A Small Heat Shock Protein Enables *Escherichia coli* To Grow at a Lethal Temperature of 50°C Conceivably by Maintaining Cell Envelope Integrity

Anastasia N. Ezemaduka,a Jiayu Yu,a Xiaodong Shi,a,cb Kaiming Zhang,b Chang-Cheng Yin,b Xinmiao Fu,a Zengyi Chang,a

State Key Laboratory of Protein and Plant Gene Research, School of Life Sciences, and Center for Protein Science, Peking University, Beijing, China; Department of Biophysics, the Health Science Centre, Peking University, Beijing, China; Jiangsu Province Key Laboratory of Anesthesiology, Xuzhou Medical College, Xuzhou, China

It is essential for organisms to adapt to fluctuating growth temperatures. *Escherichia coli*, a model bacterium commonly used in research and industry, has been reported to grow at a temperature lower than 46.5°C. Here we report that the heterologous expression of the 17-kDa small heat shock protein from the nematode *Caenorhabditis elegans*, CeHSP17, enables *E. coli* cells to grow at 50°C, which is their highest growth temperature ever reported. Strikingly, CeHSP17 also rescues the thermal lethality of an *E. coli* mutant deficient in *degP*, which encodes a protein quality control factor localized in the periplasmic space. Mechanistically, we show that CeHSP17 is partially localized in the periplasmic space and associated with the inner membrane of *E. coli*, and it helps to maintain the cell envelope integrity of the *E. coli* cells at the lethal temperatures. Together, our data indicate that maintaining the cell envelope integrity is crucial for the *E. coli* cells to grow at high temperatures and also shed new light on the development of thermophilic bacteria for industrial application.

Temperature is considered the most important single environmental factor that profoundly affects the structure and function of biomolecules. Each organism in nature has evolved to live at a certain optimal temperature range. Nonetheless, effective mechanisms have been evolved for organisms to survive under nonoptimal temperature conditions, typically termed heat shock response (1, 2) and cold shock response (3). It is of great value to understand such mechanisms of living organisms for both unveiling the nature of life and exploring biotechnological application. *Escherichia coli*, being the most extensively studied bacterium and also a popularly utilized host cell for producing pharmaceutically important recombinant proteins, is known to be unable to grow at a temperature higher than 46.5°C (4–6). It has been widely reported that heterologous overexpression of certain exogenous molecular chaperones (7–11) or an endogenous transcriptional regulator (12) is able to significantly increase the viability of *E. coli* cells undergoing heat shock treatment at lethal temperatures (around 50°C). It is also well established that preincubating *E. coli* cells (13, 14) and other organisms (reviewed in reference 15) at a sublethal temperature (e.g., 42°C) significantly increases the thermostolerance of the treated organisms at lethal temperatures. However, all these alternations usually would not allow the modified cells to permanently survive and even grow under such lethal temperatures. In two attempts at selecting heat-resistant phenotypes by using extensive experimental evolution, *E. coli* mutant strains that are able to grow at up to 48°C (16) or 48.5°C (5) were obtained, with growth at the latter temperature being partially related to the high level of expression of the molecular chaperone GroEL/GroES. In contrast, thermophilic bacteria have been found to grow effectively at optimal temperatures much higher than 50°C (17–20). They are known to possess membrane lipids with a unique composition, proteins that have higher thermostabilities and an increase in core hydrophobicity, or a higher turnover rate for their energy-transducing enzymes (21–24).

Here we observed, astonishingly, that the heterologous expression of a small heat shock protein (sHSP), HSP17, of *Caenorhabditis elegans*, referred to here as CeHSP17, enabled *E. coli* cells to grow at temperatures up to 50°C, that being their highest growth temperature ever reported. Our further studies revealed that such acquired unusual thermal growth ability might be attributed to the capacity of CeHSP17 to maintain the integrity of the *E. coli* cell envelope. Together, our observations strongly suggest the essential role of cell envelope for bacterial adaptation to thermal environments and shed new light on engineering bacterial strains that are able to grow at nonpermissive temperatures.

