

# Increased 3'-Phosphoadenosine-5'-phosphosulfate Levels in Engineered *Escherichia coli* Cell Lysate Facilitate the In Vitro Synthesis of Chondroitin Sulfate A

Abinaya Badri, Asher Williams, Ke Xia, Robert J. Linhardt, and Mattheos A. G. Koffas\*

Chondroitin sulfates (CSs) are linear glycosaminoglycans that have important applications in the medical and food industries. Engineering bacteria for the microbial production of CS will facilitate a one-step, scalable production with good control over sulfation levels and positions in contrast to extraction from animal sources. To achieve this goal, *Escherichia coli* (*E. coli*) is engineered in this study using traditional metabolic engineering approaches to accumulate 3'-phosphoadenosine-5'-phosphosulfate (PAPS), the universal sulfate donor. PAPS is one of the least-explored components required for the biosynthesis of CS. The resulting engineered *E. coli* strain shows an  $\approx 1000$ -fold increase in intracellular PAPS concentrations. This study also reports, for the first time, in vitro biotransformation of CS using PAPS, chondroitin, and chondroitin-4-sulfotransferase (C4ST), all synthesized from different engineered *E. coli* strains. A 10.4-fold increase is observed in the amount of CS produced by biotransformation by employing PAPS from the engineered PAPS-accumulating strain. The data from the biotransformation experiments also help evaluate the reaction components that need improved production to achieve a one-step microbial synthesis of CS. This will provide a new platform to produce CS.

## 1. Introduction

Chondroitin sulfates (CSs) belong to an important class of linear sulfated polysaccharides known as glycosaminoglycans (GAGs) that are widely used as nutraceuticals and pharmaceuticals.<sup>[1]</sup> These polysaccharides are composed of alternating repeating units of glucuronic acid and *N*-acetyl galactosamine. Depending on the carbon position at which the *O*-sulfate moiety is present, CS is classified into further subtypes: sulfation at positions 4, 6, 2 and 6, and 4 and 6 results in CS-A, CS-C, CS-D, and CS-E, respectively. A detailed account of the structure and types of CSs in comparison to other GAGs can be found elsewhere.<sup>[2]</sup> The role of CSs in treating arthritis and related conditions is well studied.<sup>[3]</sup> More recently, specific cellular functions of CS oligosaccharides have been uncovered.<sup>[4–6]</sup> Apart from commercial applications, both the CS polysaccharides as well as CS-derived oligosaccharides find much use in research today.

Commercially available CS is extracted from bovine/porcine/chicken cartilage which are waste products in slaughterhouse processing.<sup>[7]</sup> The extraction of CS does not compete with the use of bovine tissues in the food industry. However, the variation in the sulfation position and degree of sulfation between CS derived from different tissues, species, and even individual animals make it hard to control the chemistry of extracted CS.<sup>[8,9]</sup> Apart from this, several other factors including the possibility of contamination with animal viruses, prions, and adulteration restrain the use of CSs in the food and medical industries.<sup>[10]</sup> A single-cell system that synthesizes CS from glucose and other common media components would improve the controllability, safety, and scalability of this product.

Three major components are required to make CS: 1) chondroitin; 2) sulfotransferase enzymes; and 3) sulfate donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS). The current state-of-art workflow for animal-free synthesis is in vitro chemoenzymatic biotransformation of purified microbial chondroitin to CS using purified sulfotransferase and a pure, commercial PAPS reagent.<sup>[11,12]</sup> Compared to a theoretical one-pot, microbial biosynthesis of CS from glucose using metabolic engineering, this current biotransformation method is still very complicated, expensive, and only moderately scalable.<sup>[10]</sup>

A. Badri, A. Williams, R. J. Linhardt, Prof. M. A. G. Koffas  
Howard P. Isermann Department of Chemical and Biological  
Engineering  
Rensselaer Polytechnic Institute  
110 8th St. CBIS 4005C  
Troy, NY 12180, USA  
E-mail: koffam@rpi.edu

K. Xia, R. J. Linhardt, Prof. M. A. G. Koffas  
Center for Biotechnology and Interdisciplinary Studies  
Rensselaer Polytechnic Institute  
110 8th St. CBIS 4005C  
Troy, NY 12180, USA

R. J. Linhardt, Prof. M. A. G. Koffas  
Department of Biology  
Rensselaer Polytechnic Institute  
110 8th St. CBIS 4005C  
Troy, NY 12180, USA

R. J. Linhardt  
Department of Chemistry and Chemical Biology  
Rensselaer Polytechnic Institute  
110 8th St. CBIS 4005C  
Troy, NY 12180, USA

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A single-cell biosynthetic system might rely on a eukaryotic or a bacterial cell. While CS is part of the extracellular matrix in most animal tissues,<sup>[13,14]</sup> the manipulation, maintenance, and growth of animal cell lines are more difficult and expensive than prokaryotes. However, prokaryotes are not known to synthesize sulfated GAGs.<sup>[15]</sup> Moreover, prokaryotes that synthesize other sulfated polysaccharides are not easily amenable to the state-of-art metabolic engineering and synthetic biology techniques.<sup>[16–19]</sup> Hence, they cannot be easily engineered to prepare sulfated GAGs, such as CSs.

