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Purification, cloning, and characterization of an arylsulfotransferase from the anaerobic bacterium *Eubacterium rectale* IIIH

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A bacterium, *Eubacterium rectale* IIIH, which possessed arylsulfotransferase (ASST) activity was isolated from human feces. The ASST gene (*astA*) was cloned and the corresponding protein partially characterized. This gene shows only moderate homology to the previously sequenced ASST genes of *Klebsiella* and *Enterobacter*, which are very closely related to each other. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 305–309.

Keywords: *Eubacterium*; arylsulfotransferase; cloning; overexpression

Introduction

Arylsulfotransferase (ASST) catalyzes the transfer of a sulfate group to phenolic acceptor substrates, and is therefore speculated to play an important role in the detoxification of compounds with phenolic functional groups [9]. The enzyme was identified from both mammalian organs [8,16,17] and from the bacteria *Eubacterium* A-44 [12,13] and *Klebsiella* K-36 [11]. The mammalian version requires the donor substrate 3′-phosphoadenosine 5′-phosphosulfate (PAPS), while both bacterial enzymes can use many phenolic sulfate esters as a source of sulfate molecules [12,16]. Aside from the importance of ASST in detoxification, this enzyme has potential use in the *in vitro* O-sulfation of peptide hormones that require modification of tyrosine groups for activity, such as cholecystokinin, vasopressin, and angiotensin [13]. The genes encoding ASSTs from *Klebsiella* K-36 [3,4] and *Enterobacter amnigenus* [4] have been sequenced and show very strong homology with each other. Interestingly, a study using the *Klebsiella astA* gene as a hybridization probe indicated no substantial homology to an ASST-producing *Eubacterium* strain. However, the *astA* gene from *Eubacterium* had not been characterized so a direct comparison between this and previously sequenced *astA* genes was not possible.

Our objective in this study was to identify and clone the *astA* gene from *Eubacterium* so a comparison between the DNA and protein sequence of this and other known ASSTs could be made. In the course of this study the gene was introduced into *Escherichia coli* and overexpressed ca. 300-fold vs. the parental strain, so a plentiful and easily obtained source of the enzyme is available for future study. The *astA* gene was completely sequenced and found to encode a protein of approximately 71,000 Da. BLAST2 searches and BESTFIT comparisons indicated only limited homology between the *Eubacterium* and *Klebsiella* (and *Enterobacter*) protein sequences.

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Materials and methods

Enzymes and chemicals

Restriction endonucleases and T4 DNA ligase were purchased from Gibco BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA). 4-Methylumbelliferyl sulfate (MUS), *p*-nitrophenyl sulfate (PNS), and tyramine were purchased from Sigma Chemical (St. Louis, MO). The other compounds used were standard chemical preparations.

Strains, phage, and plasmids

Bacterial strains, bacteriophage, and plasmids used and their sources are listed in Table 1.

Culture media

For isolation of anaerobic bacteria, BBL chopped meat and brain heart infusion anaerobic blood agar medium was used (Becton Dickinson Microbiology Systems, Cockeysville, MD). *Eubacterium rectale* IIIH was grown in PYG medium (ATCC medium 1527), consisting of 0.5% peptone, 0.5% tryptone, 1% yeast extract, 0.01% resazurin (Sigma), 0.0008% anhydrous $CaCl_2$, 0.0008% $MgSO_4$, 0.004% K_2HPO_4, 0.004% KH_2PO_4, 0.04% $NaHCO_3$, 0.002% NaCl, 0.0005% hemin (Sigma), 0.0001% (v/v) Vitamin K1 (Sigma), and 0.05% L-cysteine-HCl (Sigma), pH 7.0. For solid medium, 1.5% Bacto-agar (Difco Laboratories, Detroit, MI) was included. PYG medium was boiled to drive off oxygen and placed in anaerobic bottles. Argon gas was then bubbled through the medium and the bottles were sealed with a cap containing a rubber septum before autoclaving them for 20 min at 121°C.

E. coli strains XL-1 Blue MRF′ (Bullock; Stratagene Cloning Systems, La Jolla, CA), W3110 [2], JM83 [24], and BL21 [21,22] were cultured in LB medium (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl). If required, ampicillin was included as a selective antibiotic at 100 μg/ml.

