rev	AAAGAATTCAAGAGCACCTGGCACTCG
02343_kn_for	GCTCCTTGCCGAACGCGCCGCCGGCGCGGGGGTGAGCGCATGAGCGATATCTCTAGATACCG
02343_kn_rev	GTCCGGGGCCAGGTGCCACCGGAACACCGGCAGCGCACTCATGCAACAAAAGCTGGAGCTC
cdgA_del_for	AAATCTAGAGTGGTGATCTTCACCGTCCAC
cdgA_del_rev	AAAGAATTCCTGCAGGAGATCGAGGTGC
cdgA_kn_for	CTGCACGGACAGCGATCGAGTGCACTGCGGGAGCGAGAGGTGGATATCTCTAGATACCG
cdgA_kn_rev	CTACGGGTTGCCGGCCCCCGCCTGAACCCCGGACGCGCCCAGAACAAAAGCTGGAGCTC
rmdA_del_for	AAATCTAGAGAGTTGACGATGAATACCTCCT
rmdA_del_rev	AAAGAATTCAAGTCATGTTCAGCTCACCA
rmdA_kn_for	CGCCTTCTCCGGCTTCGCGGGCGTGGGGCGTACGGCGTGAGCGATATCTCTAGATACCG
rmdA_kn_rev	CACCGATCGTCCGGCCGGTCCGCGACCGGTTTGCGCCTCAAACAAAAGCTGGAGCTC
cdgA_compl_for	AAATCTAGAGACGGTTCACGGGACACCG
cdgA_compl_rev	AAAGAATTCGTGCGGCTGCGCTCCTAC
02707_exp_for	AAAAAACATATGGGTGAGGACAGCCGGCT

02707_exp_rev	AAACTCGAGTCACGGCGAGTGCCGCCCCC
02343_exp_for	AAAAAACATATGAGCACCACGGCACTTGC
02343_exp_rev	AAACTCGAGTCATGCCGCGCCGCGGCGGC
rmdA_exp_for	AAAAAACATATGAGCGTGGAACCGGACGGG
rmdA_exp_rev	AAAAAGCTTTCACCCCGACGCGTCCAC
cdgA_exp_for	AAAAAACATATGGTGAGCGGAACGTCCGA
cdgA_exp_rev	AAACTCGAGCTACGGGTTGCCGGCCCCCG
rmdA_GGDEF_rev	AAAAAGCTTTCAGTCGGCGAGCTCGAACCG
cdgA_AAL_for	GCGGCCCTGGTCCGCTGGAACC
cdgA_AAL_rev	CACGCCGTGCACCCGG
rmdA_AAL_for	GCGGCGCTGGTGCGCTGGC
rmdA_AAL_rev	GGCGCCGCGGACGCTC
02707_AADEF_for	GCCGCCGACGAGTTCTGCCTGCTGTCC
02707_AADEF_rev	GAGCCGCGCCGCCAGG

Supplementary Table S3. Buffers used in protein purification.

Protein	Equilibration buffer	Storage buffer
$CdgE_{gh}$ $CdgE_{gh}^{AADEF}$	50 mM TrisHCl (pH 7.0) 0.5 M NaCl 5 mM Imidazole	50 mM TrisHCl (pH 7.0) 0.5 M NaCl 1 mM $MgCl_2$ 1 mM DTT 10% Glycerol
$RmdA_{gh}$ $CdgA_{gh}$ $RmdA^{GGDEF}$ $CdgA_{gh}^{AAL}$	50 mM TrisHCl (pH 7.5) 0.25 M NaCl 1 mM $MgCl_2$ 5 mM β-mercaptoethanol 5% Glycerol 10 mM Imidazole	50 mM TrisHCl (pH 7.5) 0.25 M NaCl 1 mM $MgCl_2$ 1 mM DTT 5% Glycerol