**MATERIALS AND METHODS**

*C. elegans* mitochondrion isolation. Mitochondria were isolated according to a previously described method (25), with minor modifications. Briefly, synchronized young adults of the N2 wild-type strain (obtained from the Caenorhabditis Genetics Center) were cultured using a standard method (26). Worms were washed with M9 buffer and resuspended in cold isolation buffer (10 mM Tris-MOPS [morpholinepropanesulfonic acid], 1 mM EGTA-Tris, and 0.2 M sucrose, pH 7.4) containing a 1 mM concentration of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Boehringer Mannheim). Worms were homogenized on ice by sonication. The homogenate was centrifuged at 8000 for 10 min at 4°C, with the resulting supernatant, being taken as the total lysate, was then further centrifuged at 12,000 × g for 10 min at 4°C, with the resulting supernatant being taken as the postmitochondrial supernatant and the pellet as the mitochondrion-containing fraction. Protein
**Table 1** Predicted subcellular localization of sHSPs of *C. elegans*.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Length (aa)</th>
<th>mTP</th>
<th>SP</th>
<th>Other localization</th>
<th>Location</th>
<th>TPlen (aa)</th>
<th>RC</th>
<th>TPlen, predicted presequence length for subcellular localization.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CeHSP17</td>
<td>149</td>
<td>0.593</td>
<td>0.046</td>
<td>0.353</td>
<td>Mitochondrion</td>
<td>4</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>HSP12.1</td>
<td>109</td>
<td>0.090</td>
<td>0.100</td>
<td>0.886</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP12.2</td>
<td>110</td>
<td>0.074</td>
<td>0.095</td>
<td>0.910</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP12.3</td>
<td>109</td>
<td>0.060</td>
<td>0.078</td>
<td>0.932</td>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>HSP12.6</td>
<td>110</td>
<td>0.064</td>
<td>0.127</td>
<td>0.886</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP16.1A&amp;B</td>
<td>145</td>
<td>0.747</td>
<td>0.032</td>
<td>0.263</td>
<td>3</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP16.48A&amp;B</td>
<td>143</td>
<td>0.096</td>
<td>0.083</td>
<td>0.858</td>
<td>2</td>
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<tr>
<td>HSP16.21</td>
<td>145</td>
<td>0.751</td>
<td>0.039</td>
<td>0.248</td>
<td>3</td>
<td>22</td>
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<tr>
<td>HSP16.2B</td>
<td>147</td>
<td>0.082</td>
<td>0.070</td>
<td>0.907</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HSP16.2A</td>
<td>147</td>
<td>0.332</td>
<td>0.044</td>
<td>0.706</td>
<td>4</td>
<td></td>
<td></td>
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<tr>
<td>HSP16.41</td>
<td>143</td>
<td>0.101</td>
<td>0.070</td>
<td>0.872</td>
<td>2</td>
<td></td>
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<tr>
<td>SIP-1</td>
<td>159</td>
<td>0.209</td>
<td>0.047</td>
<td>0.775</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>HSP25</td>
<td>145</td>
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<td>0.028</td>
<td>0.610</td>
<td>5</td>
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<tr>
<td>HSP43</td>
<td>368</td>
<td>0.137</td>
<td>0.064</td>
<td>0.839</td>
<td>2</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

"The prediction was performed by submitting the amino acids of the proteins to the online TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP) using default parameter settings. aa, amino acids; mTP, probability that the protein contains a mitochondrial targeting peptide; SP, possibility that the protein contains a signal peptide for secretion; RC, reliability class, from 1 to 5, where 1 indicates the strongest prediction; TPlen, predicted presequence length for subcellular localization.