Isolation of an ASST-producing bacterium

A loopful of human feces (ca. 0.1 g) was suspended in 10 ml BBL chopped meat broth and serial 10-fold dilutions were made in the

Table 1 Bacterial strains, phage, and plasmids used in this study

Strain, phage or plasmid	Relevant characteristics	Source or reference
Strains		
E. rectale IIIH	Human fecal isolate; AST^+	This work
E. coli XL-1 Blue MRF′	D(mcrA)183 D(mcrCB-hsdSMR-mrr)173 supE44 hsdR17 recA1 endA1 gyrA16 thi-1 relA1 lac [F′ proAB $lacl^qZ\Delta M15$ Tn10(Tet^r)]	Stratagene
E. coli W3110	λ^-, [F^- IN(rrnD-rrnE)	ATCC; Ref. [2]
E. coli JM83	ara Δ(lac-proAB) rspL $\Phi 80lacZ\Delta M15$	Laboratory strain; Ref. [24]
E. coli BL21	*E. coli* B F^- dcm ompT hsdS(r_{B^-} m_{B^-}) gal	Novagen; Refs. [21,22]
Phage and plasmids		
λZAPII	Phage cloning vector	Stratagene; Ref. [5]
λAST100	Ca. 4.6-kb *Eco*RI fragment of *E. rectale* cloned into λZAPII; $ASST^+$	This work
pBC-SK+	Cloning vector; Cm^r	Stratagene
pET5b	Expression vector with IPTG-regulated T7 promoter	Novagen
pAST100	5.5-kb *Bam*HI fragment from λAST100 cloned into pET5b; $ASST^+$	
pAST200	1.1 kb *Kpn*l deletion of pAST100; $ASST^+$	This work
pAST300	0.85 kb *Sal*l deletion of pAST200; $ASST^+$	This work
pSE380	Expression vector with *trc* promoter	Invitrogen
pAST500	3.6-kb *Hin*dIII fragment from pAST200 cloned into *Hin*dIII-digested pSE380	This work
pAST501	Same as pAST500 with fragment in opposite orientation	This work

same medium. Aliquots (0.1 ml) of the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were spread onto BBL brain heart infusion anaerobic blood agar medium. The plates were incubated anaerobically at 37°C for 24 h using a BBL Gas Pack Pouch, then overlayed with agarose containing a fluorescent indicator of ASST activity. Using this protocol, a fluorescing bacterium was purified (see below) and submitted for speciation based on its fatty acid profile (IEA, Essex Junction, VT).

Construction of a E. rectale genomic library

E. rectale IIIH chromosomal DNA was prepared from cultures grown in PYG medium as described [1] except the cells were treated with 10 mg/ml of lysozyme (Sigma) at 37°C for 15 min before proceeding with cell lysis. For preparation of the genomic library, chromosomal DNA was partially digested with *Eco*RI and size-fractionated on a 0.7% TAE (40 mM Tris base, 20 mM acetic acid, 10 mM ethylenediaminetetraacetic acid) agarose gel. Fragments of 5–10 kilobases (kb) were cut out of the gel and purified with GeneClean (BIO 101, La Jolla, CA). Purified fragments were ligated to *Eco*RI-digested λZAPII arms (Short; Stratagene Cloning Systems, La Jolla, CA), packaged, and used to infect *E. coli* XL-1 Blue MRF′ (Stratagene). Plaques were screened *in situ* according to the plate assay described below. Isolated plaques were diluted into SM buffer (per liter: NaCl, 5.8 g; $MgSO_4{\cdot}7H_2O$, 2 g; 1 M Tris·Cl, pH 7.5, 50 ml; 0.01% gelatin) for storage.

Subcloning and expression of the astA gene in E. coli

Restriction fragments used for subcloning into plasmid vectors pET5b (Novagen, Madison, WI) or pSE380 (Invitrogen, San Diego, CA) were obtained from a λZAPII clone expressing ASST activity or from a plasmid containing the subcloned *ast* gene, repectively. Phage particles were obtained by glycerol purification of plate lysates [15] and DNA was isolated by extraction with phenol:$CHCl_3$. Plasmid DNA was purified using the QIAquick Gel Isolation Kit (Qiagen, Chatsworth, CA). DNA fragments of recombinant bacteriophage were isolated from agarose gels and purified as described above, then ligated to plasmid DNA that had been digested with the appropriate restriction endonuclease. Ligated mixtures were used to transform *E. coli* XL-1 Blue MRF′ competent cells as described by the manufacturer (Stratagene). Transformed cells were selected on LB ampicillin agar medium. Colonies were screened for ASST activity using the overlay technique described below.