In this article, we focus on getting a step closer to preparing CS from simple sugars like glucose in an *Escherichia coli* cell-based system. Previous reports show that chondroitin can be produced using *E. coli* K4 and engineered BL21 strains.<sup>[20–22]</sup> Other reports also show successful expression of active sulfotransferase enzymes in *E. coli*.<sup>[23,24]</sup> It is important to note here that sulfotransferase expression in *E. coli* might not be as straightforward as in eukaryotic culture systems<sup>[25,26]</sup> due to the complexities associated with their post-translational modification and folding. Here, we engineered *E. coli* to accumulate PAPS, the least explored of the three critical components described above. We explore the biosynthesis of PAPS and apply standard metabolic engineering strategies to improve PAPS accumulation over 1000-fold. Having achieved this, we also utilized this PAPS to evaluate the capability for CS synthesis through in vitro biotransformation. We use this study to help identify the major barriers/limitations in the metabolic engineering of the entirely animal-free microbial synthesis of CS.

## 2. Experimental Section

### 2.1. Growth Media and Chemicals

Luria–Bertani (LB; Sigma-Aldrich) medium with appropriate antibiotics was used for cloning, selection, and overnight fermentation. Superoptimal broth with catabolite repression was used for cell recovery after transformation. M9 minimal media salts were procured from Difco, BD. Additional salts and casamino acids used in nutrient media were procured from Sigma-Aldrich/MilliporeSigma (St. Louis, MO, USA).

Standard lithium salt of PAPS was from MilliporeSigma. CS disaccharide standards were purchased from Iduron (Manchester, UK). All other nutrients and reagents for preparing samples for disaccharide analysis were purchased from Sigma-Aldrich/MilliporeSigma. High-performance liquid chromatography (HPLC)-grade solvents and salts used to prepare mobile phases were procured from Thermo Fisher Scientific (Springfield, NJ, USA).

### 2.2. Bacterial Strains and Plasmids for Accumulating PAPS

The *E. coli* MG1655 strain was the wild-type strain used in this study. A modified ePathBrick vector<sup>[27]</sup> pETM7 was used to overexpress PAPS metabolic pathway genes in *E. coli* MG1655. Genes overexpressed using the pETM7 vector were under a *lacUV5* promoter, *lacO* operator, and *rrnB* terminator and could be expressed in all wild-type *E. coli* strains. The genes selected for overexpression were individually cloned into the pETM7

vector. Combinations were then built in the monocistronic form as previously described.<sup>[27]</sup> Transformants were selected using ampicillin resistance that is conferred by the pETM7 vector, followed by colony polymerase chain reaction (PCR) and Sanger sequencing. A list of all strains, plasmids, and primers used in this study is given in Tables S1, S2, and S3, Supporting Information, respectively.

A pdCas9 plasmid carrying a nuclease-null Cas9 from *Streptococcus pyogenes* and a single-guide RNA (sgRNA) scaffold was used for clustered regularly interspersed short palindromic repeat interference (CRISPRi) repression of *cysH* (PAPS reductase). This pdCas9 was a gift from Luciano Marraffini (Addgene plasmid #46569).<sup>[28]</sup> Five different spacer sequences around the start region of *cysH* were tested for the accumulation of PAPS. Spacer sequences were cloned into pdCas9 using the BsaI-mediated golden gate cloning method.<sup>[29]</sup> Spacer incorporation was verified by Sanger sequencing. Successful transformants were selected using chloramphenicol resistance that is conferred by the pdCas9 plasmid. The plasmid carrying the spacer sequence that resulted in the highest accumulation of PAPS was selected for use in further studies. The *cysH* gene deletion was performed by the lambda Red recombinase method published by Datsenko and Wanner.<sup>[30]</sup> Deleted variants were selected based on the following three properties: 1) kanamycin resistance that was conferred by the kanamycin cassette integrated into the genome during recombination; 2) the inability to grow on minimal media without casamino acids; and 3) colony PCR and sequencing around the area of *cysH* deletion and cassette insertion. A list of primers used in this study is given in Table S3, Supporting Information.

