A DasA family sugar binding protein Ste2 links nutrient and oxidative stress to exopolysaccharides production in *Streptomyces* sp. 139

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**Abstract**

**Background:** Ebosin is an exopolysaccharide produced by *Streptomyces* sp. 139, and its biosynthetic gene cluster (*ste*) has been previously described. Ste234 has high homology to the well-known ATP-binding cassette transport system DasABC, which has been linked to the regulation of morphological differentiation, antibiotics biosynthesis and aminosugars utilization in Streptomyces. This study was conducted to evaluate the effect of the DasA family sugar binding protein Ste2 on *Streptomyces* sp. 139.

**Results:** The disruption of *ste2* results in the upregulation of transcription of genes within Ebosin biosynthetic gene cluster and a two-fold increase in Ebosin production. RNA sequencing data suggests that the disruption of *ste2* results in the decreased utilization of carbon and nitrogen sources, increased sensitivity to oxidative stress, as well as differed strain morphology, all of which have been experimentally proven.

**Conclusions:** Taken together, Ste2 controls Ebosin yields, aminosugars uptake, sensitivity to oxidative stress, and morphological differentiation of *Streptomyces* sp. 139.

**Keywords:** Ste2, DasABC, Ebosin, *Streptomyces* sp. 139, Nutrient stress, Oxidative stress

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**Introduction**

Exopolysaccharides (EPSs) are long-chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives, secreted by different microorganisms under stress conditions, and are not permanently attached to the cell surface [1]. The EPSs produced by bacteria are involved in multiple protective cellular functions that improve bacterial competition in different habitats [2]. Although a variety of bacterial EPSs have been reported, studies of EPSs from Streptomyces remain limited. In our previous work, a novel EPS produced by *Streptomyces* sp. 139, designated Ebosin (EPS 139A), has been found to possess remarkable anti-rheumatic arthritis activity *in vivo* [3, 4]. Also, its biosynthesis gene cluster (*ste*) consisting of 27 ORFs has been identified [5–7], in which Ste234 belongs to the family of ATP-binding cassette (ABC) transporters.

ABC transporters couple ATP hydrolysis to the uptake and efflux of solutes across the cell membrane in bacteria [8]. ABC transporters can uptake a wide variety of substances including sugars, peptides, and amino acids, allowing bacteria to monitor environmental and physiological changes, i.e., nutrient depletion, and providing the way to avoid possible damages [9]. ABC transporters also...
function in efflux of substances, including surface components of the bacterial cell (such as capsular polysaccharides, lipopolysaccharides, and teichoic acid), proteins involved in bacterial pathogenesis (such as hemolysin, heme-binding protein, and alkaline protease), heme, drugs and siderophores [10].

Various genes encoding oligosaccharide-uptake systems have been identified in Streptomyces: cebEFG for cellobiose and cellotriose in Streptomyces reticuli [11]; malEFG for maltose in S. coelicolor [12]; ngcEFG for N-acetylglucosamine (GlcNAc) and (GlcNAc)₂ in Streptomyces olivaceoviridis [13]; and bxlEFG for xylobiose in Streptomyces thermoviolaceus [14]. Interestingly, all of these operons encode subunits of ABC transporters, with the E and FG genes of these uptake systems encoding sugar-binding proteins (SBPs) (CebE, MalE, NgcE and BxlE) and two putative integral membrane proteins (CebFG, MalFG, NgcFG and BxlFG), respectively [15]. Deficient in aerial mycelium and spore formation (Das-RABC) was first identified in S. griseus as an ATP-binding cassette transport system involved in regulation of morphological differentiation in response to glucose and is one of the best studied ABC transporters in Streptomyces [16]. Our previous study demonstrated that ste34 in Streptomyces sp. 139 are homologous to the membrane spanning protein encoding genes dasBC [17]. The disruption of ste34 resulted in a mutant strain with dramatically decreased production of Elosin [17]. Meanwhile, the effect of Ste2, which has high homology to DasA from S. coelicolor and S. griseus, remains unclear. Here, we demonstrate that the sugar binding protein Ste2 controls nutrient uptake, exopolysaccharide yields, morphological differentiation, sensitivity to oxidative stress and microbial morphology of Streptomyces sp. 139.

Results
Homology analysis of Ste2
Whole genome sequence of Streptomyces sp. 139 is available [18]. Amino acid alignment of Ste2 with DasA has been done with Clustal Omega Software (https://www.ebi.ac.uk/Tools/msa/clustalo/) and is shown in Supplementary Fig. 1. Ste2 shows 84% identity and 91% similarity to the sugar binding protein DasA of Streptomyces coelicolor A3 (2) [19, 20], 34% identity and 49% similarity to DasA of Streptomyces griseus [16] (Supp. Figure 1A). Homology of Ste34 to DasBC has been published in our previous research [17], and the alignment is shown in Supp. Figure 1B-C. Ste3 shows 89% identity and 94% similarity to integral membrane protein DasB of Streptomyces coelicolor A3 (2) [19, 20], 35% identity and 58% similarity to DasB of Streptomyces griseus [21] (Supp. Figure 1B). Ste4 shows 87% identity and 94% similarity to integral membrane protein DasC of Streptomyces coelicolor A3 (2) [19, 20], 41% identity and 63% similarity to DasC of Streptomyces griseus [21] (Supp. Figure 1C).