Assay protocols

(1) Plate assay: To detect *E. coli* recombinants possibly possessing ASST activity, colonies were overlayed with 1.0% agarose containing 1 mM each 4-methylumbelliferyl sulfate and tyramine. Removal of the sulfate group from MUS forms 4-methylumbelliferone, which fluoresces strongly upon exposure to long-wave (320 nm) UV light. (2) Quantitative assay: An assay for ASST was adapted from that described by Miller to measure β-galactosidase activity [14]. It was composed of 0.460 ml of 20 mM tyramine, 0.033 ml of 1 M Tris–HCl, pH 8.0, 0.024 ml of 0.1 M *p*-nitrophenyl sulfate, *x* ml cell culture or enzyme fraction, and $0.480-x$ ml water (total volume 1.0 ml). If cells were used, they were first lysed by adding 0.01 ml $CHCl_3$ and 0.02 ml 0.1% SDS and vortexed for 10 s. The reaction was initiated by adding the PNS and incubated at 37°C until a pale-to-medium yellow color was seen, then terminated by addition of 0.5 ml of 1 N NaOH. Activity was calculated using the following formula:

$$\mathrm{Units} = \frac{\mathrm{OD_{410}} - (1.75 \times \mathrm{OD_{550}})}{\mathrm{t \times v \times OD_{600}}}$$

where OD_{410} = absorbance at 410 nm due to formation of *p*-nitrophenol; $1.75 \times OD_{550}$ = scattering at 550 nm due to cell debris; OD_{600} = absorbance of cell culture at 600 nm prior to assay; t = time (in minutes) of reaction; v = volume of cell culture used.

Specific activity was determined from extracts using the following formula (one unit is that activity liberating 1.0 μmol *p*-nitrophenol/min)

$$\mathrm{U/mg\ protein} = \frac{\Delta \mathrm{A/min} \times 1000}{16,200 \times \mathrm{mg\ protein/ml\ reaction}}$$

where ΔA is the change in absorbance at OD = 410 nm.

Table 2 ASST activity of *E. rectale* and recombinant *E. coli* strains

Strain	Activity (U/ml)
BL21 (pAST300)	97.2
JM83 (pAST300)	80.6
W3110 (pAST300)	130.8
XL-1 Blue MRF′ (pAST300)	97.8
E. rectale IIIH	0.4

Activities were based on the average of three independent cultures of $OD_{600}=1.0\pm0.1$.

ASST protein purification

The purification procedure was based on that described by Kim *et al* [10]. *E. coli* W3110(pAST300) cells (13.5 g) were suspended in 200 ml of 0.1 M sodium acetate, pH 5.5 (buffer A). Cells were lysed by sonication using six cycles of 30 s each with a 1-min interval between each cycle. All subsequent procedures were carried out at 4°C. Insoluble material was removed by centrifugation at 17,600×*g* for 15 min. Ammonium chloride was added to 35% (w/v) and the supernatant after centrifugation at 17,600×*g* for 30 min was retained. Additional ammonium chloride to 65% was mixed with the supernatant, and protein was pelleted at 17,600×*g* for 30 min. The precipitate was solubilized in 40 ml buffer A and dialyzed against the same buffer for 16 h with 3×2-l changes. The material was loaded onto a 26×2.6 cm DE-52 column and the enzyme was eluted with 300 ml of an NaCl gradient (0–100%) in buffer A. The gradient was added at 2 ml/min using a Pharmacia LCC-500 liquid chromatography controller (Pharmacia, Piscataway, NJ). Fractions (10 ml each) were tested for activity as described above and active fractions were pooled. The protein was concentrated using a Centricon-10 cartridge (Amicon, Beverly, MA) by centrifugation in a Beckman GS-3.7 rotor at 2000×*g* for 30 min. The material was applied to a hydroxyapatite column (7.8×2.6 cm). The protein present in the flowthrough was collected, concentrated using a Centricon-10 filter, and loaded onto an 86×2.6 cm Sephacryl S200 gel filtration column. Active fractions were again collected and stored at −20°C. The protein sample was electrophoresed through a 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide Phast gel along with protein-size standards and its specific activity determined. A summary of the purification is given in Table 3.

Protein sequencing

The N-terminus of the purified ASST was sequenced by the Princeton Protein Sequencing facility (Princeton University, Princeton, NJ).