#### 2.2.1. Growth and Harvest of PAPS-Containing Cell Lysate

The different PAPS-accumulating constructs were grown in M9G + CAA media. The composition was as follows: 1× M9 salts, 0.1 mM CaCl<sub>2</sub>, 2 mM MgSO<sub>4</sub>, 4.14 μM MnSO<sub>4</sub>, 1% glucose, 1% casamino acids, and appropriate antibiotics. In experiments with additional sulfate, varying amounts (0 mM to 50 mM) of sodium sulfate were added to the above medium. All experiments were performed in biological triplicates (3 × 125 mL flasks containing 25 mL culture each). Cells were grown at 37 °C till an OD<sub>600</sub> (optical density at 600 nm) of 0.6 and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) according to the construct design, after which growth was continued at 20 °C. The growth curve and residual glucose data of the strains used in the study are given in Figures S3 and S4, Supporting Information. At the time of harvest (≈8 h post-induction), the cells were pelleted at 4 °C. Metabolites were extracted twice into an 80% methanol solution at –80 °C for 20 min. Pooled extracts were stored at –20 °C until further analysis.

### 2.3. PAPS Analysis

The PAPS concentration in the cell lysate obtained from above was estimated using a 150 × 2 mm Develosil C-30 RP-Aqueous column (Nomura Chemicals, Japan) procured from

Phenomenex Inc. The HPLC protocol was adapted from the method described by Furuno and co-workers.<sup>[31]</sup> Mobile phase A was 100 mM potassium phosphate buffer (pH 5.8) and mobile phase B was 75% acetonitrile (in H<sub>2</sub>O) at an overall flow rate of 0.2 mL min<sup>-1</sup>. The gradient program was 40 min per sample and was set as follows: 100:0::A:B v/v at 0 min, 100:0::A:B v/v at 10 min, 50:50::A:B v/v at 12 min held for 5 min, and 100:0::A:B v/v at 20 min and held for 20 min (to elute all other components). Standard PAPS (detected using PDA detector at 260 nm) was diluted in mobile phase A eluted at 6.8 min and PAPS in 80% methanol (like cell lysate) eluted at 6.4 min.

#### 2.4. Chondroitin Production and Extraction

The nonsulfated chondroitin backbone used for the biotransformation studies was extracted from shake flask cultures of the *E. coli* K4  $\Delta kfoE$  strain developed previously in our lab.<sup>[32]</sup> Nutrient media<sup>[21]</sup> used for production were composed of the following: 3.5 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 5 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 3.5 g L<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 2 g L<sup>-1</sup> casamino acids, 100 mL of 10 $\times$  3-(*N*-morpholino) propanesulfonic acid (MOPS) mix, 1 mL of 1 M MgSO<sub>4</sub>, 0.1 mL of 1 M CaCl<sub>2</sub>, and 1 mL of 0.5 g L<sup>-1</sup> thiamine-HCl, supplemented with 20 g L<sup>-1</sup> glucose. A 10 $\times$  MOPS mixture consisted of 83.7 g L<sup>-1</sup> MOPS, 7.2 g L<sup>-1</sup> tricline, 28 mg L<sup>-1</sup> FeSO<sub>4</sub>·7H<sub>2</sub>O, 29.2 g L<sup>-1</sup> NaCl, 5.1 g L<sup>-1</sup> NH<sub>4</sub>Cl, 1.1 g L<sup>-1</sup> MgCl<sub>2</sub>, 0.5 g L<sup>-1</sup> K<sub>2</sub>SO<sub>4</sub>, and 0.2 mL of the micronutrient stock. The micronutrient stock consisted of 0.2 g L<sup>-1</sup> (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 1.2 g L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 0.1 g L<sup>-1</sup> CuSO<sub>4</sub>, 0.8 g L<sup>-1</sup> MnCl<sub>2</sub>, and 0.1 g L<sup>-1</sup> ZnSO<sub>4</sub>. Glycerol stocks of the strain were streaked onto LB agar plates. Single colonies from these plates were grown overnight and inoculated into 1 L media and were grown in a 3.84 L flask at 37 °C for 24 h and harvested by centrifugation. The polysaccharide was precipitated from the supernatant and cell lysate (obtained by autoclaving the resuspended pellet and centrifuging to collect the supernatant) using four volumes of ethanol at -20 °C for 24 h. The precipitated polysaccharide was resuspended in 20 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.0, to a final concentration of 20 mg mL<sup>-1</sup>. This partially purified extract was used in the biotransformation studies.

#### 2.5. Chondroitin-4-sulfotransferase (C4ST) Expression and Purification

C4ST was expressed and purified from *E. coli* BL21 Star (DE3) containing the pET32aLIC-C4ST plasmid as previously described.<sup>[23]</sup> The *N*-terminus of the gene contained a thioredoxin A tag to increase protein solubility and a His-6 $\times$  tag for purification. The approximate size of the fusion protein was  $\approx$ 53 kDa and its pI value was 6.85 (ExPASy). Glycerol stock of the above strain was plated on LB agar supplemented with 80  $\mu$ g mL<sup>-1</sup> ampicillin. Single colonies from the plate were used to inoculate 20 mL overnight cultures. After 14–18 h, the cells were spun down (6800  $\times$  g, 25 °C, 10 min) and resuspended in 1 L of M9 medium containing 80  $\mu$ g mL<sup>-1</sup> ampicillin in 2.8 L Pyrex Fernbach culture flasks (Corning Life Sciences). The cell

culture was grown in an incubator shaker at 37 °C, 225 rpm, and enzyme expression was induced at an OD<sub>600</sub>  $\approx$ 0.8 with 0.2 mM IPTG. The culture was incubated post-induction for 16–20 h at 22 °C, and cells were harvested by centrifugation at 4 °C (5000  $\times$  g for 10 min) and stored at -80 °C until needed.