Strain construction and validation
To elucidate the function of Ste2 in the biosynthesis of Elosin, the ste2 deletion strain (strain D2) was constructed using a double cross-over gene-replacement strategy. A number of colonies (Km’ Am’) were selected randomly and their genomic DNAs were isolated and confirmed by Southern hybridization. As shown in Fig. 1A, ste1-4 is located within two BamHI cut sites, giving a 5.8 kb fragment following BamHI digestions. When ste2 was replaced with the Kmr fragment, this BamHI fragment was 6.3 kb (Fig. 1A). As shown in Fig. 1B, a distinctive hybridization band of 6.3 kb was detected in the ste2-deleted mutant strain, designated as strain D2 (Fig. 1B, line 2), and a band of 5.8 kb was obtained for wild type strain (Fig. 1B, line 1). To study whether the effects of ste2 deletion can be reversed by the complementation of ste2, the complementary strain was constructed as described in the method section. Briefly, fragment ermE’ and ste2 were ligated together and inserted into the BamHI–HindIII-cut pKC1139 vector to yield pKC2C. After being propagated in E. coli ET12567, pKC2C was isolated and transformed into the protoplasts of strain D2. 10 transformants (Am’ Kmr’) were obtained and the existence of pKC2C was confirmed by restriction digestion pattern (not shown). The complementary strain was designated as strain C2.

Disruption of ste2 dramatically enhances Elosin production in Streptomyces sp. 139
The fermentation supernatant at 96 h for Streptomyces sp. 139, Strain D2 and Strain C2 were harvested by centrifugation, and then analyzed by ELISA for the determination of its antagonist rate for IL-1R, as well as quantified for the total yield of Elosin (Fig. 2B), respectively. The disruption of ste2 resulted in a more than 50% increase in the supernatant’s antagonist rate for IL-1R, and the complemented strain has about the same rate as the native strain. Strain D2 has an around two-fold increase in its Elosin production, while the complemented strain C2 has similar production level as the native strain.

The pH value of each sample was also analyzed. As the incubation time prolonged, the pH value of culture supernatant gradually increased from around 6.7 to 8.2. pH value of culture supernatant between Streptomyces
sp. 139, strain D2 and strain C2 did not show significant difference (Fig. 2C).

The wet mycelium weight of *Streptomyces* sp. 139, strain D2 and strain C2 at each time point were shown in Fig. 2D. Mycelium density reached maximum during 48 h to 72 h incubation period, while mycelium density of *Streptomyces* sp. 139 was higher than strain D2 and strain C2.

**Expression of genes within Ebosin biosynthetic gene cluster are significantly upregulated in D2 strain**

A RT-qPCR experiment was performed to further confirm whether genes within Ebosin biosynthetic gene cluster were regulated at certain time points (Fig. 3). The genes encoding galactosyltransferase (*ste5*) [5], chain length determinants (*Wzz*) (*ste8*), and α-D-glucose-1-phosphate cytidylyltransferase (*ste17*) [22], respectively,
were chosen for this experiment. As shown in Fig. 3, ste5, ste8, and ste17 were upregulated for at least 3-fold in D2 strain at 24 h, all of which are statistically significant \( (p < 0.05) \). At 48 h, ste5 was upregulated for around twofold in D2 strain \( (p < 0.05) \), ste8 was upregulated for about threefold in D2 strain \( (p < 0.05) \), while the upregulation of ste17 was not that significant \( (p > 0.05) \). At 96 h, there was not a significant difference in the transcription of the three genes in all strains \( (p > 0.05) \). The complemented strain has similar transcription level as wild-type strain at all time points \( (p > 0.05) \). As a conclusion, the deletion of ste2 resulted in the upregulated transcription of ste5, ste8, and ste17, especially in the first 48 h, which cooperates with the fact that the Ebosin yield is promoted in D2 strain.

