A New Arylsulfate Sulfotransferase Involved in Liponucleoside Antibiotic Biosynthesis in Streptomyces*§

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Sulfotransferases are involved in a variety of physiological processes and typically use 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the sulfate donor substrate. In contrast, microbial arylsulfate sulfotransferases (ASSTs) are PAPS-independent and utilize arylsulfates as sulfate donors. Yet, their genuine acceptor substrates are unknown. In this study we demonstrate that Cpz4 from Streptomyces sp. MK730–62F2 is an ASST-type sulfotransferase responsible for the formation of sulfated liponucleoside antibiotics. Gene deletion mutants showed that cpz4 is required for the production of sulfated caprazamycin derivatives. Cloning, overproduction, and purification of Cpz4 resulted in a 58-kDa soluble protein. The enzyme catalyzed the transfer of a sulfate group from p-nitrophenol sulfate (Km 48.1 μM, kcat 0.14 s−1) and methyl umbelliferone sulfate (Km 34.5 μM, kcat 0.15 s−1) onto phenol (Km 25.9 and 29.7 μM, respectively). The Cpz4 reaction proceeds by a ping pong bi-bi mechanism. Several structural analogs of intermediates of the caprazamycin biosynthetic pathway were synthesized and tested as substrates. However, a small class of microbial sulfotransferases is PAPS-independent and transfers a sulfate group from an aromatic donor to an aromatic acceptor molecule. These enzymes were termed arylsulfate sulfotransferases (ASSTs). The reaction of ASSTs proceeds by a ping pong bi-bi mechanism with a covalent modification of the enzyme by the sulfation of an active site histidine. In vitro studies of eight ASSTs have been published from bacteria (10–17), but for all of them their precise physiological role is yet unknown. We now report the discovery of an ASST-type sulfotransferase, which is responsible for the sulfation reaction in the biosynthesis of liponucleoside antibiotics.

Recently, we identified the genes for caprazamycin (CPZ) biosynthesis in Streptomyces sp. MK730–62F2 (18) as well as the liposidomycin (LPM) gene cluster in Streptomyces sp. SN-1061M (19). The CPZs and the LPMs are liponucleoside antibiotics of unusual structure comprising an N-alkylated glycyrlidine, an O-aminoribosyl moiety, and the characteristic diazepanone ring (20, 21). While the previously described CPZs contain a permethylated L-rhamnose, the LPMs are decorated with a sulfate group at the 2′-hydroxy of the aminoribose. The CPZs and LPMs are inhibitors of the translocase I (22, 23), an

Sulfation describes the transfer of a sulfate group onto an acceptor molecule. It alters the properties of the target substrate in terms of solubility, charge, and size considerably and is a ubiquitous reaction in living organisms. The responsible enzymes, the sulfotransferases, participate in different processes such as intracellular signaling, extracellular interaction, and detoxification of xenobiotics (1). Sulfotransferases have been extensively studied in humans and other mammals and are usually classified in the membrane-associated and the cytosolic type. In contrast, little is known about bacterial sulfotransferases. Recent reports suggested their function as important modulators of e.g. plant host interaction in Sinorhizobium meliloti (2) and virulence in Mycobacterium tuberculosis (3). Several sulfated bioactive compounds were isolated from microorganisms over the last decades, including the micafungin precursor FK463, (4), the cyanobacterial toxin cylindrospermopsin (5), and the liponucleoside antibiotic liposidomycins (6). However, in bacterial secondary metabolism, only few sulfotransferases have been studied in vitro. StaL, involved in the biosynthesis of the glycopeptide A47934, catalyzes the transfer of a sulfate group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) onto the acceptor substrate (7, 8). Another PAPS-dependent sulfotransfer reaction has been investigated in curacin biosynthesis (9).

The vast majority of known sulfotransferases from eukaryotes and prokaryotes uses PAPS as the sulfate donor substrate. However, a small class of microbial sulfotransferases is PAPS-independent and transfers a sulfate group from an aromatic donor to an aromatic acceptor molecule. These enzymes were termed arylsulfate sulfotransferases (ASSTs). The reaction of ASSTs proceeds by a ping pong bi-bi mechanism with a covalent modification of the enzyme by the sulfation of an active site histidine. In vitro studies of eight ASSTs have been published from bacteria (10–17), but for all of them their precise physiological role is yet unknown. We now report the discovery of an ASST-type sulfotransferase, which is responsible for the sulfation reaction in the biosynthesis of liponucleoside antibiotics.

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The essential enzyme in peptidoglycan biosynthesis, and they show an excellent activity against mycobacteria (24, 25). Chemical synthesis has generated a number of liponucleoside analogs for use in drug development (26–30). Comparing the biosynthetic gene clusters of the CPZs and the LPMs, we found a set of genes responsible for the sulfation of LPMs (19). Surprisingly, similar genes are located adjacent to the CPZ gene cluster. Consistently, the analysis of the corresponding producer strains led to the identification of new sulfated CPZ derivatives (19).