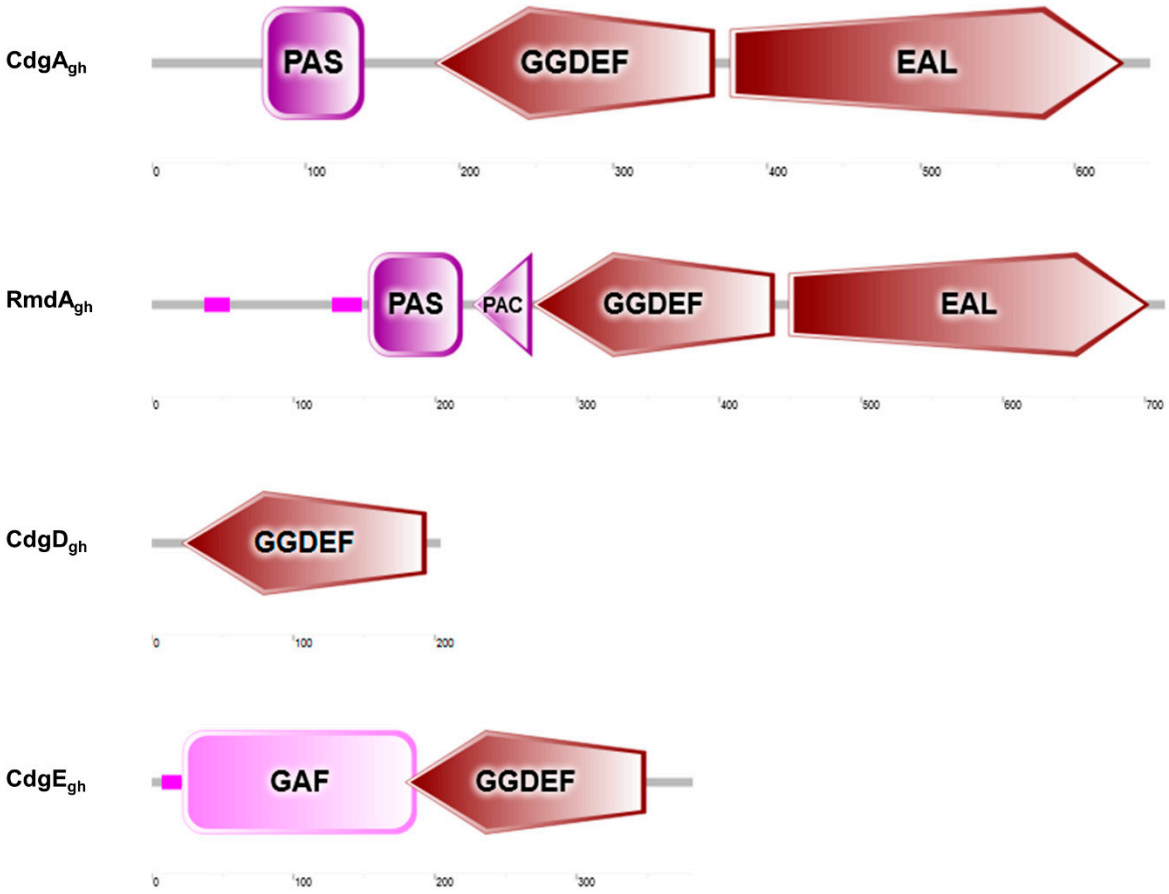

Figure S1. Domains architecture of CdgA$_{gh}$, RmdA$_{gh}$, CdgD$_{gh}$ and CdgE$_{gh}$. Domains composition was predicted by the SMART database (https://smart.embl.de).