**Strain D2 has a defect in the utilization of GlcNAc and \( (\text{GlcNAc})_2 \)**

To investigate whether Ste2 plays a role in the uptake of aminosugars, we quantified the rate of utilization of glucose (Fig. 4A-B), GlcNAc (Fig. 4C-D) and \( (\text{GlcNAc})_2 \) (Fig. 4E-F) in the supernatant of the native and D2 strain which was cultured in basic media \( (10 \text{ mM } \text{K}_2\text{HPO}_4, 10 \text{ mM } \text{KH}_2\text{PO}_4, 1 \text{ mM } \text{CaCl}_2, 0.5 \text{ mM } \text{MgCl}_2, 0.1\% \text{ (v/v) trace element solution}) \) supplemented with 250 \( \mu\text{M} \) of glucose, GlcNAc and \( (\text{GlcNAc})_2 \), respectively. Both
Fig. 3  Quantification of the transcription level of ste5 (A), ste8 (B), and ste17 (C) in Streptomyces sp. 139 wild-type, D2 and C2 strain. Strains were allowed to grow for 24 h, 48 h, and 96 h before analysis. The transcription level of ste5, ste8, and ste17 was normalized to the transcription level of hrdB, respectively. Blue: wild-type strain; black: strain D2; green: strain C2. Experiments were done in triplicate.
strains absorbed all supplemented glucose within the first two hours. However, the disruption of ste2 significantly decreased the uptake of GlcNAc and (GlcNAc)$_2$. Wild-type 139 strain utilized all supplemented GlcNAc within the first 2 h, while the D2 strain utilized only 20% supplemented GlcNAc during the same period of time, and all supplemented GlcNAc at 8 h. As for (GlcNAc)$_2$, wild-type strain utilized 80% within 2 h and nearly all supplemented (GlcNAc)$_2$ within 4 h. The D2 strain barely utilized any (GlcNAc)$_2$ within the first 4 h, around 50% until 8 h, while around 70% until 12 h. The results suggest that Ste2 plays an important role in the utilization of GlcNAc and (GlcNAc)$_2$ in Streptomyces sp. 139.

**ste2 null mutant has a highly aberrant phenotype**
A closer inspection of the native and D2 strain with a cryo-scanning electron microscope is shown in Fig. 5. On the minimum medium supplemented with glucose or R2YE agar medium, Streptomyces sp. 139 (WT) produced abundant and wild-type spores (Fig. 5A, B). In contrast, Strain
D2 failed to produce normal aerial hyphae and spores on minimum medium supplemented with glucose (Fig. 5A). Excitingly, the ste2 mutant on R2YE agar medium had highly aberrantly shaped spores, which produced spore heaps at a high frequency (Fig. 5B). To our knowledge, such a phenotype has not been established before in any Streptomyces mutant.

**Fig. 5** Scanning electron micrographs of Streptomyces sp. 139 and strain D2 grown on minimal agar medium (A) and R2YE agar medium (B).

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**Global transcriptional changes in Streptomyces sp. 139 D2 mutant**

Transcriptome analysis was done in triplicate for both the wild type strain and ste2 mutant strain. Using a cut-off of 2.0-fold difference between the wild-type and D2 mutant, 2476 genes were identified as differentially expressed. As shown in Fig. 6A, 1118 genes were upregulated and...
1358 genes were downregulated for more than 2.0-fold in D2 mutant when compared with wild-type strain, and those changes are statistically significant (Adjusted P value $< 0.001$ [23]). 4333 genes were not regulated in a statistical significantly manner. According to the National Center for Biotechnology Information (NCBI) S139 genome annotation, gene-annotation enrichment and functional annotation clustering analysis of differentially expressed genes (DEGs) were conducted [23]. As shown in Fig. 6B, the DEGs were enriched in thirty significantly changed terms in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. According to the KEGG pathways definition, most of the genes being significantly regulated are involved in metabolic pathways, following which are microbial metabolism in diverse environments, quorum sensing, carbon metabolism, ABC transporters, etc. (Fig. 6B).

According to the RNA sequencing analysis provided by Beijing Genomics Institute (Table S1), we found that 111 genes have a higher than eight-folds difference in their expression level in the ste2 mutant strain compared with wild type strain. Within the 111 genes, 86 genes were down-regulated, while 25 genes were up-regulated. Most of the genes have a close relationship with metabolic
pathways, microbial metabolism in diverse environments, quorum sensing, as well as carbon and nitrogen metabolism. It is clear that in ste2 mutant strain, some genes with their expression levels significantly differed from wild strain are involved in carbon/nitrogen fixation and cellular response to oxidative stress. For example, a gene encoding a carbohydrate ABC transporter substrate-binding protein has a 15.7-fold increase in its expression level in ste2 mutant strain. The protein is a periplasmic component within an ABC-type glyceraldehyde-3-phosphate transport system and is involved in carbohydrate transport and metabolism. A gene encoding an MBL fold metallo-hydrolase has an 18-fold increase in its expression level in ste2 mutant strain. The protein is a L-ascorbate metabolism protein and is involved in carbohydrate transport and metabolism.

