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Published in:
Nature Communications

Link to article, DOI:
10.1038/s41467-019-12022-x

Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Jendresen, C. B., & Nielsen, A. T. (2019). Production of zosteric acid and other sulfated phenolic biochemicals in microbial cell factories. Nature Communications, 10, [4071]. https://doi.org/10.1038/s41467-019-12022-x
Production of zosteric acid and other sulfated phenolic biochemicals in microbial cell factories

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Biological production and application of a range of organic compounds is hindered by their limited solubility and toxicity. This work describes a process for functionalization of phenolic compounds that increases solubility and decreases toxicity. We achieve this by screening a wide range of sulfotransferases for their activity towards a range of compounds, including the antioxidant resveratrol. We demonstrate how to engineer cell factories for efficiently creating sulfate esters of phenolic compounds through the use of sulfotransferases and by optimization of sulfate uptake and sulfate nucleotide pathways leading to the 3\textsuperscript{′}-phosphoadenosine 5\textsuperscript{′}-phosphosulfate precursor (PAPS). As an example we produce the antifouling agent zosteric acid, which is the sulfate ester of \(p\)-coumaric acid, reaching a titer of 5 g L\(^{-1}\) in fed-batch fermentation. The described approach enables production of sulfate esters that are expected to provide new properties and functionalities to a wide range of application areas.
Sulfate residues provide distinct properties to organic molecules, increasing negative charge, acidity and solubility, which may have biotechnological applications. Small phenolic compounds are naturally sulfated (O-sulfonated) in vivo; in plants controlling signaling, developmental regulation, plant–bacteria communication, and stress responses, and similarly in animals, steroid hormones are subject to sulfation and desulfation, altering activity, life-span, transport properties of the molecules, while xenobiotics are detoxified by sulfation. Several natural polymers are also subject to sulfation, having a role in intermolecular interactions, disease progression, biomaterialization, virulence, membrane integrity and osmoprotection. From a biotechnological perspective, the alterations of the physical and chemical properties of a compound through sulfation may have direct application on drugs and small molecule administration, and the properties may also have application in synthesis of novel polymers from sulfated monomers. The altered properties may also provide a means to produce an improved form of phenolic compounds that are generally poorly soluble and toxic to the production host.

Eelgrass (Zostera species) are marine plants that produce a naturally sulfated small compound, zosteric acid, the sulfate ester of p-coumaric acid. Extracts of Zostera have provided complex mixtures of compounds with a large fraction being zosteric acid, which have been shown to prevent biofouling by pre-mixtures of compounds with a large fraction being zosteric acid, the sulfate ester of p-coumaric acid, zosteric acid, the product matched the expected shift in retention time relative to the substrate were formed by the formation of a sulfate ester of p-coumaric acid catalyzed by 3′-phosphoadenosine 5′-phosphosulfate (PAPS)-dependent aryl sulfotransferases.

The genomic sequence of the zosteric acid-producing plant Zostera marina has recently been published, and while it was noted that the genome harbors 12 genes putatively encoding arylsulfotransferases, substrate prediction of these enzymes is challenging, and it was suggested that some of these might be responsible for carbohydrate sulfation. Possibly, one of these genes could confer the activity required for specific production of zosteric acid, also if employed in a microbial cell factory. Since PAPS is part of the sulfite assimilation and reduction pathways across all kingdoms of life, a microbial cell factory should be able to utilize its native PAPS supply for the process. Thus, we examined the proposed arylsulfotransferases from Zostera marina, as well as a stress-inducible sulfotransferase from Arabidopsis thaliana by heterologously expressing codon-optimized genes in E. coli, a preferred host for production of biochemicals. We used a KRX strain, for cloning and tight expression of any of the plant genes (Fig.2a). We therefore cloned genes encoding sulfotransferases from fruit fly (Drosophila melanogaster), human (Homo sapiens), rat (Rattus norvegicus), chicken (Gallus gallus), canine (Canis lupus), porcine (Sus scrofa) and equine (Equus caballus) using liver cDNA preparations. We further synthesized genes encoding sulfotransferases from the model organisms Zebrafish (Danio rerio) and the nematode Caenorhabditis elegans. Only a few bacterial PAPS-dependent sulfotranferases have been described, e.g., for the sulfation of nodular rod factors. Recently, PAPS-dependent sulfation of aromatic compounds was also described in bacteria, and we therefore also examined a few bacterial genes that are only distantly related to the plant or animal genes (Fig. 2a).