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**RESULTS**

*Caenorhabditis elegans* HSP17 is partially localized in the mitochondria. The sHSPs, as a family of molecular chaperones present in all forms of life, are more structurally diversified than the other families of molecular chaperones, such as HSP90, HSP70, and HSP60 (31–33). They are characterized by a relatively conserved α-crystallin domain of around 100 amino acids flanked by a highly variable N-terminal domain and a flexible C-terminal region (31), usually exist as multisubunit oligomers (34–37), and are able to prevent the aggregation of nonnative proteins (38, 39). Uniquely, the sHSPs of both prokaryotic and eukaryotic sources have also been found to be associated with cell membranes (34–38). Over the years, we have tried to delineate the function and mechanism of sHSPs, from largely bacterial sources, via both *in vitro* and *in vivo* studies (39–45). To study the function and mechanism of sHSPs in *C. elegans*, we first focused on CeHSP17, which remains the least characterized such proteins in terms of function and properties among the small heat shock proteins of *C. elegans* (46) and is predicted by our bioinformatics analysis to be putatively localized in mitochondria (Table 1). Consistently, we detected the CeHSP17 protein predominantly in the mitochondrial fraction of *C. elegans* extract (Fig. 1). In support of our conclusions, the mRNA level of CeHSP17 was reported to be increased in response to deficiencies of the mitochondrial respiratory chain proteins (47).

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**Protein purification and formaldehyde-mediated in vivo chemical cross-linking.** The details of the procedures used for protein purification and formaldehyde-mediated in vivo chemical cross-linking are described in the supplemental material.
The heterologously expressed CeHSP17 protein enables the *E. coli* cells to grow at the lethal temperature of 50°C. We then sought to assay whether CeHSP17 is able to function as a chaperone by examining its capacity to increase the thermotolerance of the *E. coli* cells, as is commonly observed for many other sHSPs (7–10). Remarkably, we found that the heterologous expression of CeHSP17 enabled the BL21(DE3) strain of *E. coli* to effectively resist the heat shock treatment at a temperature as high as 58°C (for half an hour), at which the control cells (carrying the empty plasmid pET21a) were found to be almost completely killed (see Fig. S1 in the supplemental material). For comparison, similar heterologous expression of other sHSPs in *E. coli* cells was reported to usually enable the cells to effectively resist a heat shock treatment only at 50°C (7–10). For instance, the overexpression of IbpA and/or IbpB (the endogenous sHSPs of *E. coli*) was reported to enable around 30% (instead of 100% as for CeHSP17) of the *E. coli* cells to survive after a heat shock treatment at 50°C for half an hour (10).

Given that the highest growth temperature for *E. coli* cells has been reported to be 46.5°C (4–6), we then asked whether the BL21(DE3) *E. coli* strain expressing CeHSP17 is able to grow at a higher temperature. Remarkably, the data presented in Fig. 2A demonstrate that the cells expressing CeHSP17 grow effectively even at 50°C, though at about half the growth rate seen when the cells were grown at 37°C. In contrast, for the control cells (carrying the empty plasmid pET21a), the cell density apparently decreased (as indicated by a decrease in light absorption at 600 nm, i.e., OD600) during incubation at 50°C and eventually became undetectable after incubation for 16 h (Fig. 2A). To rule out the possibility that this unusual CeHSP17-mediated growth property is specific to strain BL21(DE3), the *E. coli* strain that has been commonly used for overexpressing recombinant proteins (48), this protein was heterologously expressed in BW25113 cells, which are widely used for gene knockout studies (49). The data presented in Fig. 2B demonstrate that CeHSP17 also enabled the BW25113 cells to effectively grow at 50°C in a largely similar manner. Nevertheless, both BL21(DE3) and BW25113 cells expressing CeHSP17 were unable to grow but died when cultured at 51°C (data not shown).