A selected collection of genes of interest have been summarized in Table 1. Apart from genes regulating carbon metabolism, two genes encoding methionine adenosyltransferase and glutamate-ammonia ligase have been upregulated for around three- and seven-folds, respectively. It suggests that the amino acid metabolism in ste2 disruption strain is also promoted. Excitingly, two genes encoding putative N,N'-diacetylchitobiose transport system substrate-binding proteins have a 6.47-fold and 2.17-fold decrease in their expression levels in ste2 mutant strain, respectively, which corroborates with the decreased uptake of GlcNAc and (GlcNAc)2 in the D2 strain. Interestingly, a gene encoding a cyclic-di-GMP-binding protein has a 3.7-fold increase in the D2 strain. It has been previously reported that the bacterial EPSs are regulated for c-di-GMP, and c-di-GMP also plays a key role in the utilization of nitrogen source, the biosynthesis of multiple nucleoside precursors, and response to oxygen, nitric oxide, and a variety of other environmental challenges [24, 25]. However, c-di-GMP binding proteins are highly diverse, and identification of them remains a difficult and incomplete task [26], thus the increase in the expression level of the c-di-GMP-binding protein in the D2 strain needs future investigation.

The differed morphology of wild-type and D2 strain might be attributed to the differed expression of three rod shape-determining protein MreB and related proteins, in which two genes are down-regulated, while one

| Table 1 | Selected genes being significantly up- or down-regulated in the ste2 disruption strain D2. The function, KEGG orthology, and a result of BLAST are shown for each gene, respectively |
|---------|----------------------------------------------------------------------------------------------------------|
| Function | Gene ID | D2 to WT Ratio | KEGG Orthology | Blast nr |
| Carbohydrate metabolism | S139GL004816 | 15.74661713 | alpha-glucoside transport system substrate-binding protein | carbohydrate ABC transporter substrate-binding protein [Streptomyces sp. CNH189] |
| | S139GL004057 | 18.01236617 | peptidoglycan DL-endopeptidase CwlO | MBL fold metallo-hydrolase [Streptomyces sp. CNH189] |
| Amino acid metabolism | S139GL002102 | 2.958184062 | S-adenosylmethionine synthetase | methionine adenosyltransferase [Streptomyces sp. CNH189] |
| | S139GL005081 | 6.721092236 | glutamine synthetase | glutamate–ammonia ligase [Streptomyces] |
| Membrane transport | S139GL002390 | 0.154636472 | N,N'-diacetylchitobiose transport system substrate-binding protein | putative sugar transporter sugar-binding protein [Streptomyces sp. 139] |
| | S139GL002391 | 0.461712269 | N,N'-diacetylchitobiose transport system permease protein | putative sugar transporter integral membrane protein [Streptomyces sp. 139] |
| Response to oxidative stress | S139GL004328 | 0.226084044 | catalase-peroxidase | catalase/peroxidase HPI [Streptomyces sp. CB02400] |
| | S139GL003847 | 0.196900796 | ATP-binding cassette, subfamily C, bacterial CydCD | ABC transporter ATP-binding protein [Actinobacteria] |
| | S139GL000959 | 5.552515326 | peroxiredoxin [alkyl hydroperoxide reductase subunit C] | peroxiredoxin [Streptomyces sp. CNH189] |
| | S139GL001510 | 4.306959158 | aldehyde dehydrogenase [NAD+] | aldehyde dehydrogenase [Streptomyces sp. CNH189] |
| Metabolism/ regulation | S139GL000617 | 3.71384308 | cyclic-di-GMP-binding protein | DUF520 domain-containing protein [Streptomyces] |
| Cell morphology | S139GL003161 | 0.45401102 | rod shape-determining protein MreB and related proteins | hypothetical protein [Streptomyces sp. CNH189] |
| | S139GL005336 | 0.179128877 | rod shape-determining protein MreB and related proteins | rod shape-determining protein [Actinobacteria] |
| | S139GL005480 | 2.244936284 | rod shape-determining protein MreB and related proteins | rod shape-determining protein [Streptomyces] |
gene is up-regulated. MreB, the cell shape-determining bacterial actin homologue, has been demonstrated to be required for the maintenance of a rod-shaped cell and formation of spirals that traverse along the longitudinal axis of *Bacillus subtilis* and *E. coli* cells; MreB filaments also function as a cytoskeleton, serving as an organizer or tracking device for the cell wall morphogenesis in *Caulobacter crescentus* [27]. In *S. coelicolor*, MreB has been demonstrated to localize underneath the internal spore wall but not in vegetative mycelium, and has been suggested to function in the formation of environmentally stable spores [28]. The regulation of cellular shape might be a complicated process in *Streptomyces* sp. 139 strain and future work will be required.

