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RNase I Regulates *Escherichia coli* 2',3'-Cyclic Nucleotide Monophosphate Levels and Biofilm Formation

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Abstract

Regulation of nucleotide and nucleoside concentrations is critical for faithful DNA replication, transcription, and translation in all organisms, and has been linked to bacterial biofilm formation. Unusual 2',3'-cyclic nucleotide monophosphates (2',3'-cNMPs) recently were quantified in mammalian systems, and previous reports have linked these nucleotides to cellular stress and damage in eukaryotes, suggesting an intriguing connection with nucleotide/nucleoside pools and/or cyclic nucleotide signaling. This work reports the first quantification of 2',3'-cNMPs in *Escherichia coli* and demonstrates that 2',3'-cNMP levels in *E. coli* are generated specifically from RNase I-catalyzed RNA degradation, presumably as part of a previously unidentified nucleotide salvage pathway. Furthermore, RNase I and 2',3'-cNMP levels are demonstrated to play an important role in controlling biofilm formation. This work identifies a physiological role for cytoplasmic RNase I and constitutes the first progress toward elucidating the biological functions of bacterial 2',3'-cNMPs.

Introduction

Regulation of nucleotide and nucleoside pools is a crucial physiological process in all organisms. Concentrations of (d)NTPs and (d)NDPs fluctuate in *Escherichia coli* culture throughout the various phases of growth [1], and imbalanced nucleotide concentrations impair essential cellular processes, including the fidelity of DNA replication, transcription, and translation [2–4]. Numerous other biological functions also are mediated directly by nucleotide second messengers such as adenosine 3',5'-cyclic monophosphate (3',5'-cAMP), cyclic dimeric-3':5'-guanosine monophosphate (c-di-GMP), and guanosine 3'-diphosphate, 5'-triphosphate (pppGpp) in prokaryotes [5–8]. In addition, recent work has implicated primary nucleotide metabolism in the regulation of bacterial biofilm morphology, suggesting involvement in the regulation of other, unidentified processes [9–13]. Along with paradigmatic nucleotides, atypical 2',3'-cyclic nucleotide monophosphates (2',3'-cNMPs)

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B.M.F., K.S.M., and E.E.W. designed the study. B.M.F., K.S.M., J.M.G.-R., C.J., L.B., J.E.S., X.J., and E.E.W. performed the acquisition, analysis, and interpretation of the data. B.M.F. and E.E.W. wrote the manuscript.

have been quantified in mammalian organs and cells [14–17]. Further investigation demonstrated that concentrations of 2',3'-cAMP increase in response to acute organ stress in eukaryotes, suggesting an interesting link with cellular damage [17, 18]. However, less is known about these unusual nucleotides in bacteria, despite their initial detection in *E. coli* over 50 years ago [19]. A recent study quantified intracellular and extracellular 2',3'-cCMP and -cUMP levels in *Pseudomonas fluorescens* culture [20], and 2',3'-cAMP has been quantified in *Staphylococcus aureus* [21]. However, the biological relevance of these nucleotides in bacteria has not been identified. To date, physiological quantification of the four 2',3'-cNMPs of the canonical RNA nucleobases has not been reported in any prokaryote, enzymes involved in modulating 2',3'-cNMP concentrations have yet to be identified, and the physiological roles are unknown.

In vitro studies have suggested that the bacterial enzyme ribonuclease I (RNase I) may play a role in the formation of 2',3'-cNMPs [22, 23]. RNase I is an endoribonuclease in the widely distributed RNase T2 superfamily that hydrolyzes short oligoribonucleotides (oligoRNAs) *in vitro* to generate 2',3'-cNMP monomers, regardless of sequence context. The resulting 2',3'-cNMPs are then slowly hydrolyzed to 3'-NMPs in a second non-specific catalytic step [22, 23]. The gene for RNase I encodes a periplasmic localization sequence and the protein originally was isolated from the periplasm of *E. coli* [24, 25]. Thus, RNase I was suggested to function in catabolism of extracellular RNA [26, 27], but the physiological substrate(s) remains unclear due to the paucity of *in vivo* investigations.

More recently, a cytoplasmic variant of RNase I encoded by the same *rna* gene was purified from *E. coli* and characterized *in vitro*, with the cytoplasmic enzyme exhibiting differences in pH stability and thermal denaturation *in vitro*, as compared to periplasmic RNase I [28, 29]. A recent study demonstrated that *E. coli* RNase I activity is inhibited by the 16S rRNA of the 30S ribosomal subunit [30]. However, the physiological relevance of this interaction is unclear due to the use of chimeric rRNA. The study also failed to delineate which form of RNase I (i.e. periplasmic and/or cytoplasmic) binds 16S rRNA. Thus, the biological function of periplasmic RNase I remains incompletely understood, and even less is known about the role of the cytoplasmic variant. A role for cytoplasmic RNase I in the final steps of mRNA degradation was suggested, but never investigated experimentally [28]. Consequently, the physiological function of RNase I remains ambiguous, particularly the putative role of RNase I in mRNA degradation [31, 32]. Initiation of mRNA decay in bacteria typically involves endonucleolytic cleavage of the transcript by RNase E of the RNA degradosome. The inactivated transcript is subsequently degraded by 3' to 5' exoribonucleases, such as RNase II and polynucleotide phosphorylase (PNPase), with the aid of auxiliary degradosome proteins [33]. The short oligoRNAs remaining after RNase II and PNPase processing [34–36] are ultimately degraded by oligoribonuclease (oligoRNase) [37, 38], and a role for RNase I in this final catabolic step has been postulated, but never confirmed [28].

The present work quantifies 2',3'-cAMP, -cGMP, -cCMP, and -cUMP concentrations in *E. coli* and demonstrates for the first time that RNase I generates 2',3'-cNMPs *in vivo*. Experimental perturbation of RNA degradation has validated that RNase I degrades cytoplasmic RNA to generate 2',3'-cNMPs. Furthermore, studies employing a recombinant 2',3'-cyclic nucleotide phosphodiesterase have revealed a role for 2',3'-cNMPs in regulating

biofilm formation. This report constitutes the first progress toward understanding the biological functions of 2',3'-cNMP pools in bacteria and offers insight into the physiological processes regulated by RNase I, providing a foundation to further elucidate the roles of these cyclic nucleotides in processes linked to bacterial nucleotide metabolism.