In addition, we calculated the generation time of *E. coli* cells expressing CeHSP17 during exponential growth phase and found that the protein hardly affects the growth rate of *E. coli* cells at 37°C but rather allows the cells to grow at a significantly efficient rate at the lethal temperature of 50°C (Table 2). Together, these observations indicate that CeHSP17 confers the capacity for *E. coli* to grow at 50°C, which, to our knowledge, is the highest growth temperature ever reported for *E. coli* strains (4–6).

The heterologously expressed CeHSP17 protein is present and functions not only in the cytoplasm but also at the cell envelope.

To uncover the mechanism underlying the CeHSP17-mediated high-temperature growth of the *E. coli* cells, we first analyzed whether the level of soluble proteins was maintained in such high-temperature-growing cells. The data presented in Fig. 3A demonstrate that the overall level of soluble proteins in the CeHSP17-expressing *E. coli* BL21(DE3) cells cultured at 50°C (lane 11) was not only significantly higher than that of the control cells (lane 5) but, strikingly, also highly comparable with that of both the control and the CeHSP17-expressing cells cultured at 37°C (lanes 2 and 8). It is noteworthy that at 37°C the level of insoluble protein in the CeHSP17-expressing cells (lane 9) is somehow higher than that in the control cells (lane 3), most likely due to the formation of insoluble coaggregates between CeHSP17 and certain non-native proteins naturally occurring in the cells.

To identify the proteins that are protected by CeHSP17, we performed formaldehyde-mediated *in vivo* chemical cross-linking, protein purification (see Fig. S3 in the supplemental material), and/or IbpB (the endogenous sHSPs of *E. coli*). The results are shown in Fig. S4 (for half an hour).

### Table 2 Generation time of *E. coli* strains

<table>
<thead>
<tr>
<th>Bacterial strain or genotype</th>
<th>Generation time (h) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>0.6</td>
</tr>
<tr>
<td>BL21(DE3) + CeHSP17</td>
<td>0.7</td>
</tr>
<tr>
<td>BW25113</td>
<td>1.5</td>
</tr>
<tr>
<td>BW25113 + CeHSP17</td>
<td>1.5</td>
</tr>
<tr>
<td>ΔdegP</td>
<td>0.5</td>
</tr>
<tr>
<td>ΔdegP + CeHSP17</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The generation time of cells was obtained by regression analysis of the CFU-OD600 (see Table S1 in the supplemental material), which were calculated according to the standard curve of CFU-OD600 (see Fig. S2 in the supplemental material).
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CeHSP17 (56), and then mass spectrometry analysis (Table 3; also, see Table S2 in the supplemental material). Interestingly, we found that a major part of these CeHSP17-bound proteins (63 out of 96) are present in the cytoplasm and a significant portion of them (31 out of 96) are localized in the cell envelope, including the inner membrane, periplasmic space, and outer membrane (Table 3). This result implies that the heterologously expressed CeHSP17 in *E. coli* cells functions not only in the cytoplasm but also in the cell envelope. On the other hand, it was reported earlier that the overexpression of GroEL/GroES, a cytoplasmic molecular chaperone, enabled the *E. coli* cells to grow at a temperature up to only 47.5°C (5); it is conceivable that the CeHSP17 protein functions differently in terms of subcellular localization.

![FIG 3](https://www.jb.asm.org/)

**FIG 3** The heterologously expressed CeHSP17 protein is present at the cell envelope. (A) Coomassie blue staining results of 10% Tricine SDS-PAGE analysis of proteins in the supernatant (soluble) and pellet (insoluble) present in BL21(DE3) cells with or without heterologous expression of CeHSP17 and cultured at 37°C or 50°C. T, total cell lysate; S, supernatant; P, pellet. (B) Immunoblotting analysis of CeHSP17 released from the osmotic shock-treated *E. coli* cells, indicating its presence in the periplasmic space. TnaA and OsmY were monitored, respectively, as cytosolic and periplasmic marker proteins. (C) Immunoblotting analysis of CeHSP17 in the membrane fractions of the BL21(DE3) *E. coli* cells as separated by sucrose gradient centrifugation, indicating its association with the inner membrane at both 37°C and 50°C. PpiD and OmpA were monitored, respectively, as inner membrane and outer membrane marker proteins.