DNA analysis

For Southern hybridizations [20], *E. rectale* IIIH chromosomal DNA was cleaved with restriction endonuclease *Kpn*I and electrophoresed through 0.8% TAE-agarose gels. DNA fragments were transferred to Duralon UV membranes (Stratagene) using the VacuGene apparatus (Pharmacia LKB, Piscataway, NJ). DNA labeling and hybridization were performed using the ECL Direct kit (Amersham, Arlington Heights, IL). Stringent washing conditions were 0.2× SSC/0.4% SDS at 55°C. The DNA sequence of the *astA* gene was determined using the BigDye terminator kit and a model 377 DNA sequencing unit [23] (Applied Biosystems, Foster City, CA). Nucleotide and deduced amino acids were analyzed with the Vector NTI program from Informax. Related sequences were obtained from database searches (Swissprot, EMBL, and GenBank) using BLAST2. Homologies between *ast* genes from *Klebsiella*, *Enterobacter*, and *Eubacterium* were quantified using BESTFIT analysis. Alignment and consensus sequence was obtained using PRETTY. The protein sequence was scanned with SPScan in order to identify any potential signal sequences.

Results and discussion

Isolation of an ASST-producing E. rectale

The isolation of a sulfotransferase-positive bacterium, identified as *Eubacterium* A-44, was described by Kobashi *et al* [12]. Based on these results, we screened dilutions of human feces spread onto blood agar medium and grown anaerobically. Approximately 1% of the colonies present fluoresced using the agarose overlay. Six colonies were purified; all possessed identical colonial and microscopic morphology. This bacterium was found to be a strictly anaerobic, Gram-negative rod. Its fatty acid profile matched that of *E. rectale* IIIH, which was confirmed by comparison to the ATCC type culture (33656) of the same organism.

The two isolates of *E. rectale* were grown anaerobically in solid and liquid PYG medium for 24 h at 37°C. The cultures were assayed for ASST activity using the plate or quantitative assay, respectively. Strong fluorescence was seen from *E. rectale* IIIH. The ASST activity of this culture was 0.40 units, and was dependent upon the inclusion of a sulfate acceptor, tyramine, confirming that a sulfotransferase rather than a sulfatase [6] was responsible for the results obtained. Activity was lost if boiled extracts (100°C, 5 min) were used in the reaction, or if the donor substrate was omitted. No ASST activity was found in identical experiments using *E. rectale* ATCC 33656.

Cloning of the E. rectale astA gene

Chromosomal DNA from *E. rectale* IIIH was prepared and partially digested with *Eco*RI. Fragments of ca. 5–10 kb were isolated, purified, and ligated to *Eco*RI arms of phage vector λZAPII. The library was infected into *E. coli* strain XL-1 Blue

Table 3 Purification of ASST from *E. coli* W3110(pAST300)

Step	Volume (ml)	Protein (mg)	Activity (Units)	Specific activity (Units/mg)	Fold-purification
Cell extract	200	1224	8456	6.9	1.0
Ammonium sulfate fractionation	40	292	7668	26.3	3.8
DEAE-cellulose chromatography	120	129	6982	54.1	7.8
Hydroxyapatite column chromatography	18	92	6563	71.3	10.3
Sephacryl S-200 gel filtration	11	39	5733	147.0	21.3

MRF′. Out of approximately 24,500 recombinant plaques which were screened for ASST activity, one was faintly fluorescent. This plaque was diluted into SM buffer, replated, and again tested for sulfotransferase activity. Well-isolated, strongly fluorescing plaques were now visible. After another round of purification, one plaque was chosen for further work and named λAST. It was noted that ca. 10% of the plaques at each round of purification demonstrated no ASST activity. In addition, no fluorescing ampicillin-resistant colonies were obtained after attempting to excise the cloned gene as a phagemid upon co-infection of *E. coli* with λAST and a helper phage. A 5.5-kb *Bam*HI insert present in λAST containing a 4.6-kb region of *E. rectale* DNA was isolated from agarose gels, ligated to pBC-SK+ (Stratagene), and transformed into *E. coli* XL-1 Blue MRF′ cells. These colonies did not fluoresce and only deletions of the original insert were found upon restriction analysis of the isolated plasmid DNA.

Subcloning and expression of the E. rectale astA gene in E. coli

The apparent instability problem encountered upon attempting to subclone the ASST gene in λAST was overcome by using pET5b (Novagen) as a cloning vector. pET5b is present in moderate copy number and contains a tightly regulated, IPTG-inducible T7 promoter [21]. The 5.5-kb *Bam*HI fragment from the recombinant phage was ligated to *Bam*HI-digested plasmid DNA. Ligated mixtures were used to transform *E. coli* XL-1 Blue MRF′ competent cells, which were plated onto LB agar medium containing 100 μg/ml ampicillin. Stable fluorescing colonies containing the expected 5.5-kb insert were obtained upon screening for ASST activity using the overlay technique described above; this plasmid was named pAST100. In addition, a 3.65-kb *Hin*dIII DNA fragment possessing the *astA* gene was cloned into the moderate-copy-number vector pSE380 (Stratagene) in both orientations and was expressed in each instance. These results suggest that *E. coli* was unable to tolerate the presence of the ASST gene on a high-copy-number plasmid such as pBC-SK+, perhaps due to toxicity resulting from overexpression of the ASST. Any deleterious effects could be overcome by utilizing lower copy-number plasmids for expression. The fact that ASST activity was not dependent upon the *astA* gene orientation suggests that transcription in *E. coli* occurs from the native *E. rectale* promoter.