The pelleted *E. coli* cells were resuspended in 20 mL of 50 mM Tris-HCl buffer (pH 8.0, 500 mM NaCl, 30 mM imidazole) as a single-cell suspension then sonicated (with occasional cooling on ice). Cell debris was removed by centrifugation (16 000  $\times$  g for 1 h) at 4 °C and the resulting cell lysate was filtered using a 0.45  $\mu$ m Steriflip-GP filter (Millipore). The filtered supernatant was applied to Ni-NTA resin (Thermo Fisher Scientific) that was prewashed with five column volumes of buffer A (50 mM Tris-HCl, 500 mM NaCl, 30 mM imidazole, pH 7.5). The bound target protein was eluted with buffer B (50 mM Tris-HCl, 500 mM NaCl, 300 mM imidazole, pH 7.5). Imidazole was removed by buffer exchanging the elution buffer B against storage buffer (50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, pH 7.5) and stored at -80 °C until needed.

#### 2.6. In Vitro Biotransformation Reaction

The buffer chosen for the in vitro reaction was 20 mM Tris-HCl, pH 7.0, based on the work of Kang and co-workers, in which they reported very high conversion of chondroitin into CS.<sup>[11]</sup> Since the biotransformation reaction is that of a sulfotransferase enzyme, the conversion percentage reported here also represents the sulfation level of the product. The PAPS strain cell lysates (0.5 mL in 80% methanol) as well as positive and negative controls were subjected to rotary evaporation at room temperature and 100 mm Hg pressure for 3–4 h (until the methanol composition was around 50%). They were then frozen at -80 °C and lyophilized to completely remove the methanol and water. The lyophilized extracts were then resuspended in 20 mM Tris-HCl, pH 7.0. Various amounts of this lysate, chondroitin extract, and C4ST were combined to a final reaction volume of 50  $\mu$ L and incubated at 37 °C for 50 h. The reaction mixture was then heated to 90 °C for 10 min to stop the reaction and spun down to pellet the enzyme. The pellet was resuspended in 2 M NaCl to release any bound GAGs and centrifuged again. The pooled supernatants, from the above steps, were subjected to disaccharide analysis as described below.

#### 2.7. Disaccharide Analysis

The CS-containing samples from the biotransformation studies were buffer-exchanged into 200  $\mu$ L of 50 mM ammonium acetate (pH 7.4) and then depolymerized using chondroitinase ABC (20 mU in 5  $\mu$ L of 25 mM Tris, 500 mM NaCl, and 300 mM imidazole buffer, pH 7.4) at 37 °C for 12 h. The resulting disaccharides were filtered through a 3 kDa spin column and lyophilized. The freeze-dried samples were resuspended in 10  $\mu$ L of 0.1 M 2-aminoacridone (AMAC) (17:3 v/v solution in dimethyl sulfoxide [DMSO]/acetic acid [AcOH]) and mixed by vortexing for 5 min. Next, 10  $\mu$ L of 1 M NaBH<sub>3</sub>CN was added in the reaction mixture and incubated at 45 °C for 1 h. The

disaccharides formed were analyzed by liquid chromatography–mass spectrometry (LC–MS) on an Agilent 1200 LC/MSD instrument (Agilent Technologies Inc., Wilmington, DE, USA) according to published protocols.<sup>[33]</sup> Data were analyzed using Thermo Xcalibur software, and absolute and relative product and substrate levels were quantified with the help of external standards. Sample LC–MS data are provided in Figure S5, Supporting Information.

### 3. Results

In this paper, we report two major results: 1) the engineering of PAPS accumulation in *E. coli*, which led to an ≈1000-fold increase in PAPS levels; and 2) the use of the PAPS in the *E. coli* cell lysate to synthesize CS-A in an in vitro biotransformation reaction.