The enzymatic activity required for producing compounds such as zosteric acid may, however, be found in other organisms. Phenolic compounds, which are often toxic for animals, are subject to phase II detoxification, especially in the liver, where a general mechanism results in the formation of a less toxic conjugated version of the phenolic compound. One of these reactions result in the formation of a sulfate ester, catalyzed by PAPS-dependent aryl sulfotransferases. We therefore cloned genes encoding sulfotransferases from fruit fly (Drosophila melanogaster), human (Homo sapiens), rat (Rattus norvegicus), chicken (Gallus gallus), canine (Canis lupus), porcine (Sus scrofa) and equine (Equus caballus) using liver cDNA preparations. We further synthesized genes encoding sulfotransferases from the model organisms Zebrafish (Danio rerio) and the nematode Caenorhabditis elegans. Only a few bacterial PAPS-dependent sulfotransferases have been described, e.g., for the sulfation of nodular rod factors. Recently, PAPS-dependent sulfation of aromatic compounds was also described in bacteria, and we therefore also examined a few bacterial genes that are only distantly related to the plant or animal genes (Fig. 2a). These additional genes were similarly expressed in E. coli. Given that phenol sulfotransferases may have activity towards several compounds, we examined the activity of all 40 cloned sulfotransferases against p-coumaric acid, as well as the polyphenol resveratrol and the flavonoid kaempferol, which are all of interest as biotechnological products. In addition, we tested vanillic acid, a small phenolic acid. After overnight growth, culture supernatants were isolated and examined by HPLC for the depletion of substrate (Fig. 2b) and the occurrence of a respective product (Fig. 2c). The results demonstrate that a sulfotransferase was required for the conversion of all four substrates, and that all compounds could act as a substrate for at least one of the selected enzymes under the screening conditions. For the expected product of p-coumaric acid, zosteric acid, the product matched a chemically synthesized standard. For resveratrol, kaempferol and vanillic acid, emerging product peaks that matched the expected shift in retention time relative to the substrate were identified when comparing to the chromatogram of a control.
enzymes for activation of sulfate into 3′-sulfotransferase (PST) for formation from kaempferol and to a lesser extent resveratrol. H. ochraceum from animal origin, and the product of a single bacterial gene, measurable product, while 17 of the positives were sequences selected compounds, we further explored the substrate specificity of active enzymes had shown differences in activity toward four of the genes from A. thaliana, nor the gene resulted in product formation. None of the genes from Z. marina, nor the A. thaliana gene resulted in measurable product, while 17 of the positives were sequences from animal origin, and the product of a single bacterial gene, H. ochraceum DSM 14365 Hoch_6098, resulted in product formation from kaempferol and to a lesser extent resveratrol.

A range of compounds may be subjects to sulfation. As the active enzymes had shown differences in activity toward four selected compounds, we further explored the substrate specificity of E. coli strains expressing a number of selected representative enzymes: SULT1A1 from Rattus norvegicus, SULT1B1, SULT1C1 and SULT1E1 from Gallus gallus domesticus, SULT1ST1 and SULT6B1 from D. rerio and Hoch_6098 from H. ochraceum. The strains were grown in minimal media supplemented with either ferulic acid, 3-hydroxy-4-methoxy-cinnamic acid, 4-acetamidophenol, naringenin, 4-vinylphenol, 4-ethylphenol, 4-ethylguaiacol, 4-nitrophenol or 4-methylumbelliferone (4-MU). For all 13 tested compounds, at least one sulfotransferase was able to act upon it, and several enzymes also demonstrated a high degree of promiscuity (Fig. 2d). This demonstrates that a wide range of sulfated phenolic compounds can be generated using microbial cell factories.

Zosteric acid can be produced in E. coli and yeast. Zosteric acid is an attractive biochemical for production by itself because of its antifouling properties, and it may also be used as building block with interesting properties due to its charged sulfate group. Additionally, it may be a preferred intermediate in p-coumaric acid production as it is less toxic to the production organism. The growth of E. coli in minimal media is inhibited at increasing concentrations of p-coumaric acid, while this is not the case for zosteric acid (Supplementary Fig. 3). It would therefore be possible to accumulate the non-toxic zosteric acid in the fermentation broth to high concentrations and later on convert it to p-coumaric acid. Zosteric acid was shown to be stable in fermentation media, and in the presence of E. coli (Supplementary Fig. 4).

