Characterization of and functional evidence for Ste27 of *Streptomyces* sp. 139 as a novel spermine/spermidine acetyltransferase

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Ebosin, a novel exopolysaccharide produced by *Streptomyces* sp. 139, has remarkable anti-rheumatoid arthritis activity *in vivo* and its biosynthesis gene cluster (ste) consists of 27 ORFs (open reading frames). The present paper reports our study of the protein product encoded by ste27. Database searching reveals the homology of Ste27 with some spermidine/spermine acetyltransferases. To confirm the prediction, the ste27 gene was cloned and expressed in *Escherichia coli* BL21(DE3) cells and recombinant Ste27 was purified. The following enzymatic analysis revealed its ability of transferring the acetyl group from acetyl-CoA to spermidine and spermine, with spermidine being the preferred substrate. Ste27 can acetylate the N\(^1\), N\(^4\) and N\(^6\) positions on spermidine. The *K_m* values of Ste27 were determined for spermidine and spermine, as well as for acetyl-CoA, poly-L-lysine and glucosamine 6-phosphate. Upon gene knockout, the exopolysaccharide-27m produced by the mutant strain *Streptomyces* sp. 139 (ste27\(^{-}\)), compared with Ebosin, exhibited a significantly reduced binding activity to the interleukin-1 receptor. After gene complementation, the binding activity was partially restored. This demonstrated that the ste27 gene is involved in the biosynthesis of Ebosin. Molecular modelling was also carried out to predict the binding mode of Ste27 with acetyl-CoA, spermidine or spermine.

Key words: Ebosin biosynthesis, exopolysaccharide, polyamine, spermidine/spermine acetyltransferase, Ste27.

INTRODUCTION

The polyamines spermidine and spermine, and their precursor putrescine, are ubiquitous organic cations found in most cells. These molecules are essential for normal cell growth and differentiation [1]. The addition of acetyl group(s) to these polyamines appears to be necessary for their breakdown and differentiation [1]. The addition of acetyl group(s) to these polyamines appears to be necessary for their breakdown and differentiation [1]. The addition of acetyl group(s) to these polyamines appears to be necessary for their breakdown and differentiation [1]. The addition of acetyl group(s) to these polyamines appears to be necessary for their breakdown and differentiation [1]. The addition of acetyl group(s) to these polyamines appears to be necessary for their breakdown and differentiation [1].

*Streptomyces* is one group of important industrial microorganisms which produce various antibiotics and enzymes, whereas their production of EPSs (exopolysaccharides) has only been studied recently. As a novel EPS, Ebosin isolated from *Streptomyces* sp. 139 shows remarkable anti-rheumatoid arthritis activity *in vivo* and its biosynthesis gene cluster (ste) consisting of 27 ORFs (open reading frames) has been identified [8].

On the basis of database searching, the ste27 gene codes for a protein homologous with some SSATs originating from microbes. In the present paper we report the study of the biochemical function of this protein and the involvement of ste27 in Ebosin biosynthesis. After cloning and expression of the gene ste27 in *E. coli*, the purified recombinant Ste27 was confirmed to have the ability of catalysing the acetyl group from acetyl-CoA to spermidine or spermine. It prefers spermidine as the substrate and its acetylating positions on spermidine have also been identified. Using different substrates, including spermidine, spermine, acetyl-CoA, poly-L-lysine and GlcN-6-P (glucosamine 6-phosphate), a kinetic study of Ste27 was performed. Thus Ste27 was identified as a novel SSAT. For studying the function, ste27 was knocked out. The binding activity of altered EPS, EPS-27m, with IL-1R [IL-1 (interleukin 1) receptor] decreased remarkably compared with Ebosin, but after gene complementation, the activity was partially restored. Therefore Ste27 plays an essential role during the biosynthesis of Ebosin.

MATERIALS AND METHODS

Bacterial strains and growth conditions

*Streptomyces* sp. 139 was isolated from a soil sample in China and kept in the China General Microbiology Culture Collection Center (number 0405). The strain was cultured at 28°C with shaking (250 rev./min) in ether TSB (tryptic soy broth) medium supplemented with 5 mM MgCl\(_2\) and 0.5 % glycine or fermentation medium [1 % (w/v) glucose, 2 % (w/v) starch, 2 % (w/v) soya bean extract, 0.2 % tryptone, 0.2 % beef extract, 0.4 % yeast extract, 0.05 % K\(_2\)HPO\(_4\), and 0.3 % CaCO\(_3\) (pH 7.3)]. *E. coli* strain BL21(DE3) was grown at 37°C in LB (Luria–Bertani) medium.