#### 3.1. Engineering *E. coli* to Accumulate PAPS

PAPS biosynthesis is part of the cysteine/methionine biosynthesis in most cells. Genes encoding the enzymes associated with this pathway are found clustered on the *cys* operons in *E. coli*. The metabolic route for the synthesis of PAPS is not linear. Every sulfate molecule that enters the cell is activated to adenosine-5'-phosphosulfate (APS) by adenosine-5'-triphosphate (ATP) sulfurylase. APS is further phosphorylated to PAPS by APS kinase. In wild-type *E. coli*, the PAPS, generated by this pathway, is reduced to adenosine-3',5'-diphosphate (PAP) and inorganic sulfite by PAPS reductase. Sulfite is further reduced to sulfide and incorporated into amino acids by enzymes encoded by other genes in the *cys* operons. Furthermore, PAP is dephosphorylated to adenosine-5'-monophosphate (AMP) by PAP nucleotidase, thus leading to the

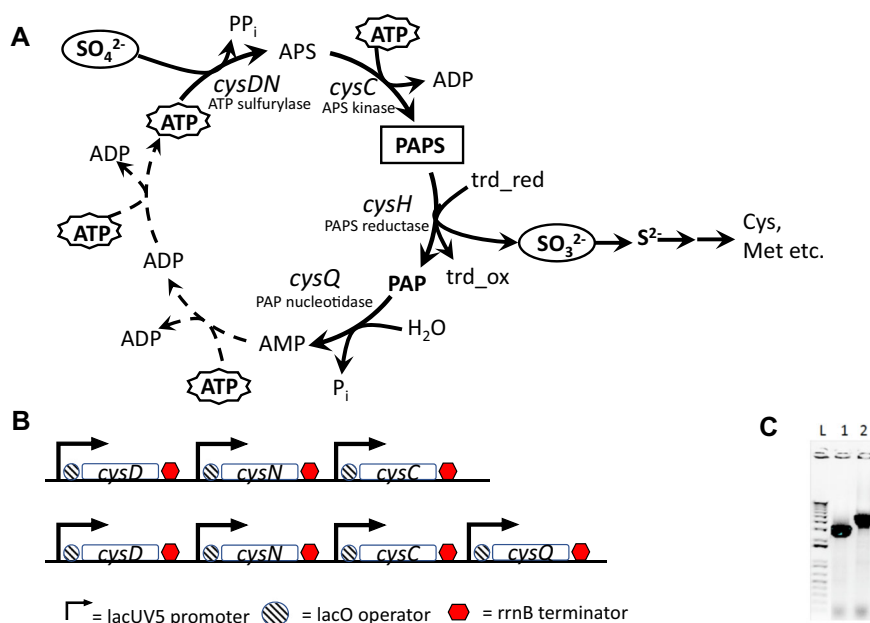
regeneration/recycle of PAPS and PAP. The pathway and associated enzymes are depicted in **Figure 1A**.

We amplified the genes *cysD*, *cysN*, and *cysC* coding for ATP sulfurylase and APS kinase from the *E. coli* BL21(DE3) genome and overexpressed them in the plasmid construct pETM7-*cysDNC* to increase the intracellular accumulation of PAPS. We also overexpressed the gene *cysQ* coding for PAP nucleotidase to aid the recycling of PAPS/PAP in vivo (pETM7-*cysDNCQ*). A schematic and a gel image of the gene assembly in the plasmid constructs are depicted in Figure 1B,C.