Among the screened enzymes, SULT1A1<sub>Rno</sub> showed the greatest production of zosteric acid from p-coumaric acid, and we therefore chose this enzyme for creating a zosteric acid microbial cell factory. p-Coumaric acid may be formed in the host organism from tyrosine by non-oxidative deamination catalyzed by a tyrosine ammonia-lyase (TAL). Thus, we combined the gene encoding SULT1A1<sub>Rno</sub> with three different TAL-encoding genes: the commonly used tyrosine ammonia-lyases from Rhodobacter sphaeroides (TAL<sub>Rsp</sub>) and Rhodobacter capsulatus (TAL<sub>Rca</sub>) as well as the recently described tyrosine ammonia-lyase from Flavobacterium johnsoniae (TAL<sub>Fjo</sub>)<sup>9</sup> in E. coli BL21(DE3). The resulting strains were grown in minimal medium with or without supplementation of tyrosine. In addition, we tested different levels of induction of the expression system. It was clear that expression of a TAL-encoding gene was required for the production of pHCA, and that this enabled the cells to further produce ZA (Fig. 3). Although the resulting titer of p-coumaric acid could be increased by the higher induction level, this was found to be detrimental to the titer of zosteric acid. Furthermore, supplementing the medium with 2 mM tyrosine allowed the strains to reach even higher titers of p-coumaric acid, while the titer of zosteric acid was largely unaffected. The highest titers of zosteric
**Fig. 2** Activity and relationship between screened sulfotransferases. The phylogenetic relationship of the chosen sulfotransferases shown together with the consumption of substrates (pHCA (p-coumaric acid), resveratrol, kaempferol, and vanillic acid) and appearance of product peaks. The substrate specificity of selected phenol sulfotransferases when the production organisms were challenged with selected compounds (p-coumaric acid, orange; resveratrol, light gray; kaempferol, yellow; vanillic acid, blue; ferulic acid, light green; 3-hydroxy-4-methoxycinnamic acid, navy blue; naringenin, rust red; 4-vinylphenol, dark gray; 4-nitrophenol, golden brown; 4-methylumbelliferone, dark blue; 4-acetamidophenol, dark green; 4-ethylphenol, light blue; 4-ethylguaiacol, light brown). The height of the bars show the relative remaining amount of compounds relative to a strain that harbors an empty expression plasmid shown (as averages of individual points plotted in circles). Source data of Fig. 2d are provided as a source data file.
acid were reached in the strain combining SULT1A1_\text{bno} with TAL_{pjo}—both under induction with 0.1 mM IPTG and 1 mM IPTG. The growth was significantly influenced by the induction with IPTG; and while it mainly affected the growth rate at 0.1 mM IPTG, it increased the lag phase at 1 mM (Supplementary Fig. 5).

In order to establish whether these results are limited to a bacterial host, we similarly expressed the TAL and PST genes in Saccharomyces cerevisiae (Supplementary Table 1). Expression of SULT1A1_\text{bno} alone enabled the production of ZA from supplemented phsCA, and in combination with the expression of TAL_{pjo}, p-coumaric acid and zosteric acid (13 \mu M ZA) were produced in minimal media with glucose. As the production was higher for E. coli, we chose this organism for further studies.

Optimization of activated sulfate reaction. Zosteric acid was only formed, when there was a supply of exogenous p-coumaric acid or if the cells were capable of synthesizing p-coumaric acid. However, increased tyrosine supply, which resulted in increased concentrations of p-coumaric acid, did not result in increasing amounts of zosteric acid being formed. In fact, increased p-coumaric acid concentration could even decrease the amount of zosteric acid formed, and similarly, increasing the concentration of sulfate in the medium had no positive effect (Fig. 4), indicating that an alternative bottleneck was found in the production pathway.

The immediate substrate for sulfation is PAPS, a sulfate- and ATP-derived nucleotide, so we attempted to overproduce PAPS pathway. However, increased tyrosine supply, which resulted in increased concentrations of p-coumaric acid, did not result in increasing concentrations of tyrosine being formed. In fact, increased p-coumaric acid concentration could even decrease the amount of p-coumaric acid formed, and similarly, increasing the concentration of sulfate in the medium had no positive effect (Fig. 4), indicating that an alternative bottleneck was found in the production pathway.