Materials and Methods

Bacterial strains, plasmids, general culture conditions, chemicals, and statistical analyses

The *E. coli* strain BW25113 (wild-type, WT) (*lacA* *rrnB*_{T14} *lacZ*_{WJ16} *hsdR*₅₁₄ *araBAD*_{AH33} *rhaBAD*_{LD78}) [39] and Keio deletion mutant *rna::kan^R* (deficient in RNase I, *rna*) in the BW25113 strain background [40] were used for all studies (Supplementary Table S1 and Figure S14), unless specified otherwise. The pKT-CNP plasmid was generated by subcloning the catalytic domain (corresponding to the final 221 amino acid residues) of the *Rattus norvegicus* CNP gene [41] (UniProtKB-P13233; codon-optimized for *E. coli*; synthesized by GenScript) into the pKT vector [42] via double digest with restriction enzymes *Nde*I and *Spe*I, placing the gene under control of the TetA promoter (inducible with anhydrotetracycline). A catalytically-inactive variant of CNPase (H73L/H152L, numbering based on catalytic domain) [41] was generated via QuikChange mutagenesis (for primer sequences, see Supplementary Table S2). To construct plasmid pACYC-*noRBS-mRNA*, polymerase incomplete primer extension (PIPE) cloning was utilized [43]. To this end, the pACYCDuet-1 vector (EMD Millipore) was amplified by polymerase chain reaction (PCR) to delete both multiple cloning sites (including the ribosome binding site, T7 promoters, and T7 terminator). The 162-bp *noRBS-mRNA* insert containing its own T7 promoter and T7 terminator (54% GC, purchased as a gBlock fragment from Integrated DNA Technologies) was PCR amplified to install 5'- and 3'-terminal regions complementary to the pACYC vector PCR product for PIPE cloning into the vector (for detailed PIPE cloning procedure and insert sequence, see Supplementary Protocol S2). T7-mediated expression was required because genes lacking a ribosome binding site are poorly transcribed by *E. coli* RNA polymerase [44]. Plasmid pCA24N-*rna* was obtained from the ASKA collection [45]. For bacterial growth, isolated colonies from Luria Broth (LB)-agar plates were cultured overnight at 37°C with 225–240 rpm shaking in 3 mL of M9 minimal medium (supplemented with 0.4% glucose and 0.2% casamino acids), unless otherwise noted. The resulting starter culture then was inoculated 1:100 into 10 mL of the same medium in 50-mL Celltreat® conical tubes (sterile, polypropylene; lids left loose for gas exchange) and incubated under the aforementioned conditions, unless specified otherwise. Kanamycin, chloramphenicol, and carbenicillin were used at working concentrations of 25, 30, and 100 µg mL⁻¹, respectively. Prior to 2',3'-cNMP extraction, cells were harvested by centrifugation at 3000 *g* at 20°C for 10 min, frozen in liquid N₂, and stored at -80°C, unless otherwise noted. Analytical standards of adenosine 2',3'-cyclic monophosphate (2',3'-cAMP) and cytidine 2',3'-cyclic monophosphate (2',3'-cCMP) (monosodium salts) were purchased from Carbosynth (Berkshire, UK); standards of guanosine 2',3'-cyclic monophosphate monosodium salt (2',3'-cGMP), uridine 2',3'-cyclic monophosphate monosodium salt (2',3'-cUMP), cyclic dimeric-3':5'-guanosine monophosphate sodium salt (c-di-GMP), and 5'-phosphoguanylyl-3':5'-guanosine sodium salt (pGpG) were purchased from BioLog (Bremen, Germany). The sodium salt of 8-bromo adenosine 3',5'-cyclic

monophosphate (8-Br 3',5'-cAMP) was obtained from Sigma-Aldrich. Adenosine 3'-monophosphate (3'-AMP) was purchased from Sigma-Aldrich as the free acid. All data represent at least three biological replicates. Statistical significance was evaluated using a two-sample *t*-test, where equal or unequal variance was assessed via an *F*-test. Data were considered statistically-significant for $P < 0.05$.

Extraction of 2',3'-cNMPs

Aliquots (10-mL) were harvested from exponential-phase WT cultures ($OD_{600} \sim 0.4\text{--}0.6$) and stationary-phase cultures (16 or 24 h post-inoculation) by centrifugation. For 2',3'-cNMP extraction, frozen cell pellets were suspended in 500 μL of ice-cold acetonitrile/methanol/water (2/2/1, v/v/v), as previously described [14]. The cells were lysed by sonication on ice and subsequently centrifuged at 4°C at 3000 *g* for 10 min. The lysate was concentrated to dryness using a vacuum centrifuge and resuspended in 250 μL of sodium phosphate buffer (50 mM, pH 7.4) containing 0.5 μM 8-Br 3',5'-cAMP as internal standard. The extracts were centrifuged at 12000 *g* for 30 min at 4°C and transferred to an LC-MS autosampler vial.

Quantification of 2',3'-cNMPs

Quantification of 2',3'-cNMPs was performed via an internal standard (IS) method, using 8-Br 3',5'-cAMP as the IS. Calibration curves for 2',3'-cAMP, -cCMP, -cGMP, and -cUMP analytes were constructed by plotting the peak area ratio of 2',3'-cNMP/IS against the concentration ratio of cNMP/IS, as described previously [14]. 2',3'-cNMP concentrations were adjusted based on the recovery efficiency of each analyte (Supplementary Figure S1) and normalized to cell density. The concentration of IS was 0.5 μM in all samples for calibration. The concentrations of authentic 2',3'-cNMP analytes ranged from 0.02 – 20 μM (a range over which the analytical response remained linear). A linear regression model was used to generate the calibration curves. All nucleotide concentrations in stock solutions were determined via UV-Vis spectrophotometry (Cary Series, Agilent Technology, Santa Clara, CA, USA).

Extraction of c-di-GMP and pGpG

WT *E. coli* were cultured overnight (18 h) at room temperature without shaking to late-exponential/early stationary phase ($OD_{600} \sim 0.7\text{--}1$ A). The nucleotides were extracted essentially as described previously [46]. The protocol was performed analogously to the 2',3'-cNMP extraction described above, except that cell pellets were suspended in 0.5 mL of ice-cold sodium phosphate buffer (50 mM, pH 7.4) with 1 mM EDTA (0.05 mL of extraction buffer added per 1 mL of bacterial culture harvested).