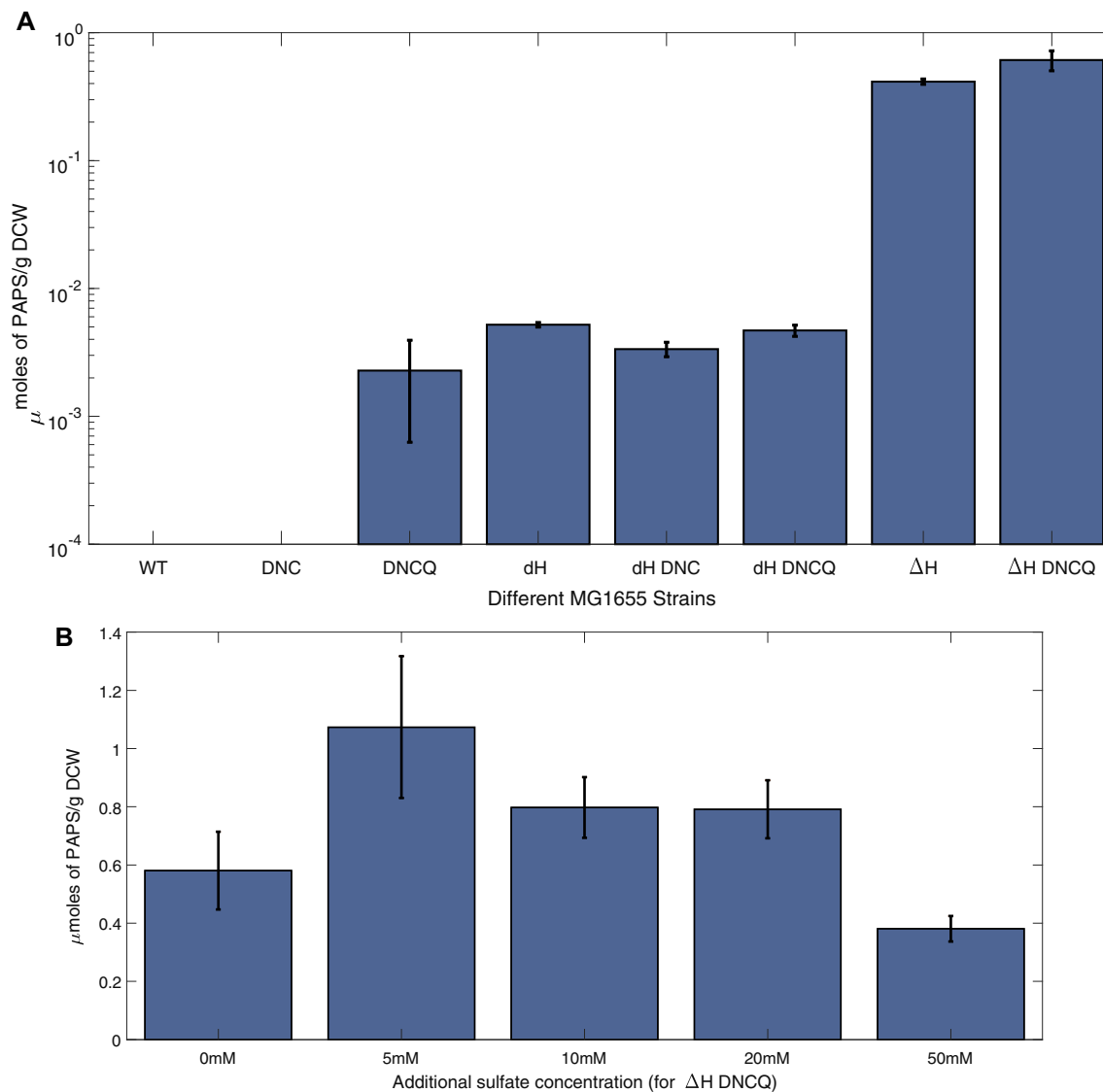
##### 3.1.1. Engineered MG1655 Strain Shows an ≈1000-Fold Increase in PAPS Accumulation

*E. coli* MG1655 overexpressing the *cysDNCQ* genes from the above-mentioned plasmid constructs accumulated detectable amounts (using the HPLC method in Section 2.3) of PAPS in contrast to the wild type and the strain overexpressing the *cysDNC* genes (**Figure 2A**). In another strategy, we explored the accumulation of PAPS in which the gene *cysH*, encoding a PAPS reductase that consumes PAPS, was subjected to repression/deletion. Repression of *cysH* using CRISPRi increased the accumulation of PAPS further (schematics of CRISPRi spacers and PAPS accumulation are given in Figures S1 and S2, Supporting Information). However, upon deletion of the *cysH*, the accumulation of PAPS increased ≈1000-fold in comparison to the overexpressing strain (Figure 2a). There were also no significant differences in the combinatorial effects of repression/deletion and overexpression.

Supplementing additional sulfate to the medium, in the form of sodium sulfate, aids the accumulation of PAPS. However, at very high concentrations (50 mM Na<sub>2</sub>SO<sub>4</sub>), additional sulfate does not correlate to higher PAPS accumulation (Figure 2B).



**Figure 1.** A) PAPS biosynthetic route in *E. coli* showing the genes (*cysDN*, *cysC*, *cysH*, and *cysQ*) and the metabolic intermediates (ATP, ADP, APS, PAPS, PAP, and AMP) of PAPS synthesis and sulfate fixation. B) Schematic of overexpression constructs pETM7-*cysDNC* and pETM7-*cysDNCQ*. C)