Abbreviations used: Am\(^r\), ampicillin-resistance; Am\(^s\), ampicillin-sensitive; CTD, C-terminal domain; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EPS, exopolysaccharide; GlcN-6-P, glucosamine 6-phosphate; IL-1, interleukin 1; IL-1R, IL-1 receptor; Km\(^r\), kanamycin-resistance; LB, Luria–Bertani; MOE, Molecular Operating Environment; Ni-NTA, Ni\(^{2+}\)-nitrilotriacetaet; NTD, N-terminal domain; ORF, open reading frame; PBST, PBS with 0.05 % Tween 20; SSAT, spermidine/spermine acetyltransferase; TNB, 2-nitro-5-thiobenzoate.

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DNA preparation and Southern blot analysis

Isolation of *E. coli* plasmid DNA and standard recombinant DNA techniques were performed as described by Sambrook and Russell [9]. *Streptomyces* plasmid and genomic DNA was isolated as described by Kieser et al. [10]. For Southern blot analysis, a DIG (digoxigenin) high-prime DNA labelling and detection starter kit II obtained from Roche was used, following the manufacturer’s protocol.

Cloning and expression of ste27 in *E. coli* BL21(DE3)

The DNA sequence of ste27 described in the present study was deposited in GenBank under accession number DQ852337. With *Streptomyces* sp. 139 genomic DNA as the template, the gene ste27 was amplified by PCR using primers P1 (5'-GCCCATGGCCAGCAAGGTGTCCG-3') and P2 (5'-GCAAGCTTCATCAACCGCAGCCCG-3') [BamHI (P1) and EcoRI (P2) restriction sites are underlined]. Amplification was performed under the following conditions: an initial denaturation at 94 °C for 4 min, then 30 cycles of 1 min at 98 °C, 1 min at 57 °C and 1 min at 72 °C, and finally 10 min at 72 °C. A 859 bp amplified DNA fragment of the ste27 gene was cloned into the plasmid pET30a digested with BamHI/EcoRI to construct the recombinant plasmid pET30a-ste27, which was then transformed into the competent cells of *E. coli* BL21(DE3). Two transformants were selected and cultured overnight at 37 °C in LB broth containing kanamycin (25 μg/ml). The culture of *E. coli* BL21(DE3) (pET30a-ste27) was diluted to 1:20 with LB broth and subjected to further incubation at 37 °C. IPTG (isopropyl β-D-thiogalactopyranoside) was added to the culture at a final concentration of 0.2 mM. After incubation for 6 h at 37 °C, the bacteria were harvested and suspended in binding buffer [5 M imidazole, 0.5 M NaCl and 20 mM Tris/HCl (pH 8.0)]. Cells were lysed by sonication.

Purification of recombinant Ste27

After cell lysis the inclusion bodies were collected by centrifugation at 4 °C. Inclusion bodies were treated with lysis buffer [50 mM Tris/HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 6 M guanidinium chloride, 8 M urea and 200 mM 2-mercaptoethanol] overnight at 4 °C and after centrifugation (15 694 g for 15 min at 4 °C), the supernatant was collected and dialysed against renaturation buffer [20 mM Tris/HCl (pH 8.0), 1 M urea, 3 M guanidinium chloride, 0.5 mM GSH, 0.5 mM GSSG, 0.5 M arginine, 2.0 mM EDTA, 80 mM NaCl and 8 M 2-mercaptoethanol] at 4 °C for 48 h. The sample was further dialysed at 4 °C for another 48 h against the same renaturation buffer, except the concentration of guanidinium chloride was increased to 1 M. After 10 h of dialysis against water at 4 °C, the protein was freeze-dried. The freeze-dried sample was suspended in 1.0 ml of binding buffer [10 mM imidazole, 0.3 M NaCl and 20 mM Tris/HCl (pH 8.0)] and loaded on to a 2.5 ml Ni-NTA (Ni²⁺-nitrilotriacetate) His-Bind resin column (Novagen) pre-equilibrated with the binding buffer. Unbound proteins were removed with 7.5 ml of washing buffer [50 mM imidazole, 0.3 M NaCl and 20 mM Tris/HCl (pH 8.0)] and the recombinant protein was eluted with 2.5 ml of eluting buffer [250 mM imidazole, 0.3 M NaCl and 20 mM Tris/HCl (pH 8.0)]. The fractions containing the recombinant Ste27 protein were collected and dialysed against water at 4 °C and freeze-dried. The purity of the protein was ascertained by SDS/PAGE and HPLC.