Quantification of c-di-GMP and pGpG

Quantification of c-di-GMP and pGpG was performed using an IS method, in analogy to that detailed above for 2',3'-cNMP quantification. The concentration of IS was 0.1 μM in all samples for calibration. The concentrations of authentic c-di-GMP and pGpG analytes ranged from 0.0125 – 0.2 μM (a range over which the analytical response remained linear).

LC-MS/MS parameters

The LC-MS/MS methodology was performed as previously described [14], with minor modifications. A Thermo Electron LTQ-FTMS was employed for sample analysis. Chromatographic analysis was performed using a Shimadzu autosampler and a Dionex Ultimate 3000 dual gradient pump. LC-MS instrumentation was controlled by Xcalibur and DCMSlink software (Thermo Scientific). Samples were separated using a reversed-phase Leapsil C18 column (2.7 μm , 150 x 2.1 mm) (Dikma Technologies, Inc; Lake Forest, CA, USA). The mobile phase consisted of water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B). The flow rate was 0.3 mL/min and the following chromatography program was employed: 0% B from 0 to 4 min, then a gradient from 0 to 1.5% B from 4 to 15 min, followed by a gradient from 1.5 to 8% B over 15 to 20 min, followed by holding at 8% B from 20 to 25 min, then a gradient from 8 to 15% B from 25 to 28 min, followed by holding at 15% B from 28 to 35 min, and finally a gradient back to 0% B from 35 to 35.1 min. The column was re-equilibrated by holding at 0% B from 35.1 to 45 min. This chromatography method separates 2',3'-cNMPs from the 3',5'-cNMP regioisomers (Supplementary Figures S8-S11) [14]. The column was washed after analysis of every 2–4 extracts using the following chromatographic method: a gradient from 0 to 100% B from 0 to 2 min, followed by holding at 100% from 2 to 10 min, then a gradient from 0% to 100% C (acetonitrile) from 10 to 12 min, followed by holding at 100% C from 12 to 20 min, followed by a final gradient from 0% to 100% A over 20 to 25 min. The column was re-equilibrated to 100% A from 25 to 40 min. 2',3'-cNMPs were quantified via 10 to 30 μL injections; pGpG and c-di-GMP were quantified via a 45 μL injection. Electrospray ionization was performed in positive-ion mode in the LTQ-FTMS using a capillary voltage of 35 V, a 5 kV needle voltage, a capillary temperature of 275°C, and a 110 V tube lens voltage. Samples were detected in the ion trap using a 1 amu isolation window, and a normalized collision energy of 35 eV. An activation Q of 0.250 was used, with an activation time of 30 ms. Nucleotides were detected based on the protonated parent ions and quantified using the protonated nucleobase fragment ions (Supplementary Figure S12). Peaks were integrated using Xcalibur software (Thermo Fisher).

Quantification of 2',3'-cNMPs in *rna* expressing pCA24N-*rna*

Cultures of *rna* harboring plasmid pCA24N-*rna* were cultured to $\text{OD}_{600} \sim 0.1\text{--}0.2$ and subsequently induced by addition of 10 μM IPTG. Incubation was continued to $\text{OD}_{600} \sim 0.5\text{--}0.6$; the cells were harvested and the 2',3'-cNMPs were extracted and quantified, as described above.

Cytoplasm/periplasm fractionation

Separation of cytoplasmic and periplasmic fractions was performed according to a published procedure, and efficiency of the fractionation procedure was evaluated via SDS-PAGE analysis as described previously [47] (Supplementary Figure S2). Samples collected during exponential growth were resuspended in 100 μL TSE buffer (200 mM Tris-HCl pH 7.8, 500 mM sucrose, 1 mM EDTA). After incubation on ice for 30 min, the suspension was centrifuged at 14000 g at 4°C for 40 min. The supernatant (final periplasmic extract) was stored at -80°C until LC-MS/MS and the pellet (spheroplast) was stored at -80°C until 2',

3'-cNMP extraction. Spheroplasts were extracted in the same way as outlined above for cell pellets.

Addition of exogenous 2',3'-cAMP

WT cultures (60-mL) were grown to OD₆₀₀ ~0.4–0.5 A in 250-mL glass Erlenmeyer flasks. Each culture then was split into two equal portions (one for 0.1 mM 2',3'-cAMP addition and one for 0.1 mM 3'-AMP addition). 10-mL samples were collected 20 min after addition of the nucleotides for 2',3'-cNMP extraction.

Quantification of 2',3'-cNMP levels following growth +/- casamino acids

WT *E. coli* were cultured in 10 mL of either M9 minimal (0.4% glucose, 0.2% casamino acids) or M9 minimal (0.4% glucose, 1.2% casamino acids) in 50-mL Celltreat® conical tubes (sterile, polypropylene). Upon reaching OD₆₀₀ ~0.4–0.6, 10-mL samples were harvested for 2',3'-cNMP extraction.

Chloramphenicol-mediated induction of RNA degradation

WT cultures (50-mL) were grown to early exponential phase in 250-mL glass Erlenmeyer flasks and split into two equal portions. One portion was treated with 200 µg mL⁻¹ chloramphenicol [48], and the other portion was treated with an equal volume of ethanol as a control. After incubation for 30 min, 10 mL were harvested from all cultures by centrifugation.

Total RNA quantification +/- chloramphenicol treatment

BW25113 were cultured as described above for **Chloramphenicol-mediated inhibition of mRNA degradation**. From these cultures, 1-mL samples were collected pre-chloramphenicol treatment and 30 min post-treatment by centrifugation at 12000 *g* at 24°C for 5 min to quantify total intracellular RNA via the *RNAsnap*TM procedure [49]. The cell pellets were suspended in 300 µL of *RNAsnap*TM extraction solution (95% formamide, 18 mM EDTA, 0.025% SDS, 1% β-mercaptoethanol) and incubated for 7 min in a 95°C sand bath. The samples were centrifuged at 14000 *g* at 24°C for 10 min and the 260 nm absorbance (A₂₆₀) of the supernatant was quantified using a NanoDropTM 1000. The total RNA concentration was calculated from the A₂₆₀ using an extinction coefficient of 0.025 µg mL⁻¹ cm⁻¹ and normalized to the OD₆₀₀- and volume-dependent cell density of each sample [50].