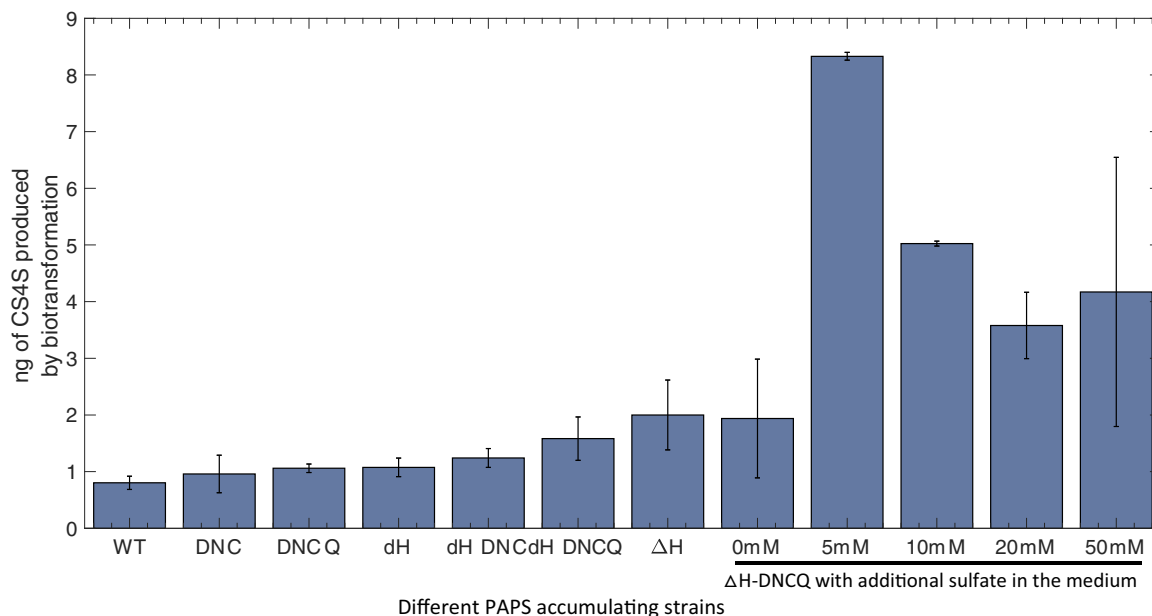
**Figure 2.** A) Intracellular PAPS levels ( $\mu\text{mol gDCW}^{-1}$ ) in different strains (WT = wild-type MG1655; DNC = MG1655 carrying pETM7-*cysDNC*; DNCQ = MG1655 carrying pETM7-*cysDNCQ*; dH = MG1655 with repressed *cysH*; dH-DNC = MG1655 carrying pETM7-*cysDNC* and repressed *cysH*; dH-DNCQ = MG1655 carrying pETM7-*cysDNCQ* and repressed *cysH*;  $\Delta$ H = MG1655 with *cysH* deletion;  $\Delta$ H-DNCQ = MG1655 with *cysH* deletion carrying pETM7-*cysDNCQ*). B) Effect of additional sulfate in media on intracellular PAPS levels ( $\mu\text{mol gDCW}^{-1}$ ) in  $\Delta$ H-DNCQ strains. All error bars indicate the standard deviation between three biological replicates. PAPS measurement was done using HPLC-UV as described in Section 2.3.  $\mu\text{mol gDCW}^{-1}$ , micromoles per gram dry cell weight.

### 3.2. In Vitro Biotransformation of Chondroitin to CS

Having obtained a considerable increase in the intracellular PAPS levels within *E. coli* MG1655, we attempted to use the PAPS extracted from these cells as a sulfate donor for the conversion of chondroitin into CS using purified C4ST. As previously described, this study uses an in vitro biotransformation to assess the possibility of achieving an entire in vivo biosynthesis of CS in *E. coli*. Of the three components required for CS synthesis, previous studies have independently established the production of two components, chondroitin<sup>[21]</sup> and C4ST,<sup>[23]</sup> in *E. coli*. In this study, we report engineered *E. coli* that can accumulate PAPS. The biotransformation described

here adjusts the individual levels of these three required components within in vitro reactions to the average levels that *E. coli* can currently produce. Sample calculations for scaling chondroitin and C4ST levels are shown in the Supporting Information section.

PAPS from the cell lysate of the strain (deletion of *cysH*, overexpression of *cysDNCQ* used in the combined strategy, and additional supplementation of 5 mM sodium sulfate to the medium) gave the highest yield of CS from a 50  $\mu\text{L}$  reaction. This highest amount of 8.3 ng of chondroitin-4-sulfate disaccharide (CS4S) is 10.4-fold higher than the PAPS in the wild-type cell lysate. **Figure 3** shows the yield of CS4S from the biotransformation setup for the different strains compared in this study.



**Figure 3.** Amount of CS produced (in ng, estimated using the LC–MS method as described in Section 2.7) in the in vitro biotransformation experiments with PAPS from cell lysates (WT = wild-type MG1655; DNC = MG1655 carrying pETM7-*cysDNC*; DNCQ = MG1655 carrying pETM7-*cysDNCQ*; dH = MG1655 with repressed *cysH*; dH-DNC = MG1655 carrying pETM7-*cysDNC* and repressed *cysH*; dH-DNCQ = MG1655 carrying pETM7-*cysDNCQ* and repressed *cysH*; delH = MG1655 with *cysH* deletion; ΔH-DNCQ = MG1655 with *cysH* deletion carrying pETM7-*cysDNCQ*). The last five entries in the plot employed lysate from ΔH-DNCQ cells grown with additional sulfate (0–50 mM) in the medium. All error bars indicate the standard deviation between three biological replicates.