We now show that Cpz4, a protein encoded close to the CPZ gene cluster, acts as a sulfotransferase in liponucleoside biosynthesis. We determined the kinetic mechanism and, for the first time, we could identify a genuine sulfate acceptor substrate of an ASST-type sulfotransferase.

EXPERIMENTAL PROCEDURES

Materials, Strains, and General Methods—Chemicals and microbiological and molecular biological agents were purchased from standard commercial sources. Streptomyces coelicolor M512 (SCP1\(^{-}\), SCP2\(^{-}\), \(\Delta\text{actIIORF4}, \Delta\text{redD}\)) and the respective derivatives were maintained and grown on either MS agar (2% soy flour, 2% mannitol, 2% agar; components were purchased from Carl Roth, Karlsruhe, Germany) or TS medium (BD Biosciences). Escherichia coli strains were cultivated in LB medium (components were purchased from Carl Roth) supplemented with appropriate antibiotics. DNA isolation and manipulations were carried out according to standard methods for E. coli (31) and Streptomyces (32).

Production, Isolation, and Analysis of Metabolites—Metabolites from S. coelicolor M512 or derivatives thereof were produced and isolated as described elsewhere (18). LC-MS/MS analysis was performed on a Surveyor HPLC system equipped with a ReproSil-Pur Basic C18 (5 μm, 250 × 2 mm) column (Dr. Maisch, Ammerbuch, Germany) coupled to a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (heated capillary temperature, 320 °C; sheath gas, nitrogen). For sample separation, a linear gradient from 2 to 40% acetone in aqueous formic acid (0.1%) over 4 min followed by a linear gradient from 40 to 100% acetone in aqueous formic acid (0.1%) over 31 min was used; the flow rate was 0.2 ml min\(^{-1}\); UV detection was at 262 nm. Positive electrospray ionization ((+)-ESI) was performed with electrospray voltage of 3.8 kV, and collision-induced dissociation spectra were recorded with collision energy of 35 eV. The respective parameters in negative mode ((-)ESI) were 4.0 and 25 eV. The same methods were used to analyze product formation in the assays with S. coelicolor M512 or derivatives thereof to 0.5 mM final concentration. After an additional 10 h cultivation at 20 °C, the cultures were harvested, and 10 ml of buffer A (50 mM Tris-HCl, pH 8, 1 mM NaCl, 10% glycerol, 10 mM \(\beta\)-mercaptoethanol) supplemented with 0.5 mg ml\(^{-1}\) lysozyme and 0.5 mM phenylmethylsulfonyl fluoride was added to the pellets. Cells were disrupted by sonication (Branson, Danbury, CT) at 4 °C. The lysates were centrifuged (55,000 \(\times\) g, 45 min), and the supernatants were applied to affinity chromatography using an Actapurifier\(^{TM}\) platform (GE Healthcare) equipped with a His-Trap\(^{TM}\) (34 μm, 1.6 × 2.5 cm) HP column (GE Healthcare) according to the platform described above. A yield of 12.5 mg of purified His\(_8\)-Cpz4 was obtained per liter of culture. Cpz4 was stored at 0 °C in aliquots.

Conditions for Photometric and Fluorometric Assays—Assays for the sulfotransferase activity of Cpz4 and mutants (125–750 nM) were performed at 30 °C in 100 mM MES, pH 6.7, containing 1 mM NaCl and 0.1 mM EDTA (reaction buffer A). Phenol as the sulfate acceptor substrate was used in concentrations of 10–160 mM. Conversion of pNS as sulfate donor substrate was followed by the addition of 500 μl 1 M Tris-HCl, pH 8 after incubation by the addition of 500 μl of 1 M Tris-HCl, pH 8.
Conversion of MUS as sulfate donor (100 – 450 μM in a reaction volume of 200 μl) was monitored on a Synergy™ HT Multi-Detection Microplate Reader (BioTek, Bad Friedrichshall, Germany) at 460 nm (excitation 360 nm). Both systems were calibrated with at least 5 different concentrations of p-nitrophenol or methyl umbelliferone for each single experiment. Sulfatase activity of Cpz4 was checked in parallel control reactions without phenol for each single assay. Concentrations of Cpz4 or variants were determined by extinction at 280 nm (ε280 = 82,850 M⁻¹ cm⁻¹). For the investigation on the effect of specific inhibitors, diethyl pyrocarbonate was added directly to the assay to give a final concentration of 3 mM. Phenylmethylsulfonyl fluoride was dissolved in methanol (100 mM) and added to the assay to give a concentration of 1 mM. 1% methanol in the assay had no effect on the activity of Cpz4.