λDE3 lysogenization of BW25113 WT

The WT BW25113 strain was lysogenized using the λDE3 Lysogenization Kit (Novagen) according to the manufacturer's instructions.

Overexpression of non-translatable mRNA pACYC-*noRBS-mRNA*

BW25113 (DE3) and BW25113 (DE3) harboring plasmid pACYC-*noRBS-mRNA* were cultured to early exponential phase (OD₆₀₀ ~0.2–0.3). All cultures then were treated with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce expression of the non-

translatable mRNA. Upon reaching an OD₆₀₀ of 0.5–0.6, the cultures were harvested by centrifugation.

2',3'-cNMP quantification in shaking and static cultures

WT cultures (10-mL) were grown in 50-mL Celltreat® conical tubes (sterile, polypropylene) at either 37°C with 225 rpm shaking to mid-logarithmic phase (OD₆₀₀ ~0.4–0.6) or at room temperature without shaking overnight to allow biofilm formation. Cells were harvested from 9 mL of culture and lysed for 2',3'-cNMP quantification as detailed above. Biofilm formation was qualitatively confirmed in the static cultures by crystal violet staining, in analogy to a published procedure [51].

Assessment of metabolic state in shaking and static cultures

WT *E. coli* were grown in 100 µL cultures in a 96-well microplate (Corning Costar, sterile, non-treated, polystyrene). One set of cultures was incubated at 37°C with shaking to exponential phase, while the other set was incubated at room temperature without shaking for 24 h. The metabolic state then was assessed using the XTT Cell Proliferation Kit II (Roche) according to the manufacturer's protocol with minor modification. Upon reaching the desired cell density, the XTT labeling mixture (50 µL) was added to each culture and the 490 nm absorbance was immediately recorded using a microplate reader. The A₄₉₀ was normalized to cell density using the OD₆₀₀ of each culture.

Biofilm quantification by Congo Red staining

Congo Red assays were conducted as previously described [52]. Individual colonies of BW25113 WT and *rna* from LB-agar plates were inoculated into 5 mL LB and cultured overnight in 15-mL plastic culture tubes. In addition, WT *E. coli* harboring plasmid pKT-*CNP* or inactive control pKT-*CNP-inact* were cultured in the same way. The overnights were inoculated 1:50 into 7 mL of YESCA (1% casamino acids, 0.12% yeast extract) containing 0.0025% Congo red in 50-mL Celltreat® conical tubes (sterile, polypropylene) (lids left loose for gas exchange). After reaching an OD₆₀₀ ~0.3–0.4, 1 mL of each culture was transferred to a 1.6-mL Eppendorf tube and either treated with vehicle or with 25 ng mL⁻¹ anhydrotetracycline (AHT) to induce expression. The cultures were incubated for 48 h at room temperature without shaking (lids left open and tubes were loosely covered in plastic wrap and foil). For biofilm quantification, samples were centrifuged at 12000 *g* for 15 min and 200 µL of supernatant were transferred to a 96-well microplate (Corning Costar; sterile, non-treated, polystyrene). The absorbance at 500 nm was recorded using a microplate reader. For normalization, each culture was disturbed by pipetting and 200 µL were transferred to a 96-well microplate prior to recording the OD₆₀₀ using a microplate reader.

Biofilm quantification by crystal violet staining

Cultures of WT and *rna* (2-mL) were incubated in 24-well Corning Costar microplates (sterile, non-treated, polystyrene) for 24 h at room temperature without shaking. Biofilm formation was quantified by crystal violet staining according to a published procedure with minor modification [51]. Non-adherent cells were poured out and the microplate was gently submerged twice in a beaker of water. A 0.1% aqueous solution of crystal violet (2.5 mL)

was added to each well and the microplate was incubated at room temperature for 15 min. The crystal violet solution was poured out and the microplate was gently submerged three times in a beaker of water to remove residual crystal violet (blotting the plate on a stack of paper towels after each wash). The plate was allowed to dry overnight at room temperature. The crystal violet in each well was dissolved by addition of 3 mL of 30% aqueous acetic acid, and the 570 nm absorbance was measured using a microplate reader and normalized to CFU (quantified by drop plating, according to published procedure [53]).

Quantification of biofilm-related gene transcript levels

Analysis of mRNA transcript levels for genes related to *E. coli* biofilm formation were quantified by the Emory Integrated Genomics Core and analyzed by the Emory Integrated Computational Core. Six *E. coli* pellets (three biological replicates of WT and three of *rna*) were submitted for extraction and expression profiling on the Affymetrix *E. coli* Genome 2.0 Array. RNA was extracted using Qiagen miRNEasy kit w/ on column DNase. Cells were lysed using 700 μ L Qiazol + 100 mg acid-washed beads (150–600 μ m) on the Qiagen tissue lyser at 30 Hz for 5 min. RNA was eluted in 30 μ L nuclease free water. 1 μ L was used to determine OD values on a Nanodrop 1000. 1 μ L was used to assess sample profiles on the Agilent 2100 using the RNA 6000 Nano assay.

Whole-Transcript Expression Analysis (Gene ST Arrays) was performed as follows. 10 ng of RNA was processed according to the GeneChip® WT Pico Reagent Kit protocol. Labeled cDNA was hybridized to the *E. coli* Genome 2.0 microarray for 16–18 hours at 45°C. Hybridized microarrays were washed and stained on an Affymetrix GeneChip 450 fluidics station using the appropriate chip dependent fluidics script. Intensity data was extracted using an Affymetrix 7G scanner and the Command Console software suite.

The obtained expression data from the microarray experiment were analyzed using ‘limma’ package in R/Bioconductor (<http://www.r-project.org>). The raw data were log₂ transformed, and Robust Multi-array Average (RMA) normalized to normalize the intensity data between the samples. The differentially expressed genes were identified on the basis of Benjamini-Hochberg (BH) multiple test adjusted *P* values (i.e. FDR) and fold changes (the increase in number of gene copies). Genes with an FDR value <0.05 and log₂ fold change ≥ 1.0 were considered significantly differentially expressed. Heat maps were created on the z-score-normalized probe signal using the R/Bioconductor function ‘hclust’ from the ‘heatmap3’ package. PCA was done using the R/Bioconductor function ‘princomp’ also applied to z-score normalized expression data.

Gene expression data obtained from the microarray experiment have been submitted to ArrayExpress at EMBL-EBI (<http://www.ebi.ac.uk/arrayexpress/>) under accession number E-MTAB-6095.