Improved production by optimizing sulfate uptake. Since higher concentrations of sulfate in the growth medium did not to further improve production (Fig. 4), we hypothesized that sulfate transport may be a limiting step in the production of zosteric acid. Sulfate can be transported across the cell membrane in bacteria by proteins belonging to several families as reviewed by Aguilar-Barajas et al.31. We therefore constructed several strains expressing different uptake systems including the native (\textit{cysP} \textit{sbp} of a \textit{cys\text{P}\text{sbp} operon were regulated by the \textit{livH}K\text{GHMF} operon regulated by the \textit{cysP}AV\text{UWA} component of NADH dehydrogenase, but none of the other genes with the \textit{cysP} operon were regulated by \textit{cysP}UWA ABC transporter. We additionally constructed an artificial operon, replacing the thiosulfate-prefering subunit encoded by \textit{cysP} with the sulfate-prefering subunit encoded by \textit{sbp}. Finally, we also examined \textit{CysP} from Bacillus subtilis (\textit{CysP}_{\text{Bsu}}), which belongs to the inorganic phosphate transporter (\textit{PIT}) family, since it was previously found to be able to restore a sulfate starving phenotype of a \textit{cysP} sbp double mutation in E. coli34.

The transporters encoded by \textit{cysP\text{UWA}} and \textit{cysP}_{\text{Bsu}} both improved the titer of zosteric acid, when expressed in combination with \textit{cysD\text{NQC}}, most notably by 3.5-fold under high concentrations of p-coumaric acid (Fig. 4). Expression of the transporters was however not consistently positive and could even hamper the production of zosteric acid. Considering the detrimental effects of overexpression of membrane proteins, we tested whether expression from a low-copy plasmid of two transporters would be beneficial. Indeed, the resulting strains (CBJ1242 and CBJ1255) significantly improved the titers of zosteric acid over the high-copy plasmids, and the low-copy

| Strain     | TAL | PST | No addition | 0.1 mM IPTG | 1 mM IPTG | 0.1 mM IPTG | 1 mM IPTG |
|------------|-----|-----|-------------|-------------|-----------|-------------|-----------|
|            |     |     | pHCA | ZA | pHCA | ZA | pHCA | ZA | pHCA | ZA | pHCA | ZA |
| CBJ1013    | None | SULT1A1<sub>bno</sub> | 82 ± 17 | 0 ± 0 | 308 ± 15 | 0 ± 0 | 480 ± 17 | 0 ± 0 | 275 ± 194 | 0 ± 0 | 1677 ± 42 | 0 ± 0 |
| CBJ1014    | TAL<sub>pjo</sub> | SULT1A1<sub>bno</sub> | 82 ± 17 | 0 ± 0 | 308 ± 15 | 0 ± 0 | 480 ± 17 | 0 ± 0 | 275 ± 194 | 0 ± 0 | 1677 ± 42 | 0 ± 0 |
| CBJ1015    | TAL<sub>pjo</sub> | SULT1A1<sub>bno</sub> | 79 ± 3 | 2 ± 1 | 164 ± 7 | 187 ± 11 | 402 ± 19 | 88 ± 14 | 222 ± 16 | 1 ± 0 | 1543 ± 16 | 165 ± 7 |
| CBJ1016    | TAL<sub>pjo</sub> | SULT1A1<sub>bno</sub> | 82 ± 17 | 0 ± 0 | 308 ± 15 | 0 ± 0 | 480 ± 17 | 0 ± 0 | 275 ± 194 | 0 ± 0 | 1677 ± 42 | 0 ± 0 |
| CBJ1246    | TAL<sub>pjo</sub> | None | 82 ± 17 | 0 ± 0 | 308 ± 15 | 0 ± 0 | 480 ± 17 | 0 ± 0 | 275 ± 194 | 0 ± 0 | 1677 ± 42 | 0 ± 0 |

Fig. 3 Production of zosteric acid directly from glucose in minimal medium in recombinant E. coli. Selected strains expressing SULT1A1<sub>bno</sub> in combination with either no tyrosine ammonia-lyase (TAL) or one of the tyrosine ammonia-lyases from Rhodobacter sphaeroides (TAL<sub>le</sub>), Rhodobacter capsulatus (TAL<sub>j</sub>), or Flavobacterium johnsoniae (TAL<sub>j</sub>), respectively, were grown in M9 medium with 0.2% glucose for 24 h. Resulting production titers (\mu M) of p-coumaric acid (pHCA) or zosteric acid (ZA) and standard deviations (± for n = 3) are shown. Source data are provided as a Source Data file.
transport genes or combinations thereof were grown in M9 medium with either low or high concentration of combination with either an empty pRSFDuet-1 plasmid, a derived plasmid harboring the acid concentration was negatively in

rather than in deep-well plates, gave similar results, as CBJ1255 (SULT1A1Rno CysPUWA low copy, CysDNCQ) and CBJ1258 (SULT1A1Rno CysPBrno CysDNCQ) reached 2.81 ± 0.33 mM (±standard deviation for n = 3) and 2.81 ± 0.48 mM zosteric acid from M9 with high level of p-coumaric acid and sulfate, respectively.