**Steady State Kinetics**—Reaction velocities of Cpz4 with pNS as the donor substrate (27–270 μM) and phenol as the acceptor substrate (6.5–160 mM) were measured in 500 μl of reaction buffer A containing 125 mM protein. Incubation time was 25 min. Reaction velocities of Cpz4 with MUS as the donor substrate (25–550 μM) and phenol as the acceptor substrate (12.5–160 mM) were monitored over 15 min in 200 μl of reaction buffer A containing 100 mM protein. Initial rates were globally fitted with GraFit 6 (Enithacus Software, Surrey, UK) to the equation $v = \frac{V[A][B]}{(K_a[B] + [B])[K_a] + K_a} + [A][B]$ describing ping pong kinetics with substrate inhibition (10). $K_a$ and $[B]$ are the initial concentrations of pNS or MUS and phenol, respectively. $K_a$ and $K_b$ are the respective Michaelis constants for pNS or MUS and phenol. The inhibition constant of phenol is $K_{ib}$. $V$ is the maximum, and $v$ is the measured initial velocity.

**Sulfation of Caprazamycin Derivatives**—Partially purified caprazamycin derivatives were obtained from the culture extract of *S. coelicolor* M512/cpzWP05 (CPZ aglycones), *S. coelicolor* M512/cpzEW07 (CPZs), and *S. coelicolor* M512/cpzLL06 (hydroxyacetyl-caprazols) as described elsewhere (18). Caprazamycins, caprazamycin aglycones, or hydroxyacyl-caprazols were dissolved in 500 μl of reaction buffer A containing 500 μM pNS and 1.5–2 μM Cpz4. The assay was performed at 30 °C overnight. Conversion of pNS was measured at 405 nm. Assays with PAPS (500 μM) as the sulfate donor substrate (instead of pNS) were performed under the same conditions but included 0.1 mM sodium ascorbate to avoid oxidation. 0.1 mM sodium ascorbate in the assay had no effect on the activity of Cpz4. Product formation was analyzed by LC-MS/MS.

For the sulfation of the synthetic caprazamycin precursor, compounds 1–6 were added to a final concentration of 200 μM to reaction buffer A containing 300 μM pNS and 125 mM protein. The reaction was measured at 405 nm after 60 min. The assays were stopped by the addition of 1 volume of methanol before LC-MS/MS analysis of product formation.

**Site-directed Mutagenesis of Cpz4**—Cpz4 variants were generated by site-directed mutagenesis using plasmid pLK03 as template and primer H180L-fw (GGCCGAGGCCATCTCTTCAGATGACATGACATCCT) and H180L-rv (AGTGATCTGGCATGTCAAGAGATCGGCTCTCGCCG), H253L-fw (CGCTGTGATGCTATGGATGCACCCCGCCG), and H253L-rv (GGCACTATGATGCGACATGCAGTGATCGGCGC). Site-directed mutagenesis using plasmid pLK03 as template and primer H180L-fw (GGCCGAGGCCATCTCTTCAGATGACATGACATCCT) and H180L-rv (AGTGATCTGGCATGTCAAGAGATCGGCTCTCGCCG), H253L-fw (CGCTGTGATGCTATGGATGCACCCCGCCG), and H253L-rv (GGCACTATGATGCGACATGCAGTGATCGGCGC). PCR and subsequent DpnI restriction was performed according to the QuikChange™ manual (Stratagene, Heidelberg, Germany). Cpz4 variants were verified by sequencing and cloned into vector pHis8 (33). Overproduction of mutant proteins was performed in 1 liter of TB as described above. The supernatants of the cell lysates were applied to affinity chromatography with 4 ml of nickel-nitrilotriacetic acid-agarose resin (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The proteins were eluted from the column with 250 mM imidazole in buffer A. Buffer exchange was carried out by PD10 columns (Amersham Biosciences) and buffer A without β-mercaptoethanol.

**Synthesis of Caprazamycin Precursor**—Compounds 1 and 3 were synthesized as previously reported in Hirano et al. (28) and compound 2 as reported in Hirano et al. (29). Compound 4 was synthesized as follows. N⁵-tert-Butoxy carbonyl-2',3'-O-isopropylidene-2',3'-O-(3-pento l yl idine)-3'''-O-tri ethylsilyl-N⁷'-desethylcaprazol 2'-O-tert-butyl ester (28) (10 mg, 0.011 mmol) was treated with 80% aqueous TFA (1 ml) at room temperature for 56 h. The mixture was concentrated in vacuo to give 4 (a TFA salt, 8.0 mg, quantitated) as a white solid.