Quantitative reverse transcription PCR (RT-qPCR) analysis

Total RNA was extracted using the Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine, CA; Cat. no. R2050) according to the manufacturer’s instructions with optional on-column DNase treatment. Subsequently, 1 mg of total RNA was used as template to synthesize cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster

City, CA; Cat. no. 4368814). Primers for all assays were designed using Primer 3 [54] (also see <http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>). For primer sequences, see Supplementary Table S6. Melting curve analysis was performed to insure single-product amplification for all primer pairs. Real time PCR was performed on the BioRad CFX384 Real Time System (BioRad, Hercules, CA) using assays specific to the genes of interest. Each reaction well contained 5 μ L of PowerUpTM SYBR Green Master Mix (Applied Biosystems; Cat. no. A25742), cDNA equivalent to 20 ng of total RNA and 250 nM each of forward and reverse amplification primers in a final reaction volume of 10 μ L. Cycling conditions were as follows: 95°C for 10 minutes for polymerase activation, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data analysis was performed using CFX Manager software from BioRad, version 3.1. The experimental C_q (cycle quantification) was calibrated against the endogenous control products DNA-directed RNA polymerase subunit beta (*rpoC*). Samples were analyzed for relative gene expression by the DDCT method [55].

Results

2',3'-cNMP levels fluctuate during *E. coli* growth

Physiological concentrations of 2',3'-cAMP, -cCMP, -cGMP, and -cUMP were quantified in wild-type (WT) *E. coli* BW25113 [39] during growth in M9 minimal medium using a sensitive LC-MS/MS-based protocol [14]. In exponentially growing *E. coli* BW25113 cultures, the various 2',3'-cNMPs exist at intracellular concentrations of approximately 10–30 μ M (Figure 1). After 16 h of growth, the four 2',3'-cNMP concentrations fall to undetectable levels (limit of detection [LOD] of the LC-MS/MS assay is approximately 150–500 fmol for the different 2',3'-cNMPs [14]). Intriguingly, the 2',3'-cNMP concentrations then increase in 24 h-old cultures to nanomolar levels, approximately 40–240 fold lower than the exponential-phase concentrations, depending on the particular nucleotide (Figure 1). *E. coli* also exhibit distinct relative concentrations of the different 2',3'-cNMPs, maintaining 2-fold and 5-fold higher concentrations of the 2',3'-cyclic purines relative to the pyrimidines in exponential phase and stationary phase (24 h-old) cultures, respectively (Figure 1). These data demonstrate that relative and absolute 2',3'-cNMP concentrations are regulated during *E. coli* growth (Figure 1).

RNase I generates 2',3'-cNMPs *in vivo* from RNA degradation

To identify the enzyme(s) involved in 2',3'-cNMP production, cyclic nucleotide levels were quantified in an *E. coli* strain deficient in RNase I (BW25113 *rna*) [40], as this enzyme generates 2',3'-cNMP monomers *in vitro* from short (~2–12-nt), unstructured oligoribonucleotides [23]. The results demonstrate that essentially all 2',3'-cNMPs produced during exponential and stationary phase growth arise from RNase I activity (Figure 2A and Supplementary Figure S11), as 2',3'-cNMP levels in the *rna* strain were below the limit of detection. To solidify the role of RNase I in 2',3'-cNMP production, the *rna* strain was transformed with a plasmid encoding the *rna* gene, which restored 2',3'-cNMP production (Supplementary Figure S7).

Although the *ma* gene encoding RNase I contains a periplasmic localization sequence [27], previous reports of a cytoplasmic RNase I variant encoded by the same gene [28, 56] necessitated separate quantification of periplasmic and cytoplasmic 2',3'-cNMP concentrations to determine the cellular localization of 2',3'-cNMPs. Importantly, 2',3'-cNMPs exist in both the periplasm and the cytoplasm, demonstrating that cytoplasmic RNase I degrades cytosolic RNA (Figure 2B). To probe the possibility that elevated 2',3'-cNMPs arise due to import following periplasmic degradation of extracellular RNA by RNase I, 0.1 mM exogenous 2',3'-cAMP was added to exponentially-growing cultures of the wild-type BW25113 strain in a separate experiment. This addition amounts to approximately 20,000 pmol exogenous 2',3'-cAMP added per 1×10^8 cells - over 4000-fold greater than the typical physiological 2',3'-cAMP concentration of ca. 5 pmol per 1×10^8 cells in exponential phase (Figure 1). 2',3'-cAMP levels were unaffected by exogenous nucleotide addition, showing no change compared to cultures treated with exogenous 0.1 mM 3'-AMP as a control (Figure 3A), further supporting that 2',3'-cNMPs are generated from RNA degradation in the cytoplasm.

Currently, the biological role of RNase I is unknown, but the nuclease is not essential for growth in *E. coli* [56]. Based on *in vitro* studies, cytoplasmic RNase I has been proposed to complete the catabolism of short oligoribonucleotides generated from mRNA degradation *in vivo*, but this function remains speculative [28]. In accord with the inability to degrade structured RNA substrates *in vitro*, RNase I is not involved in the initial inactivation of mRNA, as previously determined by quantifying the half-life of functional β -galactosidase transcript [57]. However, the function of the enzyme in the downstream degradation of short mRNA fragments resulting from transcript inactivation has not been investigated experimentally. To this end, mRNA degradation was perturbed by amino acid starvation and by overexpression of a non-translatable mRNA to probe the effect on 2',3'-cNMP levels. The role of RNase I in the degradation of rRNA also was investigated by chloramphenicol-induced ribosome turnover. Amino acid starvation has been demonstrated to induce expression of a number of *Escherichia coli* toxin-antitoxin systems, including RelE and MazF, that cleave mRNA [58, 59]. Moreover, *E. coli* lacking endoribonuclease toxin RelA display dysregulated activation of amino acid biosynthetic genes in the wake of nutrient deprivation, demonstrating the importance of toxin-antitoxin systems in responding to amino acid limitation [60]. Therefore, if 2',3'-cNMPs are formed during mRNA degradation, 2',3'-cNMP levels should be dependent on the presence of amino acids in the growth media. Indeed, *E. coli* BW25113 grown in minimal media with 1.2% casamino acids exhibit markedly lower concentrations of 2',3'-cNMPs than the same strain grown in minimal media with 0.2% casamino acids (Figure 3B).