It is important to note that the reaction we study using these biotransformations is the sulfation of the unsulfated chondroitin extracted from *E. coli* K4. Hence the conversion percentage that we discuss in detail in the following sections also represents the product's sulfation level. Though Figure 3 shows that CS was synthesized, the percentage conversion of chondroitin into CS was very low, even for the reaction with the highest titer (0.035%). This is because the substrate and enzyme concentrations were selected to reflect intracellular levels as opposed to achieving a high percentage conversion. Nonetheless, we also attempted to assess the effect of levels of C4ST as well as PAPS used in this study on percentage conversion into CS. For 5 μg of chondroitin, we added low (1:6) and high (1:60) amounts of PAPS with low (1:1) and high (1:10) amounts of C4ST and carried out the biotransformation as mentioned. Table 1 summarizes the results of this study. It is noteworthy that even the low ratio (1:1) of chondroitin to C4ST required a much higher amount of enzyme than that was used in our experiments representing the cellular environment (50:1)

**Table 1.** Effect of PAPS and C4ST concentrations on the conversion of chondroitin into CS.

Sl. no.	Chondroitin [μg]	PAPS [μg]	C4ST [μg]	% Conversion (or % sulfation)
1	5	30	5	0.31 ± 0.03
2	5	30	50	8.04 ± 0.84
3	5	300	5	1.70 ± 0.18
4	5	300	50	36.9 ± 4.7

(Figure 3). We observed 99.1% conversion when using excess amounts of enzyme, a 1:60:100 ratio for chondroitin:PAPS:C4ST.

At a 1:1 ratio of chondroitin to C4ST (by weight), a tenfold increase in PAPS resulted in an ≈5.4-fold increase in the conversion of chondroitin into CS (Table 1). The current enzyme production level (as reported previously<sup>[23]</sup>) must be increased ≈50-fold for such a condition to be met. Moreover, a tenfold increase in enzyme levels in the presence of excess PAPS translates to an ≈33.7-fold increase in the conversion level. Taken together, with the conditions that afforded a 99.01% conversion, this demands an enzyme titer requirement of 2–20 g L<sup>-1</sup> for 40–100% conversion of CS. Scaling up to this high titer of recombinant protein is almost impossible in a simple system like *E. coli*. This suggests that the best way for achieving a reasonable CS titer in vivo is to improve the specific activity of C4ST.

#### 4. Discussion

This study reports for the first time over a 1000-fold improvement in PAPS accumulation upon deletion of *cysH* and overexpression of *cysDNCQ* genes in *E. coli*. Since PAPS is the universal sulfate donor, the impact of the increase reported here would also apply to the production of many additional sulfated biomolecules. Many of these molecules are currently of commercial interest. For example, in a recent report by Yamaguchi and co-workers, a 3.84-fold increase in PAPS accumulation by CRISPRi-mediated repression of *cysH* was used to synthesize naringenin-7-sulfate entirely in vivo in *E.*

*coli*.<sup>[34]</sup> In addition to sulfated flavonoids, the findings reported in this paper can be applied directly to other sulfated GAGs like heparin, heparan sulfate, keratan sulfate, and dermatan sulfate. We also show for the first time that PAPS in the cell lysate of the engineered and wild-type *E. coli* can actually facilitate C4ST in converting chondroitin into CS. The best strain design of those presented in this study gave a 10.4-fold higher yield of CS than the wild type.

These biotransformation experiments also shed light on the components that are likely to be limiting in an entirely in vivo biosynthetic approach. Theoretically, the 200 mg L<sup>-1</sup> chondroitin production reported previously in our laboratory<sup>[21]</sup> in shake flask culture translates roughly to about 0.47 mmol g<sup>-1</sup> DCW, which is approximately 500-fold more than the best PAPS accumulation we have achieved. This emphasizes the need for PAPS recycle or an active PAPS flux inside the cell as opposed to just its accumulation in terms of intracellular titer. PAPS regeneration is a concept that has also been explored in studies focusing on in vitro chemoenzymatic approaches to synthesize CS. In such a setup, one of the key problems is the cost and instability of PAPS. Some of the most notable approaches to circumvent this include an aryl sulfotransferase-mediated regeneration of PAPS,<sup>[35]</sup> a pseudo-cell-free pathway that converts ATP into PAPS,<sup>[32]</sup> and finally a more recent approach on replacing PAPS with Na<sub>2</sub>SO<sub>4</sub> and ATP in liver fractions to synthesize sulfated steroids.<sup>[36]</sup>

The biotransformation studies showed that an ≈1000-fold increase in PAPS levels in the cell lysate translated to only an approximately tenfold increase in the CS yield. Given that chondroitin is practically in excess in all these situations, these data hint at possible limitations in C4ST levels as well as their catalytic activity. In the biotransformation experiments that tested the effect of PAPS and C4ST concentrations on CS conversion (Table 1), we also obtained data that indicated that an improvement in the specific activity of C4ST may be instrumental in successfully achieving the entire synthesis of CS in vivo.

Overall, the biotransformation studies reported here show for the first time in vitro synthesis of CS with all the three components synthesized in-house microbially from simple nutrient media and engineered *E. coli*. They also provide insights that identify improvements in in vivo PAPS recycle and C4ST activities that are vital to achieving a one-step complete microbial synthesis of CS.

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

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