Genes involved in responding to oxidative stress are regulated in a more complicated manner. A gene encoding a catalase-peroxidase (S139GL004328) is down-regulated for more than four-folds in D2 strain. A homologue of the catalase-peroxidase KatG in *E. coli* has been demonstrated to have both catalase and broad-spectrum peroxidase activity, plus NADH oxidase, INH lyase and isonicotinoyl-NAD synthase activity, in which INH lyase and isonicotinoyl-NAD synthase are responsible for the activation of isoniazid as an anti-tubercular drug [29]. It suggests the D2 strain will be more sensitive to oxidative stress than wild-type strain. A gene encoding a homologue to the bacterial heterodimeric ABC transporter CydCD is down-regulated for more than five-folds in D2 strain. In *E. coli*, CydCD is required for the biogenesis of both cytochrome *bd*-type quinol oxidases and periplasmic cytochromes. CydDC could also act as a thiol transporter [30]. Thus the disruption of *ste2* may also render the strain to be more sensitive to oxidative stress. In the meantime, genes encoding homologues of peroxiredoxin and aldehyde dehydrogenase in *Streptomyces* sp. CNH189 are upregulated for 4.3- and 5.6-folds in D2 strain, suggesting that the survival rate of D2 strain may be increased under oxidative stress. For more information, a full list of genes whose expression changed significantly is available in Table S1.

To summarize, we hypothesize that the deletion of *ste2* enhances the strain’s ability to metabolize carbohydrates and amino acids, to sense and deal with oxidative stress from hydrogen peroxide and aldehydes in the outside environment; in the meantime, the deletion of *ste2* decreases the strain’s ability to uptake aminosugars like N,N’-diacetylchitobiose. Morphology of the strain is also affected by differed expression of multiple rod shape-determining proteins.

**Strain D2 is more sensitive to hydrogen peroxide than the wild-type strain and strain C2**

Global transcriptional changes in *Streptomyces* sp. 139 D2 mutant revealed that the disruption of *ste2* may render the strain to be more sensitive to oxidative stress. We decided to test whether hydrogen peroxide has a differed effect in the growth of wild-type and D2 strain, as hydrogen peroxide is a natural source of oxidative stress. Wild-type strain and strain C2 are less sensitive to hydrogen peroxide compared with strain D2. The MIC of hydrogen peroxide...
peroxide against wild-type strain and strain C2 is 1%, while is 0.25% against strain D2. Thus Ste2 might play a role in protecting the strain from oxidative stress, which corroborates with the RNA seq analysis. To further investigate the effect of hydrogen peroxide on the growth of the three strains, we performed a strain growth test (Fig. 7). At a selected sub-MIC concentration of hydrogen peroxide (0.1%), the growth of strain D2 is much slower compared with both wild-type and strain C2. At 36 h post exposure, the OD600 of strain D2 is at around 0.3, while are at around 0.9 and 1.2 of wild-type strain and strain C2, and the difference is statistical significant ($p < 0.05$). Similar trend was presented at 48 h post exposure, in which the difference of OD600 between strain D2 and WT/strain C2 is statistical significant ($p < 0.05$). We concluded that Ste2 has a protective role against oxidative stress resulted from hydrogen peroxide, while the detailed mechanism will require future investigation.

**Discussion**

Bacterial EPSs are sources of medically important substances: many microbial EPSs are found to have anti-inflammation, anti-tumor, anti-aging and anti-rheumatic properties. They are synthesized via biosynthetic enzymes, being secreted into the surrounding environment, and are often important for the biofilm formation and pathogenicity [31]. As for the function of EPSs to their producing organisms, the regulation of oxidative stress has been linked to EPS synthesis in *Streptococcus mutans* [32]. The deletion of the diadenylate cyclase CdaA encoding gene causes decreased c-di-AMP levels, increased sensitivity to hydrogen peroxide and increased production of EPSs in *S. mutans* [32]. The function of *Streptomyces*-origin EPSs has not been well studied yet: besides from Ebosin from *Streptomyces* sp. 139, only a few other *Streptomyces*-origin EPSs have been reported, i.e., poly-β-1,6-N-acetylglucosamine from *Streptomyces coelicolor* and *Streptomyces lividans* possibly participates in the attachment of bacteria to hydrophilic surfaces [33].