Overexpression of the sulfate uptake and activation genes were then combined with expression of the phenol sulfotransferase and tyrosine ammonia-lyase for complete synthesis of zosteric acid from tyrosine or even glucose (Fig. 1 and Table 2). As previously shown, co-expression of TALp and SULT1A1Rno resulted in production of both p-coumaric acid and zosteric acid. Overexpression of sulfate uptake and activation enzymes improved the conversion to the latter, reaching 2.56 mM zosteric acid. Fed batch fermentation of a strain overexpressing SULT1A1Rno, CysPBrno and CysDNCQ, resulted in a zosteric acid titer of 3.2 mM (0.78 g L−1) from minimal salts media, and 20.4 mM (5.0 g L−1) with supplemented tyrosine (Fig. 5). The specific production reached 0.15 g L−1 OD600−1. Acetate was observed to accumulate during the early part of the fermentation, likely

Table 1 Transcriptomic response to induction of zosteric acid production

| Gene       | Fold change | FDR p-value | Gene function                                                                 |
|------------|-------------|-------------|-------------------------------------------------------------------------------|
| cysQ       | 43.5        | 1.12E−68    | Adenosine-3′(2′,5′)-bisphosphate nucleotidase                                 |
| cysC       | 10.1        | 4.32E−21    | adenylysulfate kinase                                                          |
| cysD       | 6.5         | 4.10E−09    | Sulfate adenyltransferase, CysD subunit                                       |
| cysN       | 4.9         | 1.30E−07    | Sulfate adenyltransferase, CysN subunit                                       |
| ssuA       | 6.5         | 4.24E−07    | Aliphatic sulfonate ABC transporter - periplasmic binding protein              |
| livH       | −2.7        | 2.12E−06    | Branched chain amino acid and phenylalanine transporter - membrane subunit     |
| ssuE       | 3.7         | 2.12E−04    | NADPH-dependent FMN reductase                                                  |
| sbp        | 2.4         | 1.53E−02    | Sulfate/thiosulfate ABC transporter - periplasmic binding protein Sbp          |
| cbl        | 2.1         | 1.62E−02    | Cbl DNA-binding transcriptional activator                                      |
| malM       | −2.2        | 1.62E−02    | Maltose regulon periplasmic transport                                          |
| nuoK       | −2.2        | 4.80E−02    | NADH:ubiquinone oxidoreductase, membrane subunit K                            |

Pairwise comparison of zosteric acid producing strain relative to control. Genes with false-discovery rate (FDR)-corrected p-values (t-test) smaller than 0.05
resulting in inhibition of growth. The results suggest that with the optimized strains, the supply of tyrosine was the limiting factor. Future work could focus on combination of the presented work with high-yield conversion of glucose to tyrosine by means previously reported35.

**Discussion**

Here, we present a general process for high-titer production of sulfated biochemicals through screening of sulfotransferases and optimization of sulfate uptake and activation. We link a biosynthetic pathway to a phenolic compound with phenol sulfotransferase activity in a microbial cell factory. This enabled the complete biosynthesis of a sulfated phenolic compound, zosteric acid, from a minimal medium with glucose as a carbon source. Conclusively, the sulfated product can be formed from an unsulfated precursor molecule when this is either produced in vivo or supplemented exogenously. An efficient precursor supply is required, as exemplified by the various tyrosine ammonia-lyases and p-cumaric acid levels tested, however at excessive substrate levels, there is inhibition of product formation which is consistent with formation of a dead-end complex with PAP-bound enzyme36. We demonstrate that the sulfate donor PAPS and sulfate itself becomes a limiting factor for the production of sulfated biochemicals. Therefore, we examined overexpression of genes encoding sulfate uptake, activation and recycling of nucleotides, which combined forms a sulfate assimilation cycle. We found that for production of a sulfated biochemical all of these reactions were required for the highest production titers. The sulfate pathways are naturally regulated at the genetic level in response to the availability of sulfate assimilation metabolites, however, this regulation proved insufficient in a strain that was genetically modified to produce large amounts of a sulfated biochemical. The exemplary product, zosteric acid, was found in the supernatant of bacterial cultures, suggesting that it is secreted from the cell. We have future research interest in