Compound 5 was synthesized as follows. 6-Deoxy-5'-O-[5-tert-butoxy carbonylamino-5-deoxy-2,3-O-(3-pentylidine)-β-D-ribo-pento furanosyl]-6'-N-[3-(tert-butoxy carbonylamino pro pyl)-amino-1-(uracil-1-yl)-2,3-O-isopropylidene-β-D-glycero-1-talo-heptofuranuronate (34) (13 mg, 0.015 mmol) was treated with 80% aqueous TFA (2 ml) at room temperature for 24 h. The mixture was concentrated in vacuo to give 5 (a TFA salt, 10.5 mg, quantitated) as a white solid.

Compound 6 was synthesized as follows. (2S)-2-(2,2,2-Trichloroethoxy carbonylamino)-4-pentenoic acid (35) (2.05 g, 7.09 mmol) in tetrahydrofuran (60 ml) was treated with N,N'-diisopropyl- O-tert-butyli s ourea (2.14 g, 10.6 mmol) at room temperature for 12 h. After H₂O (191 ml) was added to the reaction mixture, the mixture was further stirred for 1 h and concentrated in vacuo. The residue was puriﬁed by neutral flash silica gel column chromatography (2 × 20 cm, 5% AcOEt hexane) to afford tert-buty l(2S)-2-(2,2,2-trichloroethoxy carbonylamino)-4-pentenoate (1.10 g, 54%) as a colorless oil. A solution of tert-buty l(2S)-2-(2,2,2-trichloroethoxy carbonylamino)-4-pentenoate (380 mg, 1.12 mmol) in CH₃Cl₂ (10 ml) was treated with O₃ at 78 °C for 30 min. The resulting mixture was treated with triphenyl-phosphine (432 mg, 1.65 mmol) and stirred for 10 min. Concentrating the mixture in vacuo gave a corresponding aldehyde, which was used to the next step without puriﬁcation. A mixture of the aldehyde, tert-buty l(2S)-2-(2,2,2-trichloroethoxy carbonylamino)-4-pentenoate (1.10 g, 54%) and compound 3 (34) (800 mg, 1.12 mmol) and AcOH (674 μl, 11.2 mmol) in CH₃Cl₂ (10 ml) was treated with NaBH(OAc)₃ (714 mg, 3.37 mmol) at room temperature for 15 min. The mixture was poured onto AcOEt, which was washed with saturated aqueous NaHCO₃, H₂O, and saturated aqueous NaCl, dried (Na₂SO₄), and concentrated in vacuo. The residue was puriﬁed by neutral flash
silica gel column chromatography (2 × 20 cm, 33% AcOEt hexane) to afford the N-alkylated product as a white foam (630 mg, 54%). A part of the product (395 mg, 0.38 mmol) in MeCN (3 ml) and 1 M aqueous NaH$_2$PO$_4$ (1 ml) was treated with zinc (840 mg, 12.9 mmol) at room temperature for 10 h. After the resulting mixture was concentrated in vacuo, the residue was suspended in AcOEt. The suspension was filtered through a short neutral silica gel pad, and the filtrate was concentrated in vacuo to afford a white foam. A part of the material (120 mg, 0.14 mmol) was treated with 80% aqueous TFA (3 ml) at room temperature for 8 h. The mixture was concentrated in vacuo, and the resulting residue was triturated with AcOEt to afford compound 6 as a white solid (50 mg, 0.075 mmol). The purity of 6 was confirmed to be >98% by $^1$H NMR.

Structural assignment of compounds 4, 5, 6, and tert-butyl (2S)-2-(2,2,2-trichloroethoxycarbonylamino)-4-pentenoate was based on $^1$H, $^1$H COSY; heteronuclear multiple bond and quantum coherence NMR spectra, and ESI-MS analysis as described in supplemental Experimental Procedures. Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60F$_{254}$ plates. Normal phase column chromatography was performed on Merck silica gel 5715. Flash column chromatography was performed on Merck silica gel 60.