![TABLE 3](https://www.jb.asm.org/)

**TABLE 3** Summary of CeHSP17-bound proteins in *E. coli* according to their subcellular localization

<table>
<thead>
<tr>
<th>Subcellular location</th>
<th>Protein(s)a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periplasm (3)</td>
<td>DpsP, Skp, DegS</td>
</tr>
<tr>
<td>Outer membrane (8)</td>
<td>OmpA, OmpF, Lpp, BamA, OmpN, BamB, YiaD, YpfF</td>
</tr>
<tr>
<td>Uncharacterized (2)</td>
<td>YbfU, YbjQ</td>
</tr>
</tbody>
</table>

a The location was extracted from the EcoCyc database (http://www.ecocyc.org) (56). Protein identification details are shown in Table S2 in the supplemental material.

b Underlining indicates protein quality control factors that are potential functional partners of CeHSP17, instead of substrate proteins that were protected by CeHSP17.
We then performed sucrose gradient ultracentrifugation analysis to find out whether the CeHSP17 protein is associated with the inner and/or outer membranes of the E. coli cells. The data presented in Fig. 3C demonstrate that, in the membrane samples prepared from cells cultured at both 37°C and 50°C, the fraction distribution pattern of CeHSP17 is highly comparable to that of the inner membrane protein PpiD but apparently different from that of the outer membrane protein OmpA. To rule out the possibility that CeHSP17 oligomer cosediments with the inner membrane, we performed sucrose gradient ultracentrifugation analysis in the absence of the membrane fraction using the purified CeHSP17 protein. Data presented in Fig. S4 in the supplemental material indicate that the purified CeHSP17 protein was indeed separated with the membrane fraction during the first step of sucrose centrifugation, where it localizes in the 25% sucrose fraction, while the membrane is in the 65% sucrose fraction. Together, this indicates that the CeHSP17 protein, when expressed in E. coli cells, is at least in part associated with the inner membrane.

Subsequently, we further investigated whether CeHSP17 functions at the cell envelope of the E. coli cells. For this purpose, we examined if it is able to rescue the lethal phenotype at above 42°C for the E. coli BW25113 mutant strain lacking DegP, a protein quality control factor of the outer membrane biogenesis that functions in the periplasmic space (50–52). Remarkably, the heterologous expression of the CeHSP17 protein enabled the degP mutant strain to grow at a temperature as high as 45°C either in liquid LB (Fig. 4A) or on solid medium (Fig. 4B), whereas, in comparison, the heterologous expression of IbpB (an endogenous sHSP of E. coli) did not allow the cells to grow at such a heat shock temperature. Again, the CeHSP17 protein, when heterologously expressed in the degP mutant cells cultured at both normal and high temperatures, was found to be associated with the inner membrane (see Fig. S5 in the supplemental material), similar to its behavior in the BL21(DE3) cells (Fig. 3C).

The heterologously expressed CeHSP17 protein helps maintain the envelope and cytoplasm integrity for E. coli cells cultured at lethal temperatures. We then analyzed the protective effect of CeHSP17 on the E. coli cells by analyzing their cellular and subcellular structures using thin-section transmission electron microscopy. The data presented in Fig. 5A indicate that the cell morphology of the CeHSP17-expressing BL21(DE3) cells appears to be largely normal when cells are cultured at 50°C, as it is at 37°C. In contrast, the control BL21(DE3) cells (carrying the empty plasmid pET21a) cultured at 50°C appeared to be severely abnormal, such that their periplasmic space became hardly visible, abnormal vesicles were found to be protruding or detached from the cell surface, and heavily stained areas were clearly visualized (presumably representing protein aggregates) in the cytoplasm. We also observed similar abnormal cellular and subcellular morphology in the degP mutant cells that were cultured at 45°C, and expressing CeHSP17 significantly prevented such abnormalities (Fig. 5B). Together, these observations indicate that the recombinant CeHSP17 is able to maintain the envelope integrity for the E. coli cells cultured at lethal temperatures.