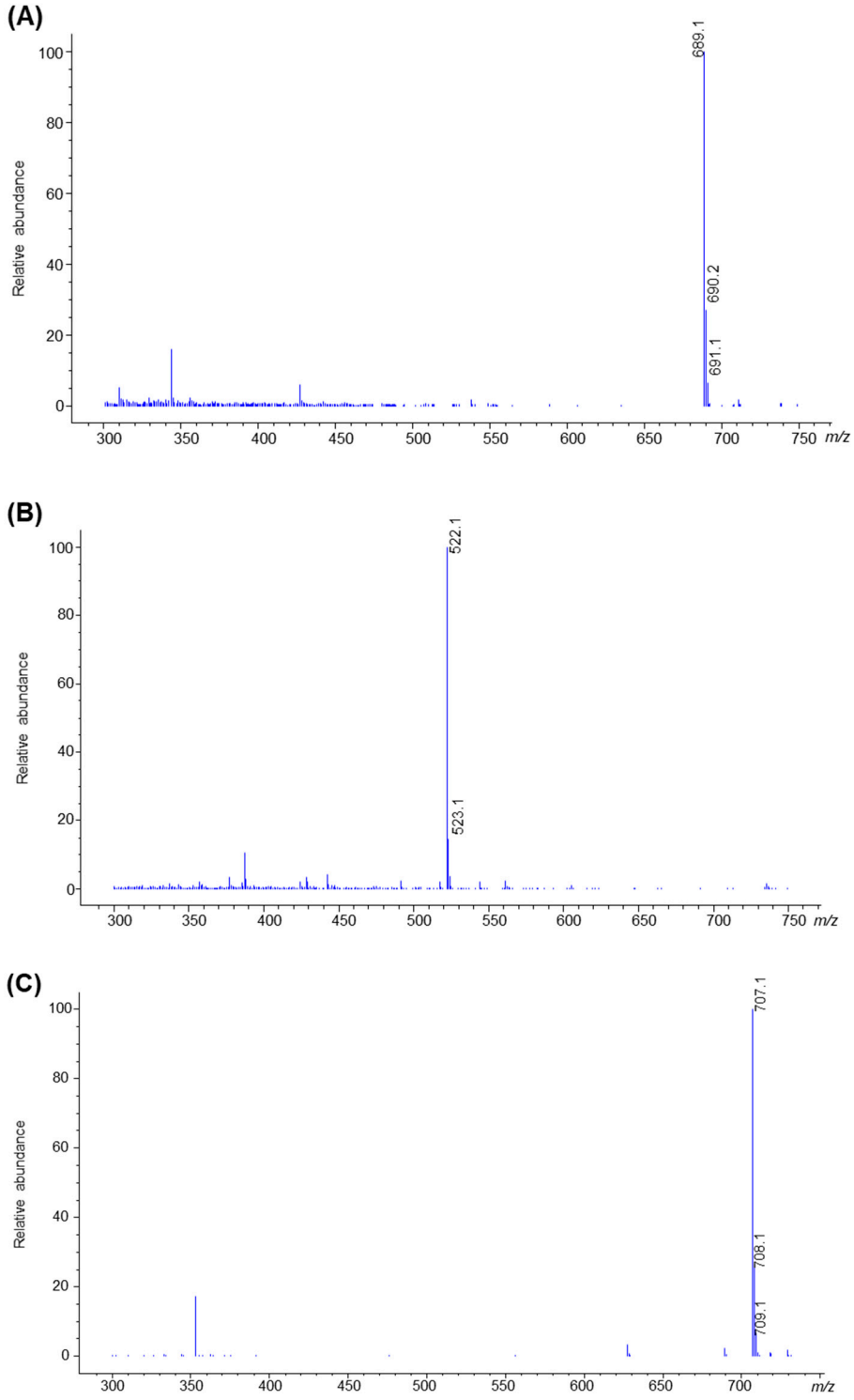

Figure 2. MS spectra of GTP, c-di-GMP and pGpG detected after the DGC and PDE *in vitro* assays. **A**) $[M-H]^-$ ion of GTP (*m*/*z* 522.1). **B**) $[M-H]^-$ ion of c-di-GMP (*m*/*z* 689.1). **C**) $[M-H]^-$ ion of pGpG (*m*/*z* 707.1).