RESULTS

Analysis of a cpz4 Deletion Mutant in S. coelicolor M512/cpzWP05—We recently showed that the heterologous host strain S. coelicolor M512 harboring the entire CPZ gene cluster (Fig. 1) not only produces the CPZ aglycones but also sulfated derivatives thereof, i.e. the LPMs (19) (Fig. 2A). In our search for the gene, which might be responsible for the sulfation reaction, we focused on cpz4 (accession number GU323955) located 4.2 kb upstream of the gene cluster (Fig. 1). Cpz4 exhibits significant sequence homology to a large number of hypothetical proteins from bacteria and fungi. A conserved protein domain (Pfam05935) was found, characteristic for the ASSTs (12). Overall, the known ASSTs show only low sequence similarity to Cpz4, e.g. 26% in the case of the ASST from E. coli CFT073 (10). To investigate the role of cpz4, we compared the previously generated deletion mutant S. coelicolor M512/cpzWP05
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FIGURE 2. HPLC-UV and HPLC-MS analysis of n-butanolic extracts from a cpz4 mutant strain. A, shown is a UV chromatogram of an extract from S. coelicolor M512 containing cosmid cpzLK09 with the intact caprazamycin gene cluster. Arrows indicate accumulated liponucleosides. B, shown is a UV chromatogram of an extract from S. coelicolor M512/cpzWP04 containing a derivative of cosmid cpzLK09 in which the genes cpz1-cpz3 are deleted. C, shown is the respective product ion chromatograms. D, shown is a UV chromatogram of an extract from S. coelicolor M512/cpzWP05 containing a derivative of cosmid cpzLK09 in which the genes cpz1-cpz4 are deleted. E, shown are the respective product ion chromatograms. UV absorption was monitored at 262 nm. Product ion chromatograms were obtained from (+)-ESI-MS mass scans for CPZ E/F aglycones with m/z 930.5 [M+H]^+. CPZ C/D/G aglycones with m/z 944.5 [M+H]^+, CPZ A/B aglycones with m/z 958.5 [M+H]^+, and for their corresponding sulfated derivatives LPM E/F with m/z 1010.5 [M+H]^+, LPM C/D/G with m/z 1024.5 [M+H]^+, and LPM A/B with m/z 1038.5 [M+H]^+. (Δcpz1-4) (18) with mutant S. coelicolor M512/cpzWP04 (Δcpz1-3). Culture extracts were applied to LC-ESI-MS/MS and analyzed for the presence of LPMs. As expected, the CPZ aglycones were accumulated by both mutants (Fig. 2). However, the sulfated LPMs (LPM E/F with m/z 1010.5 [M+H]^+ at Rt. 24.4 min, LPM C/D/G with m/z 1024.5 [M+H]^+ at Rt. 26.5 min, LPM A/B with m/z 1038.5 [M+H]^+ at Rt. 29.6 min) could only be identified in the mutant containing an intact copy of the cpz4 gene as demonstrated by HPLC-UV and HPLC-MS analysis (Fig. 2). This result proves that Cpz4 is essential for the sulfation of CPZ aglycones.

A homolog of Cpz4 was also found in the LPM gene cluster of Streptomyces sp. SN-1061M. lpmB is located at one end of the cluster, and its gene product shows 32% identity and 50% similarity to the central 300 amino acids of Cpz4. Notably, LpmB (412 amino acids) lacks 101 amino acids that form the C terminus of Cpz4 (513 amino acids). It appears likely that lpmB has a similar function as Cpz4 in LPM biosynthesis, though a conserved protein domain such as Pfam05935 could not be identified.

Overproduction and Purification of cpz4—For the biochemical investigation of the potential sulfotransferases, cpz4 and lpmB were cloned into an expression vector for the production as N-terminal His-tagged proteins and introduced into E. coli Rosetta2™ (DE3)pLys. Induction with isopropyl thiogalactoside resulted in the production of a protein with ~57.7 kDa, matching the calculated molecular mass of His8-Cpz4 (57.626 kDa) (supplemental Fig. S2). However, LpmB could not be obtained as a soluble protein. Hence, we proceeded with Cpz4 alone. Ni^{2+} affinity chromatography and subsequent gel chromatography resulted in 25 mg of protein from 2 liters of culture, showing ~95% purity in SDS-PAGE (supplemental Fig. S1). This protein was used for further biochemical studies.

Analysis of Cpz4 as an Arylsulfate Sulfotransferase—Identification of a conserved protein domain typical for ASSTs suggested that Cpz4 may catalyze a PAPS-independent sulfation reaction. Accordingly, Cpz4 was incubated with either pNS or MUS as sulfate donor substrates and phenol as an acceptor substrate. Product formation was monitored by LC-ESI-MS/MS analysis. In the total ion current chromatograms, a peak appeared at ~18.3 min both in assays with pNS and in assays with MUS as the donor substrate (Fig. 3A). The formation of this product was dependent on the presence of active Cpz4. The mass spectrometric data of the new compound (Fig. 3B) corresponded to the expected product, phenol sulfate, with m/z 173 [M-H]^- and MS2 fragments with m/z 93 (phenol) and m/z 80 (sulfate). These experiments showed that Cpz4 can act as an arylsulfate sulfotransferase.