| Table 2 Production of zosteric acid from glucose or tyrosine |
|------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Strain          | TAL       | PST      | Transport   | Activation | Supplement | pHCA /mM | ZA /mM |
|-----------------|-----------|----------|-------------|------------|------------|----------|--------|
| CBJ262          | TAL<sub>pyo</sub> | SULT1A<sub>1exo</sub> | None        | None       | 10 mM tyrosine | 3.47 ± 0.98 | 0.29 ± 0.16 |
| CBJ264          | TAL<sub>pyo</sub> | SULT1A<sub>1exo</sub> | None        | CysDNSQ    | 10 mM tyrosine | 1.61 ± 0.5  | 2.23 ± 0.94 |
| CBJ266          | TAL<sub>pyo</sub> | SULT1A<sub>1exo</sub> | CysPUWA     | CysDNSQ    | None       | 0.52 ± 0.21 | 0.25 ± 0.09 |
|                 |           |          |             |            | 2 mM tyrosine | 0.40 ± 0.26 | 1.38 ± 0.23 |
|                 |           |          |             |            | 10 mM tyrosine | 1.00 ± 0.44 | 2.56 ± 0.87 |

Resulting production titers (µM) of p-coumaric acid (pHCA) or zosteric acid (ZA) are shown as averages with standard deviations (n = 3). Source data are provided as a Source Data file.

Fig. 5 Production of zosteric acid in fed-batch bioreactor cultivation. Growth and production in a 1-L fermenter under fed-batch conditions in minimal salts media without tyrosine (a) or with supplementation of exogenous tyrosine (b).

Identification of the possible transport mechanisms for this and other sulfated compounds. Synthesis of sulfated phenolic compounds using enzymes enables specific sulfation of selected groups using a sulfate carrier as donor through the action of either bacterial arylsulfate sulfotransferases or PAPS-dependent sulfotransferases. As a biocatalytic process, this is limited by the supply of excess sulfate donors. These could come in the form of PAPS or an existing phenol–sulfate conjugate such as p-nitrophenol sulfate (p-NPS)<sup>10</sup>. Using the metabolic capabilities of a whole-cell factory, the obstacle of having a specific sulfur source is readily overcome.

In the present work, we chose to perform the screening of sulfotransferase candidates in vivo, even though p-NPS or 4-methylumbelliferyl sulfate (MUS)<sup>41</sup> could be used in enzymatic assays due to the generation of color and fluorescence of their respective non-sulfated counterparts, p-nitrophenol and 4-methylumbelliferone. Both compounds were included in this study as substrates for sulfation, but as shown in Fig. 2, the various enzymes did not have identical preference for these compounds, arguing for using in vivo rather than in vitro screening for the sulfotransferases suitable to be used in a microbial cell factory.

Previously characterized PAPS-dependent phenol sulfotransferases are predominantly mammalian, and their natural function is the clearance of exogenous compounds, since sulfate conjugates are less toxic and more soluble, thereby facilitating their excretion. The enzymes also function to activate and stabilize hormones. Here, we have included bacterial enzymes as well. The sulfation of an aminoribose-moiety of a secondary metabolite in Streptomyces occurs through a sulfation of an intermediate: a triketide pyrone. The reaction is catalyzed by a PAPS-dependent sulfotransferase Cpz8, and subsequent transfer of the sulfate group by a PAPS-independent sulfotransferase Cpz4 to the aminoribose-moiety of an intermediate in the synthesis of sulfated caprazamycin antibiotics<sup>16,42</sup>. A similar transfer of
sulfate from a donor molecule rather than PAPS occurs in the biosynthesis of similar fatty acid nucleoside antibiotics. We included the codon optimized cpz8 gene as well as the homolog lipC, and the closest homolog to Cpz8 in eukaryas; namely the gene encoded by SPPG_07427 in the fungus Spizellomyces punctatus DAOM BR117. We also included the Halangiun ochraceus DSM 14365 Hoch_6098 and Rubrobacter radiotolerans RradSPS_0172, the closest homologs to SPPG_07427 by BLASTP at the time of searching. Interestingly, Hoch_6098 showed reactivity towards resveratrol, and this appears to be a functional bacterial PAPS-dependent phenol sulfotransferase.