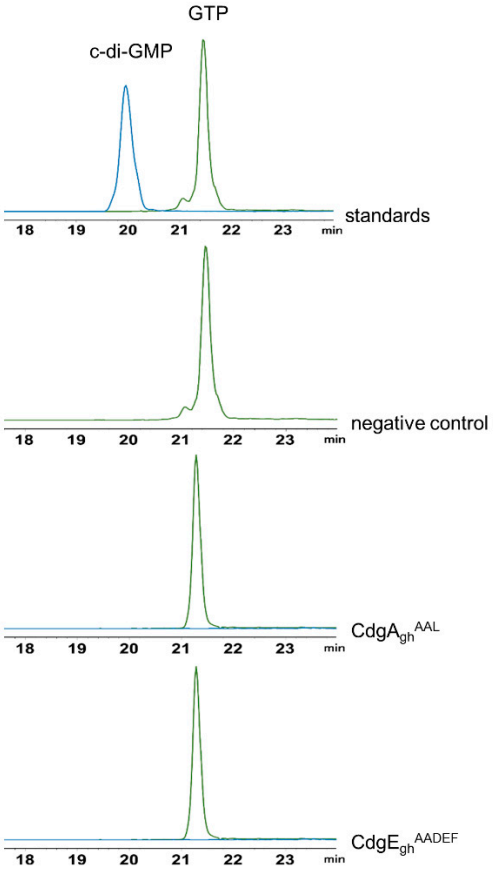

Figure 3. LC-MS chromatograms of CdgA$_{gh}^{AAL}$ and CdgE$_{gh}^{AADEF}$ diguanylate cyclase *in vitro* assays. No conversion of GTP into c-di-GMP ([M-H]$^-$ ion of c-di-GMP (*m*/*z*= 689.1)) was observed in both reaction mixtures containing CdgA$_{gh}^{AAL}$ and CdgE$_{gh}^{AADEF}$, respectively.