To further solidify a function for RNase I in mRNA degradation, a plasmid-borne gene lacking a ribosome-binding site (pACYC-*noRBS-mRNA*) was overexpressed to increase the intracellular mRNA concentration, with the expectation that 2',3'-cNMP levels would increase upon expression of the mRNA substrate. In accord with the hypothesis, *E. coli* cultures expressing the non-translatable mRNA displayed ~2–2.7-fold higher 2',3'-cNMP levels compared to control cultures lacking the plasmid, providing additional validation that 2',3'-cNMPs arise from RNase I-mediated degradation of mRNA (Figure 3C). Collectively, these experiments identify a novel role for RNase I in mRNA catabolism.

The role of RNase I in ribosome decay was interrogated by treating WT *E. coli* cultures with chloramphenicol to stall translation and concomitantly increase rRNA turnover [48, 61]. Cultures treated with chloramphenicol displayed higher 2',3'-cNMP levels relative to concentrations in the ethanol-treated control cultures (Figure 3D), demonstrating that the increased 2',3'-cNMP levels arise from rRNA degradation. Moreover, a control experiment confirmed that chloramphenicol treatment altered the total RNA concentration, as expected (Supplementary Figure S6). These studies provide evidence that cytoplasmic RNase I is involved in degradation of mRNA and rRNA to yield 2',3'-cNMPs. Additional work is underway to determine whether RNase I also degrades tRNA.

RNase I modulates biofilm formation

Several reports have established intriguing links between nucleoside/nucleotide pools and bacterial biofilms [9–13], which are microbial communities of aggregated cells growing in an extracellular matrix of polysaccharides, nucleic acids, and other biopolymers [62]. Therefore, the roles of RNase I and 2',3'-cNMPs in biofilm formation were interrogated. Levels of 2',3'-cNMPs for *E. coli* BW25113 WT cells grown in shaking *versus* static culture first were investigated. Significant differences in 2',3'-cNMP levels were observed; quantification yielded approximately 15-fold lower levels of all 2',3'-cNMPs for cells grown in static, biofilm-forming cultures, as compared to shaking cultures (Figure 4A). To test the possibility that the decreased 2',3'-cNMP levels observed in sessile culture were simply a result of reduced metabolism compared to planktonic cells, the metabolic state of the cultures was assessed via a colorimetric tetrazolium-based assay. Although the static/20°C cultures exhibited approximately 1.3-fold decreased metabolism relative to the shaking/37°C cultures (Supplementary Figure S3), the metabolic difference is not sufficient to explain the 15-fold lower 2',3'-cNMP concentrations in the static cultures (Figure 4A). Furthermore, compared to WT BW25113, which is known to form a relatively poor biofilm [63], biofilm formation increased over 10-fold in the *rna* strain (Figure 4B and Supplementary Figure S5), which does not have observable levels of 2',3'-cNMPs. These results demonstrate that 2',3'-cNMP concentrations are correlated with biofilm formation, with sessile cells having low levels of 2',3'-cNMPs.

One possible explanation for the dysregulated biofilm formation in the *rna* mutant is aberrant c-di-GMP signaling. Previous work with *Pseudomonas aeruginosa* demonstrated that deletion of oligoRNase, which degrades 2–5-nucleotide RNAs, increases the concentration of 5'-phosphoguananylyl-3':5'-guanosine (pGpG), the immediate degradation product of the important biofilm regulator c-di-GMP [64, 65]. Accumulation of pGpG inhibits c-di-GMP-specific phosphodiesterases, thereby increasing the c-di-GMP concentration in *P. aeruginosa* lacking oligoRNase, resulting in upregulated biofilm production [64, 65]. However, *E. coli* contains both oligoRNase and RNase I [66], while *P. aeruginosa* lacks a known homolog of RNase I. Due to the similar capability of oligoRNase and RNase I to hydrolyze short oligoRNAs *in vitro* [28, 67], the effect of RNase I deletion on intracellular pGpG and c-di-GMP levels in *E. coli* was investigated. Intriguingly, neither c-di-GMP nor pGpG levels were altered in *rna* relative to WT *E. coli* (Figure 4C), suggesting alternative c-di-GMP-independent mechanisms for RNase I and/or 2',3'-cNMPs in modulating biofilm formation in this bacterium.

To independently investigate the role of RNase I *versus* the role of 2',3'-cNMPs in biofilm formation, the catalytic domain of a mammalian 2',3'-cyclic nucleotide phosphodiesterase (CNPase, UniProtKB-P1323) [68] was developed as an inducible tool to hydrolyze 2',3'-cNMPs in WT *E. coli* expressing RNase I. WT cells harboring plasmid pKT-*CNP* or inactive variant pKT-*CNP-inact* as a control were assayed for biofilm formation via Congo red assay. Biofilm formation increased in WT cultures expressing CNPase relative to control cultures expressing the inactive CNPase variant, both in the presence of the inducer (anhydrotetracycline, AHT) and under basal expression conditions in the absence of AHT (Figure 4B), thus demonstrating a functional link between 2',3'-cNMPs and biofilm formation. Importantly, expression of active CNPase in sessile cultures decreased levels of the 2',3'-cyclic purine nucleotides below the quantification limit of the LC-MS/MS assay, while reducing concentrations of 2',3'-cCMP and -cUMP 25-fold and 14-fold, respectively, compared to levels in cells expressing the inactive CNPase control (Supplementary Figure S4). These results further indicate that decreasing 2',3'-cNMP levels upregulates biofilm formation.