Enzymatic assays

The DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)]-coupled spectrophotometric assay (Figure 1) for SSATs [11] was used to establish the enzymatic activity of Ste27. After transferring the acetyl group from acetyl-CoA to spermidine or spermine to produce an acetyl polyamine and free form of CoA, which was used to measure SSAT activity after the DTNB reaction.

Figure 1 Schematic diagram showing the colorimetric assay for SSAT activity

SSAT catalyses the transfer of the acetyl group from acetyl-CoA to spermidine or spermine. The DTNB 

![Figure 1](image-url)
of the optimum reaction time, the reaction was carried out at 28°C in the same buffer (pH 7.5) containing Ste27 (20 μg), acetyl-CoA (50 μM) and spermidine (200 μM), but for different periods (5–30 min).

Kinetic studies were carried out using concentration ranges of 20–200 μM spermidine or spermine, 5–50 μM acetyl-CoA, 25–200 μM poly-L-lysine and 10–80 μM GlcN-6-P respectively. The values of $K_m$ and $K_{ac}$ were calculated using Origin 8.0 (OriginLab).

**Identification of enzymatic product**

The 300 μl reaction mixture containing Ste27 (200 μg), spermidine (2 mM), acetyl-CoA (0.5 mM) and EDTA (1 mM) in 0.1 M Tris/HCl buffer (pH 7.5) was incubated at 28°C for 30 min and then subsequently the reaction was terminated and the products were extracted. The reaction products were analysed with TLC (silica gel 60; Merck). The extracted samples were spotted on to the TLC and developed with chloroform/methanol/ammonia/water at a ratio of 1:4:1:1 (by vol.). The enzymatic products were identified by comparison with known standards and were visualized by staining with iodine vapour. MS analysis of the enzymatic product was performed on an Autospec Ultima-TOF mass spectrometer (Micromass).

**Disruption of the ste27 gene**

To understand the function of the ste27 gene in the biosynthesis of Ebosin, knockout mutants were generated. A 1.59-kb internal fragment (F1, upstream of ste27) and another 1.35-kb internal fragment of ste27 (F2, downstream of ste27) were amplified by PCR from the chromosome DNA of Streptomyces sp. 139 using primer pairs P3 (5′-CCGAACCTGCTGAGGATTGAAAGTCGAC-3′) and P4 (5′-GGCTCTAGAGCGACCGACCATGCACTG-3′) [EcoRI (P3) and XbaI (P4) restriction sites are underlined], and P5 (5′-CGCTCTAGATTGGGGAATTCGCTTC-3′) [XbaI (P5) and HindIII (P6) restriction sites are underlined], and P6 (5′-GGGGAATTCGCTCCGCGGCTTACAGTGTTCGGTCA-3′) [XbaI (P5) and HindIII (P6) restriction sites are underlined]. PCR amplification was performed under the following conditions: an initial denaturation at 94°C for 4 min, then 30 cycles of 1 min at 94°C, 1 min at 57°C and 1.5 min at 72°C, and finally 10 min at 72°C. A 1.23-kb fragment F3 carrying the Km' (kanamycin-resistance) gene was digested with XbaI from plasmid pUC19-Km'. The fragment containing F1, F3 and F2 was cloned into pKC1139 [12] to construct plasmid pKC27m. After propagation in E. coli ET12567 [13], pKC27m was introduced into Streptomyces sp. 139 by [poly(ethylene glycol)]-mediated protoplast transformation [11]. After incubation at 28°C for 16–20 h, the plates were overlaid with soft R2YE (0.7% agar) containing apramycin (50 μg/ml). Plasmid pKC27m bears a temperature-sensitive Streptomyces replication origin [13] that is unable to replicate at temperatures above 34°C. Therefore the transformants were first incubated at 28°C for 2 days until pinpoint-sized colonies became visible and plates were then shifted to 37°C for further incubation. Mutants resulting from a double crossover via homologous recombination grew out of the original pinpoint-size colonies over several days.