Producing sulfated conjugates as end-products in biotechnology has the potential to overcome the problems of toxic effects of the non-conjugated products. For example, p-coumaric acid is highly toxic to E. coli, making it challenging to produce high titers without two-phase fermentations. Likewise, polyphenolic compounds are difficult to produce in large amount due to various inhibitory effects. While polyphenols are for example reported to have anti-cancer effects through targeting mitochondrial ATPases, they also target the ATPase of E. coli, a biotechnological production organism which has been employed for the production of flavonoids and resveratrol. Also, the end-product could be subject to either chemical degradation, or catabolism of the host organism. To circumvent these issues, formation of a sulfate conjugate could protect both the end-product and the production host. This study offers guidance to the further development of microbial cell factories for production of a wide range of sulfated compounds as final products or as soluble, non-toxic derivatives.

Methods

Chemicals, strains and media. All chemicals were purchased from Sigma-Aldrich, except trans-resveratrol 3-sulfate sodium salt (Santa Cruz Biotechnology, USA) and zosteric acid (ChirioBlock, Germany). E. coli strains were routinely grown in rich medium, 2XYT or LB, with appropriate antibiotics for selection of plasmids; 34 µg mL⁻¹ chloramphenicol for pLSyS, 100 µg mL⁻¹ ampicillin for pETDuet-1-derived plasmids, 50 µg mL⁻¹ spectinomycin for pCDFDuet-1-derived plasmids, and 50 µg mL⁻¹ kanamycin for pKSFDETU-Derived plasmids. For growth experiments, M9 minimal medium with 0.4% glucose, 2 mM pHCA, 1 mM IPTG, 100 mg mL⁻¹ ampicillin for selection for pLysS and plasmids encoding phenol sulfotransferases. Genes encoding tyrosine ammonia-lyases and phenol sulfo transferase were amplified using the oligonucleotides listed in Supplementary Data 2 and inserted alone or in combination by uracil excision cloning into the vector pCBJ132 after the Phe/Arg promoter or the PTE1 promoter, respectively. The finished plasmids (Supplementary Data 3) were transferred into S. cerevisiae CEN.PK102-5B selecting for growth on synthetic dropout medium plates lacking uracil. Stains are listed in Supplementary Data 4.

Growth conditions. Screening of phenol sulfoxo transferase activity: strains containing plasmids were grown overnight in 2xYT with chloramphenicol and ampicillin for selection for pLSyS and plasmids encoding phenol sulfoxo transferases. Inner and outer rounds for enzymatic activity, 50-180 µg mL⁻¹ of cultures (KRX background) were used to inoculate 950 µL M9 minimal medium with 4 mM MgSO₄, 1 mM IPTG, 100 µM kaempferol, 10 µM resveratrol, 50 µM vanillic acid. The plates were incubated at 37 °C in a water bath. They were inoculated to OD600 0.001-0.005 and the exponential phase growth rates were determined by following the density at 600 nm.

For testing of inhibitory effect of phenol conjugate on E. coli MG1655 was grown in chemically defined M9 minimal medium with 0.2% glucose as a carbon source without further addition or with the addition of either 10, 20, 25, 30, 35 or 40 mM p-coumaric acid (pHCA), or with 20 or 40 mM of zosteric acid. All media preparations had been adjusted to pH 7. Cells were grown at 37 °C with agitation and the exponential phase growth rates were determined by following the optical density at 600 nm.