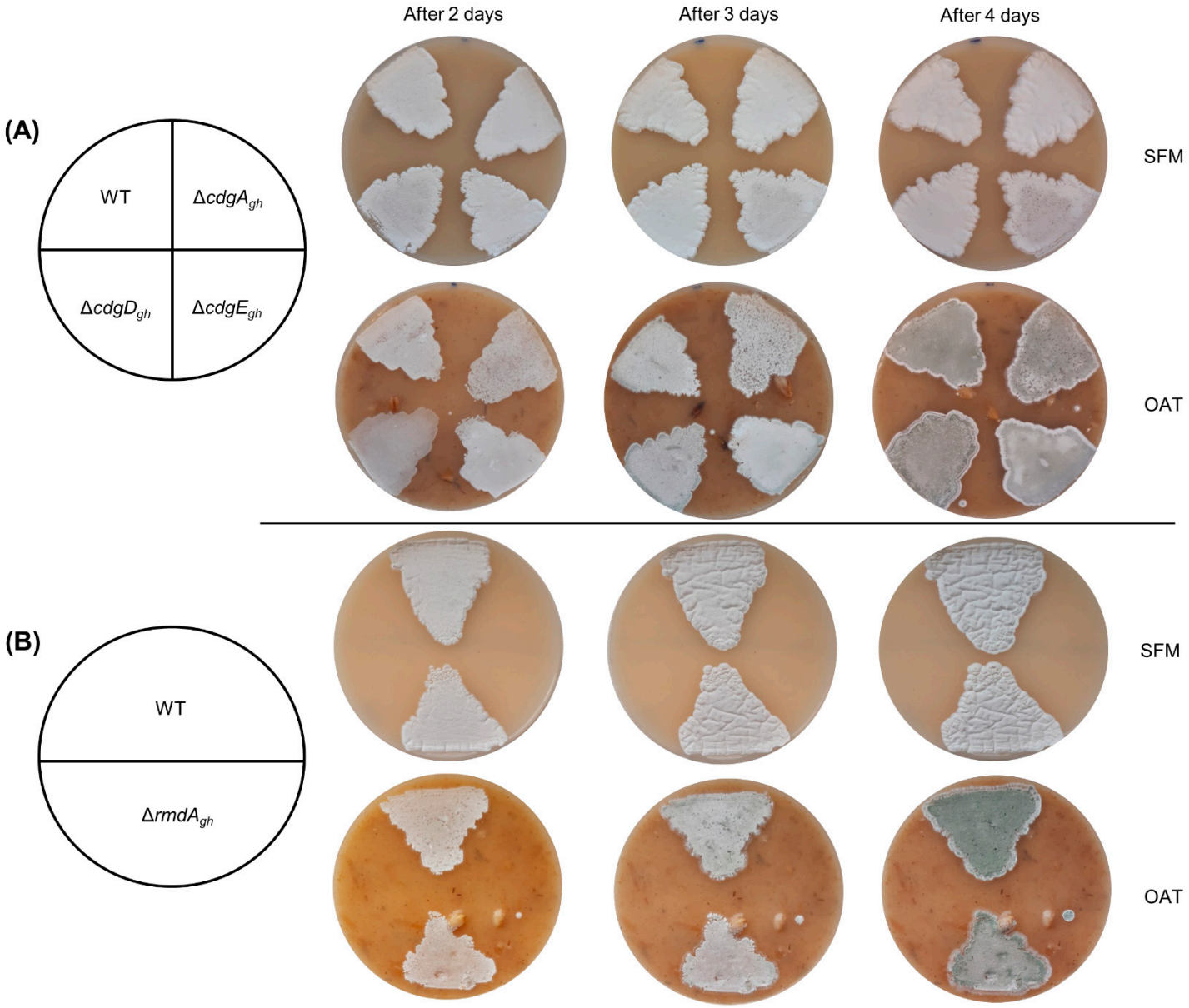

Figure 4. Phenotype evaluation of *S. ghanaensis* wild-type (WT), *S. ghanaensis* Δ*cdgA*$_{gh}$ (Δ*cdgA*$_{gh}$), *S. ghanaensis* Δ*cdgE*$_{gh}$ (Δ*cdgE*$_{gh}$), *S. ghanaensis* Δ*cdgD*$_{gh}$ (Δ*cdgD*$_{gh}$) and *S. ghanaensis* Δ*rmdA*$_{gh}$ (Δ*rmdA*$_{gh}$). In comparison to the control strain (WT), no obvious differences in morphological development were observed upon inoculation of *S. ghanaensis* Δ*cdgA*$_{gh}$, *S. ghanaensis* Δ*cdgE*$_{gh}$, *S. ghanaensis* Δ*cdgD*$_{gh}$ (**A**) and *S. ghanaensis* Δ*rmdA*$_{gh}$ (**B**) in SFM and oatmeal (OAT) agar after four days of growth.

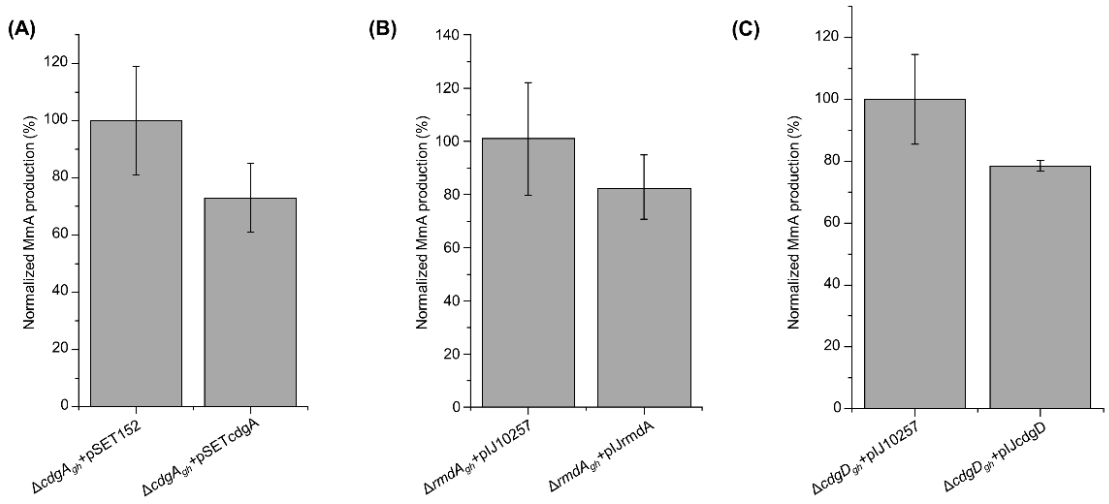

Figure 5. Complementation experiments in *S. ghanaensis* null-mutants. **A**) *S. ghanaensis* Δ*cdgA*$_{gh}$ carrying an empty copy of pSET152 (Δ*cdgA*$_{gh}$+pSET152) and complemented strain (Δ*cdgA*$_{gh}$+pSETcdgA). **B**) *S. ghanaensis* Δ*rmdA*$_{gh}$ carrying an empty copy of pIJ10257 (Δ*rmdA*$_{gh}$+pIJ10257) and complemented strain (Δ*rmdA*$_{gh}$+pIJrmdA). **C**) *S. ghanaensis* Δ*cdgD*$_{gh}$ carrying an empty copy of pIJ10257 (Δ*cdgD*$_{gh}$+pIJ10257) and complemented strain (Δ*cdgD*$_{gh}$+pIJcdgD). The mean value of moenomycin mass peak area of each *S. ghanaensis* null-mutant carrying the empty vector was taken as 100%. Error bars represent standard deviations.