Quantitation of ASST activity in E. coli

Specific deletions in pAST100 were created by removal of small *Kpn*I and *Sph*I fragments, forming pAST300 (see Table 1). This plasmid was transformed into four *E. coli* strains: XL-1 Blue MRF′, W3110, JM83, and BL21. Single AST+ colonies of these isolates as well as XL-1 Blue MRF′ (pAST300) were inoculated into 50 ml LB+ampicillin liquid medium in a 250-ml flask and grown at 30°C, 250 rpm, for 18 h. ASST activity was quantified from lysed cells at OD_{600}=1.0 as described in Materials and Methods. As shown in Table 2, all recombinant *E. coli* strains were significantly more active than the parental strain. In the best case, W3110(pAST300) demonstrated a 300-fold improvement in ASST activity as compared to *E. rectale* IIIH. Given the relative difficulty in large-scale fermentation of a strict anaerobe such as *E. rectale*, the ability to make active enzyme in *E. coli* assures that an easily obtainable supply of enzyme will be available for future work.

ASST protein purification and protein sequencing

ASST was purified 21-fold to a specific activity of 147 U/mg from cultures of W3110(pAST300) as summarized in Table 3 and subjected to SDS–polyacrylamide gel electrophoresis. The subunit molecular weight was estimated to be 75,000 Da based on comparison to size standards. The formation of *p*-nitrophenol in our quantitative assay was dependent on the inclusion of tyramine in the reaction mixture, similar to results obtained using enzyme extracted from *E. rectale*. The molecular weight of the isolated ASST was determined to be 75,000 Da by comparison to protein-size standards after electrophoresis through a 12.5% SDS–polyacrylamide gel (data not shown). The N-terminus of the purified ASST was analyzed at the Princeton Protein Sequencing facility (Princeton University). A sequence of M-S-V-K-Y-S-F-E-D-J-I-V-N-R-Q-Y was obtained.

DNA sequence analysis and comparison

DNA sequence analysis of the cloned *astA* gene revealed an open reading frame (ORF) that contained an amino acid sequence corresponding exactly to the amino terminus of the ASST enzyme. This ORF encodes a protein of 620 amino acids with a molecular weight of 70,919 Da, in good agreement with that observed after SDS–polyacrylamide gel electrophoresis. A putative promoter region containing −35 and −10 sequences as well as a Shine–Delgarno motif [7,18] was also identified (data not shown).

Two sequences related to the *E. rectale* ASST were obtained from database searches (Swissprot, EMBL, and GenBank) using BLAST2. The amino acid sequence of the *E. rectale* IIIH *astA* gene was compared to ASSTs from *Klebsiella* A-44 [3,4] and *Enterobacter amnigenus* AR-37 [4] using BESTFIT analysis. While the latter two ASST proteins are nearly homologous to each other (88.63% similarity and 83.78% identity), these genes show only modest homology to the *Eubacterium* version (44.35% similarity and 33.48% identity). This may explain the lack of hybridization observed by Baek *et al* [4] using a probe derived from the *Klebsiella astA* gene vs. *Eubacterium* A-44 chromosomal DNA. Another significant difference between the *Klebsiella*/*Enterobacter* and *E. rectale* ASSTs is the location of the protein in the respective host bacteria. The *Eubacterium* protein sequence was scanned with SPScan in order to identify any potential signal sequences. Unlike the *Klebsiella* or *Enterobacter* ASST protein, the *Eubacterium* ASST protein does not appear to have a signal peptide associated with it, indicating that it is cell-associated. This result agrees with our finding that cell extracts were required in purification of the protein, and that the N-terminus of purified ASST is identical to that predicted from the DNA sequence of the cloned gene. These data suggest that significant divergence has occurred between the ASSTs present in the anaerobic bacterium and that possessed by aerobic organisms.

Nucleotide sequence accession number

The sequence of the *E. rectale astA* gene has been deposited in GenBank with accession number AF230529.

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