DasRABC (Deficient in aerial mycelium and spore formation) was first identified in *S. griseus* as an ATP-binding cassette transport system involved in regulation of morphological differentiation in response to glucose [16]. Overexpression of *dasA* caused ectopic septation in very young substrate hyphae after only one day of growth and subsequent sporulation in response to glucose, while disruption of *dasA* or *dasR* resulted in growth as substrate mycelium [16]. In a later study, DasA has been demonstrated to act as a link between chitin utilization and morphogenesis in *S. coelicolor* [34]. The interruption of *dasA* resulted in an extraordinary and unique phenotype, i.e., spore chains showed extensive germination. The premature germination could possibly be linked to the disorder of GlcNAc utilization in *dasA* mutants [34]. Since then, *dasRABC* has been extensively studied and has been linked to the *Streptomyces* antibiotics biosynthesis as well as aminosugars utilization. For example, DasR is able to bind directly to the promoters of all genes encoding pathway-specific regulators of each all known antibiotics produced by *S. coelicolor*, including undecylprodigiosin, actinorhodin, cpk-tertolyketide and cda-calcium dependent antibiotic [35]. Moreover, DasR could sense the nutritional signals and regulate the biosynthesis of secondary metabolites [35, 36]. The *dasABC* gene cluster has been demonstrated to encode an ABC transporter for the uptake of N, N'-Diacyltchitobiose ((GlcNAc)$_2$) in *S. coelicolor* A3(2) [37]. Notably, DasA protein had the highest affinity for (GlcNAc)$_2$, and the disruption of *dasA* resulted in a four-fold decrease in the uptake rate of (GlcNAc)$_2$. GlcNAc and (GlcNAc)$_2$ serve as both carbon and nitrogen sources, and are the preferred nutrient sources for *Streptomyces* [38]. In this study, ste2, which is homologous to *dasA*, has also been demonstrated to play an important role in the utilization of carbon/nitrogen sources including GlcNAc and (GlcNAc)$_2$ in *Streptomyces* sp. 139.

Our previous study has linked Eboisin production to Ste1 (GntR family regulator DasR) and demonstrated that Ste1 serves as a transcription repressor during Eboisin biosynthesis by binding to the promoter 1 and 3 regions in the ste gene cluster with high affinity [39]. In this study, we focused on Ste2 and found Ste2 to be the negative regulator during Eboisin biosynthesis. The disruption of *ste2* results in a two-fold increase in Eboisin production. Real-time qPCR reveals that genes within Eboisin biosynthetic gene cluster are significantly upregulated in the *ste2* disruption strain. RNA sequencing analysis suggests that the disruption of *ste2* possibly results in the increase of sensitivity to oxidative stress as well as the fixation of carbon and nitrogen sources, and the increased level of Eboisin being secreted would in turn help the bacterial strain to avoid cellular damage resulted from oxidative stress. Transcripomnic analysis reveals that the disruption of *ste2* results in the increase of sensitivity to oxidative stress, which is confirmed by a hydrogen peroxide sensitivity test. The *ste2* disruption strain has a defect in aminosugars uptake as well as has a different morphology compared with the native strain. A list of genes has been found to be affected by the disruption of *ste2*, which will be of future interest to study EPSs biosynthesis and the function of EPSs to its producing strain. To the best of our knowledge, this is the first study which links the *Streptomyces*-origin EPSs production to the cellular morphology, nutrient utilization, and cellular response to oxidative stress.
Conclusion

Our findings revealed that the sugar binding protein Ste2 controls Ebosin production, aminosugars utilization, sensitivity to oxidative stress, and morphological differentiation of *Streptomyces* sp. 139. Our work points out the possible connection between environmental stress sensing, nutrient uptake and EPSs biosynthesis, thus will be useful for future work aimed at understanding the function of EPSs to their producing organisms and illustrating the biosynthesis of microbial EPSs that are of medical importance.

Materials and methods

Bacterial strains, plasmids, primers and growth conditions

Bacterial strains, plasmids, and primers used in this work are listed in Table 2. *E. coli* DH5α was routinely used for vectors construction. All primers were provided by Invitrogen (Beijing, China). *Streptomyces* sp. 139 and mutant strains were cultured in solid minimal (10 mM K₂HPO₄, 10 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM MgCl₂ supplemented with 0.1% v/v trace element solution) or R2YE medium [40] plus agar (1.5% w/v), and were incubated at 28°C with shaking (250 rpm) either in Tryptic Soy Broth (TSB, USA) supplemented with 5 mM MgCl₂ and 0.5% glycine or in fermentation medium (1% glucose, 2% starch, 2% soybean extract, 0.2% tryptone, 0.2% beef extract, 0.4% yeast extract, 0.05% K₂HPO₄, 0.3% CaCO₃, pH 7.3). *E. coli* strains were grown aerobically at 37°C in Luria–Bertani (LB) medium or LB solidified with agar (1.5% w/v) supplemented with the appropriate antibiotics (100 μg/mL ampicillin, 100 μg/mL kanamycin or 50 μg/mL apramycin).

General DNA manipulation

Isolation of *E. coli* plasmid DNA and standard recombinant DNA techniques were performed as previously described [41]. *Streptomyces* plasmid and genomic DNA was isolated as previously mentioned [40].