**Complementation of the ste27 disruption mutant**

Using genomic DNA of Streptomyces sp. 139 as a template, a 900-bp ste27 fragment was amplified by PCR with the primers P7 (5′-GGCTCTAGACGTCACGGGGCGACCAGGGCGGCTG) and P8 (5′-GCAAGCTTATCACCAGCCGCCGCGG) [BamHI (P7) and EcoRI (P8) restriction sites are underlined]. A 0.45-kb fragment of the ermE* promoter was isolated from the plasmid pGEM-3zf-ermE* [14] digested with HindIII/BamHI. The ermE* fragment and the 900-bp ste27 fragment were ligated and this was inserted into plasmid pKC1139 and digested with EcoRI/HindIII to create pKC27c, which was then transformed into E. coli ET12567. After culturing the recombinant strain with Am* (ampicillin-resistance) (50 μg/ml), the plasmid pKC27c was isolated and transformed into the protoplasts of Streptomyces sp. 139 (ste27Δ). The complementing strain was named as Streptomyces sp. 139 (pKC27c).

**Isolation of Ebosin and its derivatives**

Ebosin and its derivatives were isolated from the supernatant of the fermentation culture of Streptomyces sp. 139 and mutants as described previously [15].

**Assay for EPS activity**

An ELISA method was used to analyse the competitive binding activity of isolated EPSs with IL-1 for IL-1R. A 100 μl aliquot of IL-1 (0.01 μg; Peprotech) was coated on to a 96-well immunoplate (Nunc) at 4°C overnight. To each well, 250 μl of 3% BSA in PBS [0.024% KH₂PO₄, 0.363% Na₂HPO₄, 0.02% KCl and 0.8% NaCl (pH 7.4)] was added and the plate was kept at 4°C for 4 h, followed by washing three times with PBS and PBST (PBS with 0.05% Tween 20) respectively and blotted dry. The EPSs were then diluted in PBS and 50 μl was added to each well, and then 50 μl of IL-1R (1.100; R&D Systems) was also added to each well. The plate was incubated at 4°C for 3 h. After binding, the plate was washed three separate times with PBS and PBST, and then 100 μl of a diluted solution (1:1000) of a goat polyclonal anti-human IL-1R antibody (R&D Systems) was transferred into each well and the plate was allowed to stand at 4°C for 1 h. The plate was washed again as described above, and 100 μl of a dilute solution (1:1000) of secondary antibody [rabbit polyclonal anti-goat IgG antibody conjugated with SA (streptavidin)–HRP (horse-radish peroxidase); Promega] was added to each well and the plate was kept at 4°C for 1 h. After final washes as described above, 100 μl of TMB (3,3′,5,5′-tetramethylbenzidine) solution was transferred into each well and the reaction took place at room temperature for 1 h (the solution turned blue in colour) before being stopped by the addition of 100 μl of 2 M HCl. The absorbance at 450 nm was recorded as a measurement of the reaction.

**Molecular modelling**

The homology work for Ste27 was performed using the molecular modelling software Discovery studio 2.5 (Accelrys). The templates for Ste27 protein (NCBI number AB147990) were obtained using the BLAST-search protocol of the sequence analysis module. The proteins (R)-oxynitrile lyase isoenzyme 1 (PDB code 3GD[P] [16] and a putative acetyltransferase (PDB code 20ZH) were selected as the templates of the Ste27 NTD (N-terminal domain) and CTD (C-terminal domain) respectively. The multiple sequence alignment between the templates and the objective protein were carried out using the Align multiple module of the Protein Modeling module (N-terminal domain) and CTD (C-terminal domain) respectively. The multiple sequence alignment between the templates and the objective protein were carried out using the Align multiple sequence module of the Protein Modeling module according to the alignment profile.