Biochemical Properties of Cpz4—The biochemical properties of Cpz4 were determined with pNS as the sulfate donor and phenol as the acceptor substrate. Reaction velocities were spectrometrically measured at 405 nm, the absorption maximum for the product p-nitrophenol. The aryl sulfotransfer reaction was
strictly dependent on the presence of active Cpz4 and pNS. However, a low formation of p-nitrophenol was detected in assays without phenol, indicating a certain sulfatase activity of Cpz4. Therefore, hydrolysis of pNS was monitored in parallel assays without phenol, and the amount was subtracted from the initial results for all subsequent experiments. The reaction (as described under “Experimental Procedures”) showed a linear dependence on the amount of Cpz4 (up to 250 nM) and the incubation time (up to 90 min). In the absence of NaCl, pH values ≤7.0 resulted in precipitation of the protein in the assay. Inclusion of 1M NaCl prevented precipitation and allowed the assay of the protein in a range from pH 4.0 to 11.0. Maximal activity was observed at pH 6.7 with half-maximal values at pH 5.0 and 7.6. Low concentrations of Mg2+ (1, 5, 10 mM) had no significant effect on the reaction velocity. The presence of 20 mM Mg2+ or 2 mM Ni2+ in the assay led to the precipitation of the protein. Notably, the addition of 0.1 mM EDTA increased product formation of Cpz4 by 2.5-fold and was, therefore, routinely included in the assay.

Influence of Inhibitors on Cpz4
Activity—While PAPS-dependent sulfotransferases follow a sequential mechanism (1), the ASSTs, e.g. from Eubacterium A-44 (36) and from E. coli CFT073 (10), have been shown to react by a ping pong bi-bi mechanism with sulfation of an active site residue of the enzyme. Until recently, it had been assumed that the sulfate is transferred first to a histidine residue and subsequently to a tyrosine residue (1). However, Malojcic et al. (10) could only show the formation of a sulfohistidine in the E. coli ASST. Specific inhibitors were added to assays containing Cpz4, pNS, and phenol. In the presence of 1 mM phenylmethylsulfonyl fluoride, which targets nucleophilic hydroxy groups, the turnover of pNS by Cpz4 was not affected. In contrast, the addition of 3 mM diethyl pyrocarbonate, which specifically modifies histidine side chains (31), led to an ~90% decrease of the activity. These findings suggest that one or more histidine residues may be involved in the catalysis of the reaction. However, other explanations, e.g. an impact of diethyl pyrocarbonate on the flexibility or stability of the enzyme, cannot be ruled out completely.

Site-directed Mutagenesis—A multiple sequence alignment of characterized and putative ASSTs including Cpz4 (data not shown) revealed three highly conserved histidine residues, i.e. His-180, His-253, and His-308. The corresponding histidine residues in the ASST from E. coli CFT073 were reported to be essential for the catalysis of the sulfotransfer reaction (10). In this enzyme, the sulfate group is covalently bound to His-436 and further stabilized by His-252 and His-356. Consequently, each of these histidines was replaced with leucine in Cpz4 by site-directed mutagenesis. Heterologous production of the mutant enzymes as His-tagged proteins and subsequent purification led to 17.4 mg (H180L), 1 mg (H253L), and 6.2 mg (H308L) of soluble protein from 1-liter cultures. Activities of all three mutant enzymes against pNS and phenol were reduced to ~5% in comparison to the wild type enzyme. On the basis of these findings and the results obtained from the addition of specific inhibitors, we would assume a catalytic role of the histidines in Cpz4 similar to the ASST from E. coli CFT073.

Kinetic Analysis of Cpz4—Applying a constant concentration of phenol (75 mM) and varying concentrations of pNS, a plot of product formation over substrate concentration gave a hyper-
A bolic curve, as expected for Michaelis-Menten kinetics. Using different concentrations of phenol with 25 \( \mu \)M pNS, the reaction velocities increased up to a phenol concentration of 75 mM and thereafter declined, indicating substrate inhibition. To investigate the reaction mechanism, Cpz4 was incubated with different fixed concentrations of phenol (13 mm (○), 26 mm (×), 39 mm (□), and 52 mm (●)) shows parallel lines indicating a ping pong kinetics. B, shown are double-reciprocal plots of reaction velocities, obtained with pNS and phenol over phenol concentrations (13, 26, 39, 52, 75, 100, and 160 mM phenol). Partial regression of velocities from data sets with fixed initial concentrations of pNS (27 \( \mu \)M (■), 54 \( \mu \)M (◇), 108 \( \mu \)M (×), 189 \( \mu \)M (□), and 270 \( \mu \)M (●)) shows parallel lines up to a phenol concentration of \( \sim 50 \) mM. C, shown are initial velocities with MUS at initial fixed concentrations of 27 \( \mu \)M (○), 54 \( \mu \)M (◇), 108 \( \mu \)M (×), 189 \( \mu \)M (□), 270 \( \mu \)M (●) over phenol (0, 6.5, 13, 26, 39, 52, 75, 100, and 130 mM). This data set was globally fitted (under "Experimental Procedures") to obtain the depicted kinetic parameters for Cpz4 with MUS and phenol as substrates. D, shown are initial velocities with MUS at initial fixed concentrations of 25 \( \mu \)M (○), 50 \( \mu \)M (△), 75 \( \mu \)M (●), 100 \( \mu \)M (□), 150 \( \mu \)M (■), 200 \( \mu \)M (▲), 250 \( \mu \)M (◇), 300 \( \mu \)M (△), 350 \( \mu \)M (○), 450 \( \mu \)M (◇), and 550 \( \mu \)M (×) over phenol (12.5, 20, 36, 60, 70, 100, 130, and 160 mM). This data set was globally fitted (under "Experimental Procedures") to obtain the depicted kinetic parameters for Cpz4 with MUS and phenol as substrates. Dotted lines in C and D indicate linear dependence of the reaction velocities and phenol concentration on low phenol concentrations.