Transcriptomic analysis. Strains CBJ1041 (BL21(DE3)/pCBJ136 + pCBJ72) and CBJ1055 (BL21(DE3)/pETDuet-1 + pKSFDETU-Derived) were grown in 50 mL M9 minimal medium with 0.4% glucose, 2 mM pHCA, 1 mM IPTG, 100 mg mL⁻¹ kanamycin and 50 µg mL⁻¹ kanamycin in 250 mL baffled shake flasks in a shaking, 37 °C water bath. They were inoculated to OD600 = 0.05 from an overnight culture. At OD600 = 0.7, cells were harvested for isolation of RNA by mixing 1000 µL with 250 µL ice-cold STOP solution (phenol saturated with 0.1 M citrate buffer, pH 4.3, diluted to 5% in 99% ethanol). 1000 µL samples were also withdrawn at the same time for HPLC analysis. Samples were kept cold, centrifuged at 6500 x g, 4 °C, for 5 min, followed by removal of supernatant and snap freezing in liquid N₂ before storing at −80 °C. The HPLC samples were centrifuged twice at 13,000 × g for 3 min for isolation of supernatant. Cells were lysed with lysozyme in TE buffer and total RNA was isolated using the RNeasy mini kit (Qiagen). The RNA quality was assessed by running RNA Nano prokaryote on a Bioanalyzer (Agilent) and RNA was quantified with a Qubit RNA assay (Invitrogen). Libraries were prepared and run on a NextSeq sequencer (Illumina) using a Mid Output Kit. Paired-end reads were mapped to the chromosomal sequence of BL21(DE3) and counted and normalized to fragments per kilobase of published reads (FPKM), before fold-change and false-discovery rate (FDR) adjusted p-values were calculated for the pairwise comparison. Gene ontology enrichment analysis was performed by a PANTHER Overrepresentation Test, the GO Ontology database released 2019–01–01 using Fisher's exact test with FDR correction.

Fed batch fermentation. Fermentations were performed in Sartorius 1-L fermenters. Initial batch media (500 mL) contained 2 g L⁻¹ KH₂PO₄, 5 g L⁻¹
(NH₄)₂SO₄, 2 g L⁻¹ MgSO₄·7H₂O, 2 mL of 1000× M9 vitamin solution, 25 µL of a 1 M CaCl₂ solution, 5 g L⁻¹ glucose, 2 g L⁻¹ yeast extract (Oxoid LP0021), 1 mL of trace element solution, 100 µg mL⁻¹ ampicillin, 50 µg mL⁻¹ spectinomycin, and 50 µg mL⁻¹ kanamycin. The temperature was adjusted to 37 °C, and pH was adjusted with 2 M NaOH to pH 7.0. 10 mL of an overnight culture of CB1292 was used to inoculate the media, and oxygen saturation was kept at minimum 40% dissolved oxygen using a cascade controlling stirring and sparging of air. At OD₆₀₀ = 4.5, the cultures were induced with IPTG to 100 µM. At OD₆₀₀ = 6, the fed-batch was initiated. The feed contained 2 g L⁻¹ KH₂PO₄, 15 g L⁻¹ (NH₄)₂SO₄, 2 g L⁻¹ MgSO₄·7H₂O, 1 mL of 1000× M9 vitamin solution, 25 µL of a 1 M CaCl₂ solution, 420 g L⁻¹ glucose, 2 g L⁻¹ yeast extract (Oxoid LP0021), 1 mL of trace element solution, 100 µg mL⁻¹ ampicillin, 50 µg mL⁻¹ spectinomycin, 50 µg mL⁻¹ kanamycin and 100 µM IPTG. pH was maintained by automatic adjustment with 2 M NaOH, and manually with slow addition of 1 M HCl 2–5 mL samples were taken manually for determination of OD, and sampling for glucose, acetate, tyrosine, pHCA and zosteric acid. For the culture with tyrosine addition, multiple points were taken manually for determination of OD, and sampling for glucose, acetate, tyrosine, and confers pathogen resistance in Arabidopsis.

Bioinformatic analysis. Phylogenetic tree was constructed using muscle alignment of protein sequences and neighbor-joining algorithm with a JTT substitution matrix with 1000 bootstrap replications in MEGA7.6.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Next-generation sequencing data is available at the NCBI Sequence Read Archive (SRA) with the BioProject ID PRJNA487499. Plasmids are available upon request from the laboratory or from Addgene. The source data underlying Figs. 2d, 3 and 4 and Table 2 are provided as a Source Data file.

Received: 16 July 2018 Accepted: 19 August 2019
Published online: 06 September 2019

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Acknowledgements
This work was funded by a grant from the Novo Nordisk Foundation (grant number NNF10CC1016517) to the Technical University of Denmark as well as a grant to Christian Bille Jendresen (grant number NNF15OC0015246).

Author contributions
C.B.J. designed and performed the experiments and wrote the manuscript. A.T.N. supervised the work. Both authors read, corrected and approved the final manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-12022-x.

Competing interests: C.B.J. and A.T.N. have filed provisional applications on this work and are co-founders of Cysbio ApS.

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Peer review information: *Nature Communications* thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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