The binding mode for acetyl-CoA, spermidine or spermine towards the binding site of the homology model was generated through molecular docking using MOE (Molecular Operating Environment) module.
Environment) version 2009.10. In general, the docking was performed through the ‘DOCK’ module in MOE using the Alpha-Triangle placement method. Refinement of the docked poses was carried out using the Forcefield refinement scheme and scored using both the affinity dG and London dG scoring system. The pose with the highest docking score was returned for further analysis.

RESULTS

Gene expression and purification of the Ste27 protein

After cultivation of *E. coli* BL21(DE3) (pET30a-ste27), the cell lysate was analysed by SDS/PAGE with Coomassie Blue staining. On the gel, an intense protein band appeared with a molecular mass in agreement with the expected size of 35.1 kDa for Ste27 (Figure 2A).

Inclusion bodies of recombinant protein Ste27 were lysed, renatured and purified with Ni-NTA His-Bind resin column chromatography. The combined fractions containing recombinant Ste27 protein were dialysed against water and freeze-dried, then the protein purity was ascertained by HPLC to be 91.43% (results not shown).

SSAT activity of Ste27

The assumed SSAT activity of purified Ste27 was determined. The amount of CoA over time in the reaction increased remarkably with the increasing concentration of the protein (2.5–25 μg), indicating that the Ste27 protein has the enzyme activity of a SSAT, catalysing spermidine or spermine and acetyl-CoA to produce CoA and acetylspermidine or acetylspermine (Figure 2B).

When different amounts of spermidine (25–200 μM) were added to the reaction mixture containing a fixed amount of Ste27 (20 μg) and acetyl-CoA (50 μM), the increase in CoA was found to be correlated with the increase in spermidine concentration, but this flattened at 150 μg (Figure 2C). In reaction mixtures containing various amounts of acetyl-CoA (5–50 μM), Ste27 (20.0 μg) and spermidine (200 μM), the increase in CoA, measured by absorbance at 412 nm, was proportional to the concentration of acetyl-CoA and the curve tended to be flat above 35.0 μM (Figure 2D).

Optimal pH, temperature and reaction time of Ste27

When the SSAT activity of Ste27 was measured in reaction mixtures of 0.1 mM Tris/HCl buffer with various pH values (6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0), the optimum pH was found to be 7.5. The activity at pH 6.0 was 51.7% of that at pH 7.5 (Figure 2E). The optimum temperature measured in 0.1 mM Tris/HCl buffer (pH 7.5) at different temperatures (15°C, 22°C, 28°C, 37°C and 42°C) was found to be 28°C. The enzyme showed 52.4% activity at 15°C compared with at 28°C (Figure 2F). At the optimal pH and temperature conditions, the optimum reaction time was determined to be 10 min (Figure 2G).

Substrate specificities and kinetics of Ste27 catalysis

For studying the substrate specificities of Ste27, various substrates, including spermidine, spermine, acetyl-CoA, GlcN-6-P, poly-L-lysine and some amino acids, were tested. The results showed that the amount of CoA over time in the reaction increased remarkably with increasing concentrations of spermidine, spermine, acetyl-CoA, poly-L-lysine and GlcN-6-P, but the same was not observed with the amino acids tested (glycine, glutamate, aspartate, leucine, alanine or arginine). These results demonstrate that Ste27 protein can catalyse the acetylation of spermidine, spermine, acetyl-CoA, GlcN-6-P and poly-L-lysine.

On the basis of the Lineweaver–Burk equation, the *K*<sub>m</sub> values of SSAT encoded by ste27 were calculated using Origin 8.0 to be 56.523 ± 5.560 μM for acetyl-CoA, 65.140 ± 4.573 μM for spermidine, 215.448 ± 13.200 μM for GlcN-6-P, 338.110 ± 56.793 μM for spermine and 859.855 ± 227.057 μM for poly-L-lysine (Table 1). Ste27 has therefore been shown to be a SSAT, with spermidine being the preferred substrate.
Table 1  Kinetic constants for the SSAT activity of Ste27 from *Streptomyces* sp. 139