**FIGURE 4.** Kinetic analysis of the Cpz4-catalyzed sulfate transfer reaction from the aromatic donor substrates pNS and MUS to phenol. A, shown is a double-reciprocal plot of reaction velocities, obtained with pNS and phenol, over pNS concentrations (27, 54, 108, 189, and 270 \( \mu \)M pNS). Partial regression of velocities from data sets with fixed initial concentrations of phenol (13 mm (○), 26 mm (×), 39 mm (□) and 52 mm (●)) shows parallel lines indicating a ping pong kinetics. B, shown are double-reciprocal plots of reaction velocities, obtained with pNS and phenol over phenol concentrations (13, 26, 39, 52, 75, 100, and 160 mM phenol). Partial regression of velocities from data sets with fixed initial concentrations of pNS (27 \( \mu \)M (■), 54 \( \mu \)M (◇), 108 \( \mu \)M (×), 189 \( \mu \)M (□), and 270 \( \mu \)M (●)) shows parallel lines up to a phenol concentration of \( \sim 50 \) mM. C, shown are initial velocities with MUS at initial fixed concentrations of 27 \( \mu \)M (○), 54 \( \mu \)M (◇), 108 \( \mu \)M (×), 189 \( \mu \)M (□), 270 \( \mu \)M (●) over phenol (0, 6.5, 13, 26, 39, 52, 75, 100, and 130 mM). This data set was globally fitted (under "Experimental Procedures") to obtain the depicted kinetic parameters for Cpz4 with MUS and phenol as substrates. D, shown are initial velocities with MUS at initial fixed concentrations of 25 \( \mu \)M (○), 50 \( \mu \)M (△), 75 \( \mu \)M (●), 100 \( \mu \)M (□), 150 \( \mu \)M (■), 200 \( \mu \)M (▲), 250 \( \mu \)M (◇), 300 \( \mu \)M (△), 350 \( \mu \)M (○), 450 \( \mu \)M (◇), and 550 \( \mu \)M (×) over phenol (12.5, 20, 36, 60, 70, 100, 130, and 160 mM). This data set was globally fitted (under "Experimental Procedures") to obtain the depicted kinetic parameters for Cpz4 with MUS and phenol as substrates. Dotted lines in C and D indicate linear dependence of the reaction velocities and phenol concentration on low phenol concentrations.
time (up to 25 min). The reaction was dependent on the presence of MUS and phenol. No sulfatase activity could be observed in a control without phenol. A dataset was generated applying various phenol concentrations (12.5-160 mM) against constant initial MUS concentrations in a range from 25 to 550 μM (Fig. 4D). Double-reciprocal plotting of the reaction velocities over substrate concentration confirmed the ping pong bi-bi kinetics with substrate inhibition of reaction velocities over substrate concentration confirmed. Kinetic parameters were calculated as $V_{\text{max}} = 15.3\, \text{nmol s}^{-1}$, $K_{m,\text{MUS}} = 34.5\, \mu\text{M}$, $K_{m,\text{phenol}} = 29.7\, \text{mm}$, and $K_{l,\text{phenol}} = 41.7\, \text{mm}$, $K_{ca}$ was determined as 0.15 s$^{-1}$.

The $K_m$ values for pNS (48.1 μM) and MUS (34.5 μM) are similar to the $K_m$ for MUS published for the ASST from E. coli CFT073 i.e. 44.5 μM (10). However, catalytic efficiency for phenol ($k_{cat}/K_m$) is low at 5.5 and 5.1 M$^{-1}$ s$^{-1}$, as may be expected for an artificial substrate.

Sulfation of Caprazamycin Derivatives Extracted from Bacterial Cultures — To investigate the role of Cpz4 in the sulfation of liponucleosides, CPZ aglycones were partially purified and tested as possible substrates. Extracts from S. coelicolor M512/cpzWP05, which does not produce the sulfated LPMs, were incubated with Cpz4 and pNS. In a second experiment, pNS was replaced by PAPS to test the sulfate donor specificity of the sulfotransfer reaction. Therefore, we conclude that Cpz4 is a specific sulfotransferase for the biosynthesis of sulfated liponucleosides. It requires both the fatty acyl and the uridyl moiety of the liponucleoside for an efficient structural recognition of the substrate.