| Table 2 | Strains, plasmids, and primers used in this study |
| --- | --- |
| **Bacterial strains** |
| *Streptomyces* sp. 139 | Ebosin producing strain |
| *Streptomyces* sp. 139 D2 | ste2 knockout mutant of *Streptomyces* sp.139 |
| *Streptomyces* sp. 139 C2 | ste2-complemented strain |
| *E. coli* DH5α | F⁻ recA1 endA1 hsdR17 thyA-1 supE44 gyrA96 relA1 (lacYZA- argF) U169λ− (φ80dlacZΔM15) |
| *E. coli* ET12567 | methylation-deficient *E. coli*, dam− dcm− hsdM |
| *E. coli* BL21 (DE3) | F− ompT hsdSB(rB− mB−) dcm gal λ(DE3) |
| **Plasmids** |
| pKC1139 | Shuttle plasmid (*E.coli*–*Streptomyces*); pSG5, pBR322; aac[3]IV lacZα oriTRK2; Amr 43 |
| pKC2D | pKC1139 derived plasmid carrying F1, F2 and Km' fragments; Km' Amr' |
| pKC2C | pKC1139 derived plasmid carrying 0.45-kb ErmE' promoter fragment and ste2; Amr' |
| pGEM-3Zf-ErmE | Resource of ErmE' promoter; Ap' |
| pET30a | T7 promoter, His-tag, Km' |
| pET30a-ste2 | pET30a derived plasmid carrying ste2; Km' |
| **Primers** |
| P1 (EcoRI) | CTGGAATTCTGCGCCCTTGGCCCTGGAT |
| P2 (XbaI) | GCCCTCTAGACTCGCCTGGCTTCTCT |
| P3 (XbaI) | GCAATCTAGAGCGAGGGGAGAGGAGGAG |
| P4 (HindIII) | AGCGAGCTCTAGGATGGGAGGAGGAGGAG |
| P5 (BamHI) | CGCGGGATCCTAGGTCGGAGAAGAGGAG |
| P6 (HindIII) | CCCAAGCTTATTACCTCTGAGGGCCAG |
| ste5 forward | GCTGATCTCGCTGCTGGTCGTCG |
| ste5 reverse | CTGCGCGCGACGGGAGGACGG |
| ste6 forward | TCTGCGCGCGAGGAGGAGGAG |
| ste6 reverse | CGAAGTGCGGGGAGGAGGAG |
| ste17 forward | CTGCGCGCGAGGAGGAGGAG |
| ste17 reverse | CGAAGTGCGGGGAGGAGGAG |
| hrdB forward | TGGTGCAGGTGCTCATCAGAAG |
| hrdB reverse | TGGACCTCTGATGACCTTTC |

Reference:

1. Our lab
2. This study
3. Novagen
Disruption of ste2 in Streptomyces sp. 139

Primers are listed in Table 2. Using Streptomyces sp. 139 chromosome as template, the 829-bp upstream region of ste2 (designated F1) was amplified with primers P1 and P2, while the 749-bp downstream region of ste2 (designated F2) was amplified with primers P3 and P4. P1 contains an EcoRI restriction site, P2 contains an XbaI restriction site, P3 contains an XbaI restriction site and P4 contains a HindIII restriction site. The PCR protocol is as follows: initial denaturation at 98°C for 3 min, 30 cycles of 20 s at 98°C, 30 s at 62°C, 1 min at 72°C and finally 10 min at 72°C. A 1.2-kb fragment containing the kanamycin resistance gene (designated F3) was obtained by digesting DNA of Streptomyces griseus SS-1198PR with XbaI [42].

After ligating the three DNA fragments (F1, F3, then F2), the resulting 3-kb fragment was inserted into the EcoRI–HindIII sites of pKC1139 [43] to create the ste2 disruption vector pKC2D. After propagation in E. coli ET12567 [44], pKC2D was introduced into Streptomyces sp. 139 by polyethylene glycol (PEG)-mediated protoplast transformation [40]. Briefly, plates were incubated at 28°C for 20 h, before being overlaid with soft R2YE (0.7% agar) containing 40 μg/L of kanamycin. pKC2D bears a temperature-sensitive Streptomyces replication origin which is unable to replicate when temperature reaches 34°C. The transformants were first incubated at 28°C for two days until pinpoint-size colonies appeared, and were shifted to 37°C for further incubation. Mutants resulted from the double-crossover homologous recombination grew out of the original pinpoint-size colonies within a few days. The disruption of ste2 on the chromosome was confirmed by Southern blot. For Southern blot analysis, a DIG high prime DNA labeling and detection starter kit (Roche, USA) was used following the manufacturer’s instructions. All transcripts were normalized to hrdB (RNA polymerase principal sigma factor) transcript quantities.

Complementation of ste2 disruption strain

Using Streptomyces sp. 139 chromosomal DNA as template, ste2 was amplified with primers P5 and P6. Digested with EcoRI and BamHI, a 0.45-kb fragment of ermE promoter was isolated from plasmid pGEM-3zf-ermE [45]. Fragment ermE and ste2 were ligated together and inserted into the BamHI–HindIII-cut pKC1139 vector to yield pKC2C. After being propagated in E. coli ET12567, pKC2C was isolated and transformed into the protoplasts of strain D2. The complementing strain was designated as strain C2.