Curli production is upregulated in the *rna* mutant

To provide mechanistic insight into the hyper-biofilm phenotype of the RNase I-deficient strain, which lacks 2',3'-cNMPs, the effect of *rna* deletion on transcript levels of biofilm-associated genes was investigated. Analysis of the transcriptome indicated 1.5-fold higher expression of curli structural gene *csgB* and 1.8-fold increased expression of curli accessory gene *csgC* in *rna* compared to WT *E. coli* (Figure 5). Thus, the upregulated biofilm production in the mutant strain is due, at least in part, to increased curli synthesis. Curiously, *rna* displays decreased expression of the divergently transcribed *csgDEFG* operon, which activates *csgBAC* transcription (CsgD) and regulates curli transport and assembly (CsgEFG) (Figure 5) [69]. Upregulated expression of curli genes is consistent with the increased Congo red staining observed in the *rna* mutant and in WT cells expressing active CNPase (Figure 4B), as Congo red primarily binds to amyloid curli fibers and cellulose [70]. Notably, decreased expression of the *pgaABCD* locus in *rna* indicates that elevated poly-*N*-acetyl- β -1,6-D-glucosamine (PNAG) production is not contributing to the hyper-biofilm phenotype [71, 72]. To validate the surprising downregulated expression of the PNAG biosynthetic operon, quantitative reverse transcription PCR (RT-qPCR) was performed to quantify abundance of the *pgaA* transcript. The *rna* mutant displayed reduced *pgaA* expression, further confirming that increased PNAG synthesis is not responsible for hyper-biofilm production in RNase I-deficient *E. coli* (Supplementary Table S5).

Discussion

Absolute and relative nucleotide concentrations are maintained by elaborate regulation of *de novo* synthesis and salvage pathways. These processes are vital not only in primary metabolism, but also in the coordination of specialized signal transduction cascades which rely on nucleotide second messengers. The present work demonstrates that 2',3'-cNMP concentrations are regulated over the *Escherichia coli* growth curve and are generated by RNase I-catalyzed degradation of mRNA and rRNA (Figures 1, 2, and 3), presumably, based on the inability of RNase I to digest structured RNA substrates [28], as one of the final steps

in RNA catabolism. RNase I homologs exist in several classes within Proteobacteria, indicating that 2',3'-cNMPs likely govern certain biological processes in other bacterial taxa. In addition, genes encoding other RNase T2 superfamily enzymes are conserved across bacteria, eukaryotes, and viruses [73], alluding to possible 2',3'-cNMP-dependent pathways in diverse kingdoms of life. The present results suggest that 2',3'-cNMP pools constitute a previously unknown facet of primary nucleotide metabolism and/or a novel nucleotide second messenger signaling system. 2',3'-cNMPs and the corresponding 3'-NMPs resulting from enzymatic hydrolysis possibly function as intermediates in a novel salvage pathway, as the nonspecific nucleotidase SurE in the cytoplasm accepts 3'-NMPs as substrates [74]. Analysis of previously published NTP, NDP, NMP, and nucleoside concentrations in *E. coli* fails to suggest many obvious parallels between the 2',3'-cNMP ratio and other nucleotide/nucleoside pools [1, 75]. However, the finding that 2',3'-cNMP levels decrease in stationary phase *E. coli* cultures relative to exponential phase cultures mirrors the previously observed growth-dependent fluctuation in dNTP concentrations [1] (Figure 1). The present study also reveals an increased concentration of 2',3'-cAMP and 2',3'-cGMP compared to 2',3'-cCMP, and -cUMP in exponential and stationary phase cultures (Figure 1). The elevated 2',3'-cAMP level could be due to poly-A polymerase (PAPase) activity, as 3'-polyadenylation of mRNA facilitates exonucleolytic degradation in bacteria [33]. The different 2',3'-cNMP concentration ratios observed in exponential and stationary phase *E. coli* cultures (Figure 1) cannot be explained by preferential activity of RNase I because the enzyme does not display strong sequence or nucleobase specificity *in vitro* [28]. These results allude to a complex regulation of 2',3'-cNMP metabolism, which likely intersects with processes governing other nucleotide levels. Understanding the regulation of 2',3'-cNMP concentrations will require further investigation of growth-dependent relationships between 2',3'-cNMP, 3'-NMP, and other nucleotide/nucleoside concentrations.

Recent studies indicate that changes in nucleoside and nucleotide metabolism can alter biofilm formation [9–13], demonstrating the importance of exploring 2',3'-cNMP pools in the context of overall nucleotide metabolism. This work demonstrates that decreasing 2',3'-cNMP levels increases biofilm formation in *E. coli* due to upregulated production of curli, the major protein constituent of biofilms (Figures 4B and 5, Supplementary Figure S5). Intriguingly, pyrimidine auxotrophy impairs synthesis of curli fibers, and conditions favoring UMP synthesis via pyrimidine salvage, as opposed to *de novo* UMP biosynthesis, also modify the biofilm matrix by increasing cellulose production [9]. In addition, the pyrimidine antimetabolite cancer drug 5-fluorouracil inhibits *E. coli* biofilm formation by up-regulating expression of AriR, a transcriptional repressor of biofilm-related genes [11]. Though some of the effectors involved in these processes have been identified in certain bacterial species, such as the CytR transcription factor in *Vibrio cholera* that de-represses pyrimidine metabolic genes in response to cytidine [12], additional mechanistic details of the pathways connecting nucleoside/nucleotide levels to biofilm formation remain elusive. Notably, published data have shown that CytR is not involved in modulating pyrimidine-dependent biofilm phenotypes in *E. coli* [9], suggesting that additional unknown factors mediate this process in certain species. These findings suggest that disrupting normal 2',3'-cNMP regulation may alter biofilm formation by perturbing primary nucleotide/nucleoside metabolism, perhaps ultimately impacting c-di-GMP signaling. Although the total c-di-GMP

concentration does not differ between WT and *rna* *E. coli* (Figure 4C), it remains possible that cells lacking RNase I and 2',3'-cNMPs exhibit dysregulated levels of spatially isolated c-di-GMP pools, as local concentrations of this second messenger mediate biofilm formation [76]. Alternatively, the aberrant biofilm phenotype observed in *rna* and in WT *E. coli* expressing CNPase (Figure 4B) potentially could be elicited by a novel second messenger signaling pathway mediated directly by 2',3'-cNMPs, as the micromolar 2',3'-cNMP concentrations in exponential phase *E. coli* cultures are similar to the basal level of 3',5'-cAMP [75, 77], a canonical second messenger.