Product formation was verified by LC-ESI-MS/MS analysis in negative mode. Mass peaks for the sulfated products 1a and 2a were found with $m/z$ 878.4 [M-H]$^-$ at Rt. 26.2 min and $m/z$ 694.4 [M-H]$^-$ at Rt. 35.2 min, respectively. Ions with $m/z$ 848, 622, 578, and 466 in the 1a MS$^2$ spectrum (Fig. 6B) match the expected fragments containing a sulfated aminoribose. The ion with $m/z$ 349 may derive from an uridyl diazepanone substructure. The lack of an ion with $m/z$ 429, corresponding to a sulfated analog of the $m/z$ 349 fragment, suggests that the sulfation of compound 1 takes place at the aminoribosyl group, similar to the sulfation of LPMs. MS$^2$ fragmentation pattern of product 2a (Fig. 6C) displays an analogous situation.

**DISCUSSION**

In this study we show for the first time the physiological function of an arylsulfate sulfotransferase type enzyme. Gene deletion experiments proved the requirement of cpz4 for the biosynthesis of sulfated liponucleoside antibiotics, as in the absence of cpz4, the LPMs were not produced. Purified Cpz4 protein accepted different liponucleoside derivatives and synthetic caprazamycin precursors as substrates for a sulfotransfer reaction. Therefore, we conclude that Cpz4 is the sulfotransferase responsible for the biosynthesis of sulfated caprazamycins in Streptomyces sp. MK730–62F2. We isolated the hydroxacyl caprazols, the caprazamycin aglycones, and the intact caprazamycins from suitable engineered producer strains. All of them were sulfated by Cpz4. In this case, conversion rates could not be quantified as the substrates represent mixtures rather than pure compounds. Structural analogs of putative intermediates of the caprazamycin biosynthetic pathway were synthesized and investigated as substrates of the enzyme. Clearly the fatty acyl-containing caprazol derivative (1, Fig. 6) was sulfated with much higher reaction velocity than non-acylated analogs or phenol. Compound 2 lacking the acyl moiety was con-
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Recently, we observed that the rhamnosyltransferase Cpz31 from the CPZ gene cluster is able to glycosylate not only the CPZ aglycones but also the LPMs (data not shown). Therefore, we would favor the latter hypothesis. However, the physiological or ecological function of the sulfated liponucleosides is still unknown (10).

Cpz4 represents one of the few biochemically characterized sulfotransferases from bacterial secondary metabolism. However, in sharp contrast to StaL from Streptomyces toyocaensis (7), CurM-ST from the curacin gene cluster, (9), and to most...
other known sulfotransferases (1), Cpz4 does not utilize PAPS as the preferred sulfate donor. Instead, it efficiently uses aromatic sulfate donor substrates such as pNS or MUS resembling the ASSTs isolated from other bacteria and fungi. A genuine sulfate donor of the ASST-type sulfotransferases remains to be identified. Kinetic investigations showed that the reaction catalyzed by Cpz4 follows a ping pong bi-bi mechanism as has been reported for previously investigated ASSTs (10, 15, 36, 39). X-ray structural analysis and biochemical characterization of the ASST from *E. coli* CFT073 indicated the transient sulfation of an active site histidine residue (10). The results obtained for Cpz4 by site-directed mutagenesis and by use of inhibitors may suggest a similar role for one of the conserved histidine residues in Cpz4.

For none of the ASSTs reported so far, the genuine substrates are known. They have been biochemically investigated using phenol or other synthetic aromatic compounds as acceptor molecules. Cpz4 now represents the first ASST-type enzyme shown to preferentially sulfate the hydroxy group of the sugar moiety of a glycosidic substrate rather than a phenolic hydroxy group. While this report was under review another ASST-type sulfotransferase involved in A-90289 biosynthesis has been identified (40). Notably, a BLAST search identifies numerous genes with significant sequence homology to Cpz4 in bacterial and fungal genomes. Almost all of them are annotated as hypothetical proteins, and it remains to be shown whether they are structural genes for sulfotransferases. A sequence homology-based clustering analysis (41) of Cpz4, functionally characterized ASSTs and homologs thereof, separates two major clusters (supplemental Fig. S6). One of them comprises the *E. coli* ASST (10), and all of the other five previously characterized sulfotransferases (12–14, 16, 17). Within this group, the ASSTs from enterobacteria form a well defined subcluster, whereas the enzyme from *Eubacterium* A-44 (17) and putative ASSTs from other Firmicutes form another subcluster. A second, clearly separated cluster comprises Cpz4 and LpmB as well as further hypothetical proteins mainly from actinobacteria and fungi. The biochemical and physiological investigation of these potential ASSTs represents an exiting challenge for future research.
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