Isolation and activity analysis of Ebosin

Ebosin was isolated from a total of 1 L of the supernatant of the fermentation culture of Streptomyces sp. 139, Strain D2 and Strain C2 at 28 °C for 96 h in triplicate, as previously described [46]. An enzyme-linked immunosorbent assay (ELISA) was used to analyze the binding activity of isolated Ebosin with interleukin-1 (IL-1) for IL-1R [46].

Determination of glucose, GlcNAc, and (GlcNAc)2 concentrations.

To investigate the responses of the cells to sugars, Streptomyces sp. 139 and its mutant strain Streptomyces sp. 139 D2 were cultivated by following a method described previously [39], with some modifications. Spores of Streptomyces sp. 139 strains formed on agar medium were inoculated into 100 mL TSB medium in a 500-mL baffled Erlenmeyer flask and grown for 48 h at 28 °C on a rotary shaker at 200 rpm. Mycelia were harvested by centrifugation (3,000 rpm; 10 min), washed with MM (A minimal medium (MM) (10 mM K2HPO4, 10 mM KH2PO4, 1 mM CaCl2, 0.5 mM MgCl2 supplemented with 0.1% (vol/vol) trace element solution) without carbon sources, suspended in 25 mL MM, and divided into several aliquots (0.8 g/25 mL). Each aliquot was supplemented with various carbon sources, i.e., 250 μM of Glc, GlcNAc, (GlcNAc)2 (TCI, Japan). The culture was further grown at 28 °C on a rotary shaker at 200 rpm. Portions of the culture fluids were sampled periodically, centrifuged to separate the supernatants and the mycelia, and stored at -80 °C. The supernatants were subjected to measurements of sugar (Glc, GlcNAc, (GlcNAc)2) concentrations, whereas the mycelia were used for total RNA preparation.

RT-qPCR analysis

Streptomyces sp. 139, mutant strain D2 and the complemented strain C2 were cultured at 28 °C for 48 h in TSB. 10 mL of the culture were inoculated into 50 mL fresh TSB and were cultured at 28 °C for 24 h, 48 h, and 96 h, respectively. At each time point, the mycelia of the three strains were collected by centrifugation (3,000 g; 10 min) and washed with PBS (NaCl 137 mM, KCl 2.7 mM, Na2HPO4 10 mM, KH2PO4 2 mM, pH 7.4), respectively. Total RNA of each mycelia sample was isolated with the TRIzol System (TransGen) according to the manufacturer’s instructions. Remaining DNA was removed by DNase (TaKaRa). cDNA was synthesized with SuperScript III First-Strain synthesis system kit for RT-PCR (TransGen) according to the manufacturer’s instruction. Primers for each gene are listed in Table 1. 50 ng cDNA was used for each qPCR reaction with FastStart Universal SYBR Green Master kit (Roche) following the manufacturer’s instructions. All transcripts were normalized according to hrdB (RNA polymerase principal sigma factor) transcript quantities.
Microscopy
Surface-grown aerial hyphae and spores of *Streptomyces* sp.139, Strain D2 and strain C2 were examined by scanning electron microscopy (SEM) after four days of growth on minimal and R2YE agar, respectively. The agar blocks containing spores and hyphae were cut and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.0 for overnight at 4 °C, washed three times in 0.1 M phosphate, post-fixed in 1% osmium tetroxyde, 2–4 h in 0.1 M phosphate, then 15–20 min in ethanol 30%, 50%, 70%, 85%, 95% and 100%, respectively. Each specimen was rinsed, dehydrated, coated with platinum-gold and examined with a FEI QUANTA 200 scanning electron microscope [47].

RNA sequencing
*Streptomyces* sp. 139 and its mutant Strain D2 was cultured in 10 mL TSB medium at 28°C for 36 h in triplicate. The culture was inoculated 1:10 (vol/vol) into 50 ml fresh TSB medium and incubated at 28 °C for 24 h. After centrifugation at 5,000 rpm for 10 min, the cell pellet was harvested, flash frozen in liquid nitrogen, and sent out for RNA extraction and RNA sequencing (Beijing Genomics Institute, Shenzhen, China). RNA sequencing data have been deposited in NCBI SRAs under the BioProject accession PRJNA647684 and can be accessed with the following link: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA647684.

H₂O₂ sensitivity experiments
A minimum inhibition concentration (MIC) test of H₂O₂ against *Streptomyces* sp. 139 wild-type, D2 and C2 strain was performed in TSB according to the CLSI protocol [48]. The MIC of H₂O₂ against wild-type, D2 and C2 strain was 1%, 0.25% and 1%, respectively. The effect of H₂O₂ on the growth of strains was tested at a sub-MIC concentration (0.1%). The initial concentration of strains was adjusted to OD600 0.1. Bacterial strains were cultured at 28°C with shaking and their growth was monitored with an 800TS microplate reader (BioTek Instruments Inc.) at 0 h, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 36 h and 48 h. Experiments were done at least in triplicate.

Supplementary Information
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