Ongoing work seeks to investigate potential 2',3'-cNMP-mediated signal transduction and elucidate the roles of the different 2',3'-cNMPs in modulating bacterial phenotypes. Gene expression data reported herein demonstrate that *E. coli* lacking RNase I exhibit aberrant expression of several transcripts relevant to biofilm production (Figure 5 and Supplementary Table S4). Published phenotypic and deep sequencing investigations using *E. coli* deficient in RNase II, PNPase, or RNase R have linked these processive exoribonucleases to biofilm formation via perturbation of biofilm-associated transcripts [78, 79]. Although the mechanistic intricacies of the uniquely altered transcriptome in these different RNase mutants remain ambiguous, RNase II, PNPase, and RNase R directly impact transcript half-life [80, 81], suggesting that altered mRNA decay is directly influencing the biofilm phenotype. Conversely, previous work has shown that *rna* deletion does not directly affect transcript stability [57], and the present study demonstrates that RNase I deletion does not perturb global c-di-GMP or pGpG levels (Figure 4C). These data allude to a more complex regulatory mechanism involving 2',3'-cNMPs, which is further demonstrated by the finding that inducing hydrolysis of 2',3'-cNMPs upregulates biofilm formation in WT cells expressing RNase I (Figure 4B).

The present study quantifies 2',3'-cNMPs in *E. coli*, demonstrates that RNase I generates 2',3'-cNMPs via mRNA and rRNA degradation, and identifies a role for 2',3'-cNMPs in regulating biofilm formation. The identification of RNase I as the enzyme responsible for generating 2',3'-cNMP pools provides the first insights into the phenotypic consequences of aberrant 2',3'-cNMP concentrations and RNase I levels on biofilm formation in bacteria. Additional experiments are in progress to elucidate the mechanisms that control the relative concentration ratios of 2',3'-cNMP pools and the link to biofilm formation in *E. coli* and in other bacterial taxa. Given the importance of biofilm formation in virulence and pathogenesis of numerous bacterial species [5, 7], elucidation of the mechanism(s) by which RNase I and 2',3'-cNMPs alter biofilm formation will provide insight into new methods to alter bacterial phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2',3'-cNMP	2',3'-cyclic nucleotide monophosphate
RNase I	ribonuclease I
3',5'-cAMP	adenosine 3',5'-cyclic monophosphate
c-di-GMP	cyclic dimeric-3':5'-guanosine monophosphate
(p)ppGpp	guanosine 3'-diphosphate, 5'-(tri)diphosphate
oligoRNase	oligoribonuclease
oligoRNAs	oligoribonucleotides
PNPase	polynucleotide phosphorylase
LC-MS/MS	liquid chromatography tandem mass spectrometry
pGpG	5'-phosphoguanylyl-3':5'-guanosine
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase

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Figure 1. Growth phase-dependent 2',3'-cNMP concentrations in *E. coli*.

Concentrations of 2',3'-cCMP, -cAMP, -cGMP, and -cUMP in *E. coli* BW25113 WT quantified via LC-MS/MS during mid-exponential (exp) and stationary phase growth (16 h and 24 h post-inoculation) in M9 minimal medium (0.4% glucose, 0.2% casamino acids). 2', 3'-cNMPs were below the limit of detection (LOD) in the 16 h cultures. Unless otherwise specified, data are the mean of at least three biological replicates, error bars denote standard deviation. All differences between exponential and stationary phase measurements and between purine and pyrimidine concentrations are statistically significant ($P < 0.05$). All subsequent 2',3'-cNMP quantifications were performed in exponentially growing cultures in M9 minimal media (supplemented with 0.4% glucose and 0.2% casamino acids), unless otherwise noted.

Figure 2. 2',3'-cNMPs are produced by RNase I and accumulate in both the cytoplasm and periplasm.

(A) Sum of 2',3'-cAMP, -cCMP, cGMP, and cUMP concentrations in the total cell extract of WT and *rna* during mid-exponential and late stationary phase growth (24 h post-inoculation). The *rna* mutant did not contain detectable 2',3'-cNMP levels (LOD ca. 150–500 fmol for the different 2',3'-cNMPs). **(B)** 2',3'-cNMP concentrations quantified in WT *E. coli* from total cell extract, cytoplasmic extract, and periplasmic extract.

Fig 3. 2',3'-cNMPs are generated through RNA degradation in the cytoplasm.

(A) 2',3'-cNMP levels in BW25113 WT cultures following addition of 0.1 mM exogenous 2',3'-cAMP or 0.1 mM exogenous 3'-AMP control quantified 20 min after nucleotide addition. (B) Effect of amino acid starvation on 2',3'-cNMP concentrations in BW25113 WT. BW25113 WT were cultured either in M9 minimal (0.4% glucose, 0.2% casamino acids [CA]) or in the same medium containing 1.2% casamino acids and the 2',3'-cNMPs were quantified. (C) 2',3'-cNMP levels increase upon expression of a non-translatable mRNA. 2',3'-cNMPs were quantified in BW25113 (DE3) overexpressing a non-translatable transcript lacking a ribosome binding site (pACYC-*noRBS-mRNA*) and compared to levels in BW25113 (DE3) lacking the construct. (D) Addition of chloramphenicol induces rRNA cycling and increases 2',3'-cNMP levels. 2',3'-cNMPs were quantified in BW25113 following treatment with either chloramphenicol (cam) or ethanol as a control. All fold-changes reported (B, C, D) are statistically significant ($P < 0.05$). Graphs of all quantified 2',3'-cNMP levels can be found in Supplementary Figure S13.

Fig 4. RNase I and 2',3'-cNMPs regulate biofilm formation.

(A) 2',3'-cNMP levels are decreased in static *E. coli* BW25113 cultures relative to shaking cultures. BW25113 WT were cultured either at 37°C with shaking to exponential phase or at 20°C without shaking for 24 h prior to 2',3'-cNMP quantification. All fold-changes are statistically significant ($P < 0.05$). (B) Elevated biofilm production in the *rna* mutant and in WT cultures overexpressing a 2',3'-cyclic nucleotide phosphodiesterase (CNPase) relative to control. The biofilm formation of WT cultures harboring plasmid pKT-CNP or inactive variant pKT-CNP-inact as a control was quantified by Congo red staining after 48 h of static growth at 20°C in the presence or absence of 25 ng mL⁻¹ anhydrotetracycline (AHT) and compared to biofilm production in the WT and *rna* strains. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (C) Cyclic-di-GMP and pGpG levels quantified in *E. coli* BW25113 WT and *rna* strains grown in static cultures at 20°C for 18 h in M9 minimal (0.4% glucose, 0.2% casamino acids).

Figure 5. Deletion of *rna* alters expression of biofilm-related genes.

Heatmap of expression levels of biofilm-related genes for *E. coli* BW25113 WT and *rna* strains. Heatmap was created using Heatmapper [82]. Raw gene expression data can be found in Supplementary Table S4.