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ ($\mu$M)</th>
<th>$V_{max}$ ($\mu$M·min$^{-1}$·mg$^{-1}$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$·s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermidine</td>
<td>65.140 ± 4.573</td>
<td>147.15 ± 0.049</td>
<td>0.858 ± 0.057</td>
<td>1.317 × 10$^4$</td>
</tr>
<tr>
<td>Spermine</td>
<td>338.110 ± 56.793</td>
<td>158.25 ± 0.440</td>
<td>0.922 ± 0.209</td>
<td>2.726 × 10$^3$</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>56.523 ± 5.560</td>
<td>233.000 ± 0.255</td>
<td>1.358 ± 0.100</td>
<td>2.403 × 10$^4$</td>
</tr>
<tr>
<td>GlcN-6-P</td>
<td>215.448 ± 13.200</td>
<td>319.25 ± 0.608</td>
<td>1.860 ± 0.433</td>
<td>8.633 × 10$^3$</td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td>859.855 ± 227.057</td>
<td>678.200 ± 8.592</td>
<td>3.953 ± 0.770</td>
<td>4.597 × 10$^3$</td>
</tr>
</tbody>
</table>

Identification of the enzymatic product of Ste27

After performing the reaction catalysed by Ste27 as described above, the enzymatic product was extracted and analysed by TLC. As the substrate control, spermidine and acetyl-CoA along with the product were spotted on to the silica gel plate. After TLC, the plate was visualized by staining with iodine vapour. The $R_f$ values of the enzymatic product, spermidine and acetyl-CoA were 0.28, 0.02 and 0.32 respectively (Figure 3B). The mass spectrum of the extracted enzymatic product yielded the molecular parent ion at $m/z$ 272.3 which matched the molecular mass of 271.3 kDa for spermidine acetylated at the N1, N4 and N8 positions (Figure 3A). This result indicates that Ste27 works as a novel SSAT which can acetylate at the N1, N4 and N8 positions on spermidine. Therefore the enzymatic product in the reaction catalysed by Ste27 should be $N_1,N_4,N_8$-acetylspermidine.

Disruption of *ste27* and complementation of the knockout mutant

Km' Am' (ampicillin-sensitive) colonies (five colonies in total) were randomly selected and the isolated genomic DNA of mutants and *Streptomyces* sp. 139 wild-type strain were digested with NcoI. The evidence of gene replacement of *ste27*, as shown in Figure 4(A), was further supported by Southern hybridization using a 0.8-kb fragment (upstream of *ste27*) as a probe. The hybridization signals appeared with the expected sizes of 2.61 kb (wild-type strain) and 1.94 kb (*ste27*− mutant) (Figure 4B). This result indicated that the colony with Km' Am' phenotypes had integrated the Km' cassette into the *ste27* gene, which therefore has been deleted. The knockout mutant strain was named *Streptomyces* sp. 139 (*ste27*−).

After pKC27c was introduced into the knockout mutant strain, two transformants (Am' Km'') were selected and the existence of plasmid pKC27c was demonstrated by the restriction digestion pattern of the isolated plasmid (results not shown). The complementary strain was named *Streptomyces* sp. 139 (pKC27c).

Bioactivity of EPSs *in vitro*

ELISA results indicated that the competitive binding ratio of EPS-27m produced by *Streptomyces* sp. 139 (*ste27*−) with IL-1 for IL-1R was 2.62% (at 0.128 ng/μl), 9.83% (at 0.64 ng/μl) and 18.12% (at 3.20 ng/μl) respectively.
Molecular modelling of Ste27