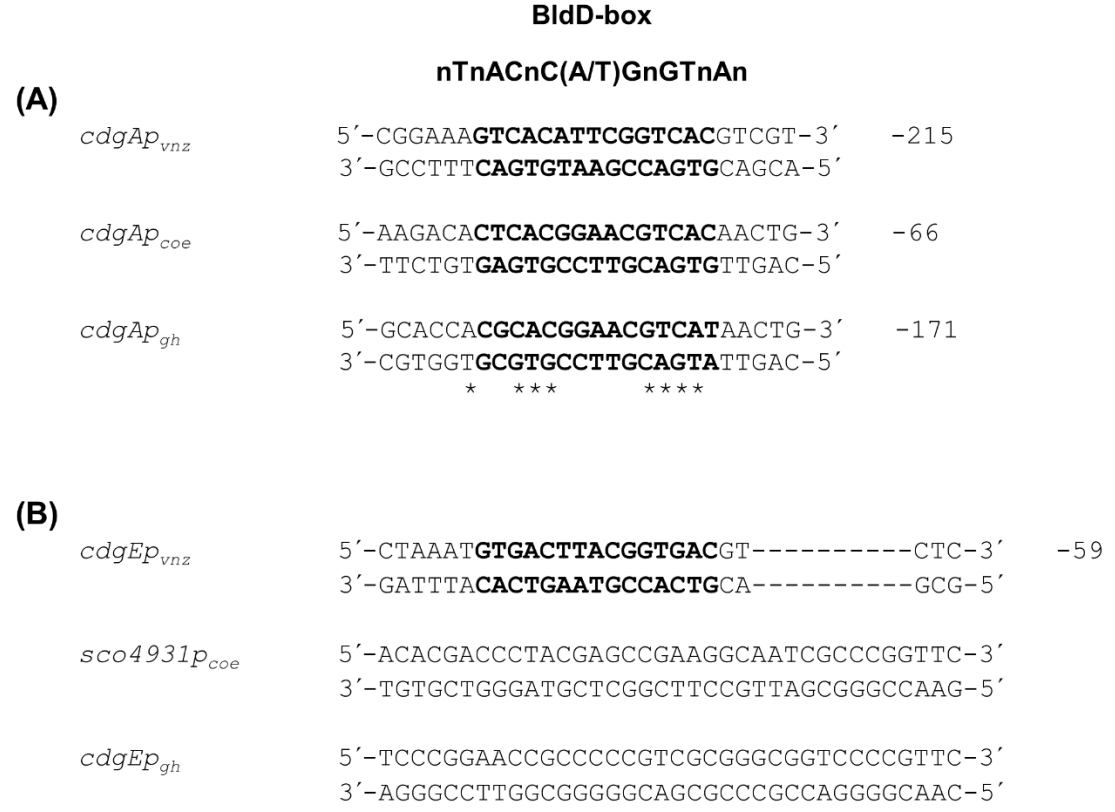

Figure 6. Multiple sequence alignment of putative BldD-binding site (BldD-box) in the promoter of *cdgA*$_{gh}$ and *cdgE*$_{gh}$ and their orthologs. **A**) Alignment of BldD-boxes (in bold) identified in the promoter region of *cdgA* in *S. venezuelae* (*cdgAp*$_{vnz}$) [7,8] and *S. coelicolor* (*cdgAp*$_{coe}$) [9] shows a putative BldD-box also present in the promoter of *cgdA*$_{gh}$ (*cdgAp*$_{gh}$). Asterisks indicate identical nucleotides. **B**) A BldD-box was identified in the promoter of *cdgE* in *S. venezuelae* (*cdgEp*$_{vnz}$) [7,8] but not in the promoter regions of its orthologs from *S. coelicolor* (*cdgEp*$_{coe}$) and *S. ghanaensis* (*cdgEp*$_{gh}$). BldD-box was determined by den Hengst et al. (2010) [9] and the numbers represent the distance from the putative start codon of the corresponding downstream gene. .

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