DISCUSSION

Here we report our unexpected observation of the continuous growth of E. coli cells at 50°C by simply overexpressing CeHSP17 (Fig. 2), a mitochondrion-localized sHSP from C. elegans (Fig. 1). Prior to this study, the highest growth temperature for E. coli cells was reported to be around 48.5°C, as achieved by experimental evolution (5, 16). Here, we found that the CeHSP17 protein is, at least partially, localized at the E. coli cell envelope (Fig. 3B and C) and helps to maintain the envelope integrity upon culturing at the lethal temperatures (Fig. 5A and B), although it also protects the cytoplasmic proteins (Fig. 3A and Table 3) and maintains the cytoplasm integrity (Fig. 5A and B). In line with our observations, other sHSPs, such as Synechocystis Hsp17 (36), Mycobacterium tuberculosis Hsp16.3 (37), and mammalian α-crystallin (38), have been found to be associated with the membrane under nonoptimal or stress conditions. Together, our study reveals an essential role of the envelope for the E. coli cells to survive and grow at a lethal temperature and sheds light on the future development of thermophilic microbes for industrial applications.
A key step during the early evolution of life was most likely the emergence of a cell membrane, or a cell envelope in general, that separates cellular contents from their surroundings. Accordingly, under stress conditions of external sources (e.g., elevated temperature), the integrity of the cell envelope would have to be maintained for the cellular contents to be protected or even to continue functioning. In this regard, the cell envelope would be expected to play a role equal to, if not more important role than, that of the cytoplasm for cells to survive and grow under such harsh conditions, in spite of the fact that earlier studies largely focused on how the cytoplasm is protected (53). In support of our hypothesis, overexpression of the cytoplasmic chaperone GroEL/GroES was reported to increase the maximal growth temperature of *E. coli* from 46.5°C to 47.5°C (5), significantly lower than the temperature (50°C) that we reached by overexpressing CeHSP17, which seems to function not only in the cytoplasm but also at the cell envelope.

For the *E. coli* cells to grow at 50°C, all the cytoplasmic components would have to perform their functions effectively to allow such processes as DNA replication and transcription, protein translation, and metabolism to actively occur. It would be difficult to imagine that CeHSP17 enables the cytoplasmic components to function at 50°C through simply providing direct protection to them. Instead, it is more likely that CeHSP17 protects the cell envelope, which in turn serves as an effective thermal insulator such that the cytoplasmic temperature is kept significantly lower than 50°C, although our attempts to demonstrate this did not succeed due to technical difficulties. It should be noted that protective structures outside the spores formed by certain bacteria are able to serve as a thermal insulator for protecting the cells even against boiling temperatures (54). Other unresolved issues regarding the unique growth properties of such CeHSP17-expressing *E. coli* cells include the following. First, what biomolecules in *E. coli*, e.g., membrane lipids and/or proteins, are directly bound and thus potentially protected by CeHSP17? Second, are there any other members of the sHSP family that also possess properties similar to those of CeHSP17, given that many other sHSPs were also found to be associated with cell membranes? Last but not least, what does this unique property of CeHSP17 indicate with regard to its physiological function in *C. elegans*? Given that dormant (dauer) *C. elegans* organisms are known to be able to survive under extreme stress conditions (55), the question of whether CeHSP17 plays a role in such dauer-related survival merits further investigation.

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A.N.E. and X.F. designed the research; A.N.E., J.Y., X.S., and K.Z. performed the research; A.N.E., C.-Y., X.F., and Z.C. analyzed the data; A.N.E., X.F., and Z.C. wrote the paper.

REFERENCES