To investigate further the function of Ste27, we used molecular modelling to predict the binding mode of acetyl-CoA, spermidine or spermine with Ste27. Homology models were built for Ste27 using the crystal structure of \((R)\)-oxynitrile lyase isoenzyme 1 (PDB code 3GDP) as the template of the NTD (residues 1–175) and that of a putative acetyltransferase (PDB code 2OZH) as the template for the CTD (residues 176–270). The sequence alignment results between the templates and the Ste27 sequence are shown in Figure 5(A). Both templates have high similarity with the objective sequence, with the similarity approximately 30% for the Ste27 NTD and approximately 45% for the CTD respectively, suggesting a high credibility of the homology model. The model is shown in Figure 5(B), and two separate domains were generated. Notably, the CTD structure of the model is well aligned with the crystal structure of N-\(\alpha\)-acetylation of ribosomal protein S18 [17] (PDB code 2CNT, a member of the acetyltransferase family), especially well-aligned at the CoA-binding site motif (Figure 5C), with an RMSD (root mean square deviation) of 1.42 Å (1 Å = 0.1 nm). Therefore we hypothesize that the similar cavity on the Ste27 model is the catalytic site. Molecular docking towards the site was performed to verify the possible binding mode of spermidine, spermine or acetyl-CoA, and the results are shown in Figures 5(D) and 5(E). For acetyl-CoA, it fits the binding site quite well (Figures 5D and 5E). In addition, it is assumed to form hydrogen-bond interactions with Arg205, Arg216, Phe243 and Arg253, as well as hydrophobic interactions with Pro208, Val209, Phe243 and Val250 at the predicted binding site (Figure 5F). Interestingly, from the results of molecular docking, both spermidine and spermine were found to bind at the edge of the other site of the cavity, rather close to the acetyl group of acetyl-CoA (Figures 5D and 5E), which indicate the possibility that spermidine or spermine could be acetylated at the predicted catalyst site with the aid of acetyl-CoA. This would be consistent with our previous experimental data described above. Specifically, it is assumed that spermidine forms hydrogen-bond interactions with Glu95, Glu96, Ala161 and Asp245, as well as hydrophobic interactions with Ala97, Ala161 and Leu246 at the site (Figure 5F), whereas spermine forms hydrogen-bond interactions with Glu95, Glu96, Ala161, Phe243 and Asp245, as well as hydrophobic interactions with Ala97, Ala161, Leu164, Phe243 and Leu246, which indicates that both spermidine and spermine should bind at the unique position with high specificity (Figure 5F).

DISCUSSION

The homoeostasis of polyamines is tightly regulated to maintain normal cell growth and proliferation [18–20]. In eukaryotes, acetylation is a rate-limiting step in polyamine catabolism [21]. The mammalian SSAT1 preferentially recognizes and acetylates the primary propyl amines of spermidine or spermine, that is, they

13.25% (at 3.2 ng/\(\mu\)l), which were obviously lower than those of Ebosin (54.28%, 50.12% and 41.59% respectively) at same concentrations (Figure 4C). But the competitive binding ratio of EPS-27c isolated from the Streptomyces sp. 139 (pKC27c) with IL-1 for IL-1R were 13.77% (at 0.128 ng/\(\mu\)l), 31.9% (at 0.64 ng/\(\mu\)l) and 39.35% (at 3.2 ng/\(\mu\)l), which were remarkably restored compared with EPS-27m, but still lower than those of Ebosin at the same concentrations (Figure 4C).
transfer the acetyl group from acetyl-CoA to the N1 position of spermidine or spermine [22]. Increasing evidence has indicated that SSAT1 may be involved in various physiological and pathological events including carcinogenesis [23], pancreatitis [22] and lipid metabolism [24]. These findings make SSAT1 an attractive target for studying new drugs. Many polyamine analogues, such as PG11047 and DENSpm (diethylnorspermine), have been developed for cancer therapy [25,26].

There have been some more detailed studies of microbial SSAT1s. In E. coli, SpeG acetylates spermidine at the N4 or N8 position with apparently equal frequency [4,6]. Woolridge et al. [27] reported that overexpression of the BldD gene in Bacillus subtilis causes acetylation of the polyamines spermidine and spermine. The BldD protein encoded by the BldD gene acetylates both spermidine and spermine at primary propyl amine moieties, with spermine being the preferred substrate, to form their respective monoacetylated derivatives. The Bacillus BldD was found to be a SSAT, which participates in the export of polyamines [28]. Amino acid sequence analysis showed that SSAT and BldD have 51% similarity and 29% identity [7], whereas SpeG and BldD have 51% similarity and 24% identity. PaiA, as a novel SSAT, was also identified in Bacillus subtilis, which shares only weak homology with both BldD and SpeG [7]. PAAI, another novel polyamine acetyltransferase, was isolated from yeast, which acetylates spermidine, spermine and putrescine. It also can acetylate the monoacetylated derivatives of these compounds, leading to the diacetylated products [30]. Polyamine acetylation in bacteria has been associated with a variety of chemical and physical stresses [7].

The filamentous, soil-inhabiting Gram-positive bacterial genus Streptomyces is characterized by the ability to produce a wide variety of secondary metabolites, especially antibiotics. Various acetyltransferases involved in the biosynthesis of antibiotics have been identified. For example, the oate gene, located in the clavulanic acid gene cluster in Streptomyces clavuligerus, which encodes the ornithine acetyltransferase, is a modulator of clavulanic acid biosynthesis [31]. An arylyamine NAT (N-acetyltransferase) responsible for the N-acetylation of exogenous 3-amino-hydroxybenzolic acid in Streptomyces griseus has also been found to be involved in the biosynthesis of xenobiotic compounds [32].

Ebosin, a novel EPS with remarkable anti-rheumatoid arthritis activity in vivo, was isolated from Streptomyces sp. 139. A gene cluster (ste) consisting of 27 ORFs has been identified as being responsible for its biosynthesis [8]. Database searches revealed that the deduced protein Ste27 is homologous with PaiA (SSAT) from Bacillus subtilis str. 168 with 22% identity and 41% similarity over a 69-amino-acid region (GenBankapsulation number CAB15205.1). For BldD (a SSAT) of Bacillus subtilis (GenBankapsulation accession number BAA12354.1) the identity and similarity are 23% and 47% respectively over a 53-amino-acid region. Ste27 also shares 23% identity and 35% similarity over a 60-amino-acid region compared with the zSSAT1 of zebrafish (GenBankapsulation accession number BC150358.1). Therefore Ste27 may be a SSAT.

Recently, Lin et al. [33] described a colorimetric SSAT activity assay, which is quick, easy and reliable. In the present study, we also employed a colorimetric assay method, similar to that of Lin et al. [33]. The results showed that Ste27 has the enzyme activity of a SSAT, catalysing spermidine or spermine and acetyl-CoA to produce CoA and acetyl spermidine or acetyl spermine, with spermidine being the preferred substrate. As a novel SSAT, it can acetylate the N1, N4 and N8 positions on spermidine. The values of Km and Vmax for various substrates, including spermidine, spermine, acetyl-CoA, GlcN-6-P and poly-L-lysine, were determined. The optimal pH and temperature in reactions was also assessed.

With the aid of molecular modelling tools, an attempt was also made to predict the binding mode of this novel acetyltransferase with its substrates. The results appear to match well with previously worked-out binding models and are consistent with our experimental findings.

For understanding the role of ste27 in the biosynthesis of Ebosin, a gene-knockout approach was taken, which resulted in a significant reduction in the competitive binding activity with IL-1 for IL-1R of EPS-27m. These results demonstrated the activity of Ste27 as a SSAT and function during Ebosin biosynthesis.

EpsH encoded by epsH in the EPS gene cluster from Streptococcus thermophilus Sf6 showed strong homology with acetyltransferase of the NodL-LacA-CysE family [34]. Acetyltransferases of this type have also been found in other biosynthesis gene clusters of polysaccharides [35]. Hence it is not surprising that a gene encoding a SSAT is involved in the biosynthesis gene cluster (ste) of Ebosin. Most polysaccharides (approximately 80%) were found to contain amino acids, uronic acid and various other non-carbohydrate components [36]. Polyamines, such as spermidine, spermine and putrescine, are positively charged small molecules that bind tightly to both RNA and DNA and also to some proteins [37,38]. Whether spermidine or spermine is present in the molecule of Ebosin when binding needs further investigation. As a novel SSAT, the role of Ste27 in the acetylation of polyamines for their catabolism and/or excretion from cells should also be studied.

AUTHOR CONTRIBUTION
Yang Zhang identified the enzymatic product of Ste27 and performed the experiments investigating the binding activity of IL-1R of the EPSs and the assays of kinetics for Ste27 catalysis. Jinming Zhou carried out the molecular modelling of Ste27. Ming Chang generated the mutant Streptomyces sp. 139 (ste27−), the complementary strain and performed cloning and expression of ste27 in E. coli. Liping Bai and Chen Yao purified the protein. Junjie Shan and Rong Jiang isolated the EPSs. Lianhong Guo cultivated the strains. Ren Zhang and Jiangbo Wu participated in the data analysis. Yuan Li was in charge of the experimental design.

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