Acetylation of CspC Controls the Las Quorum-Sensing System through Translational Regulation of rsaL in Pseudomonas aeruginosa

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ABSTRACT Pseudomonas aeruginosa is a ubiquitous pathogenic bacterium that can adapt to a variety of environments. The ability to effectively sense and respond to host local nutrients is critical for the infection of P. aeruginosa. However, the mechanisms employed by the bacterium to respond to nutrients remain to be explored. CspA family proteins are RNA binding proteins that are involved in gene regulation. We previously demonstrated that the P. aeruginosa CspA family protein CspC regulates the type III secretion system in response to temperature shift. In this study, we found that CspC regulates the quorum-sensing (QS) systems by repressing the translation of a QS negative regulatory gene, rsaL. Through RNA immunoprecipitation coupled with real-time quantitative reverse transcription-PCR (RIP-qRT-PCR) and electrophoretic mobility shift assays (EMSAs), we found that CspC binds to the 5’ untranslated region of the rsaL mRNA. Unlike glucose, itaconate (a metabolite generated by macrophages during infection) reduces the acetylation of CspC, which increases the affinity between CspC and the rsaL mRNA, leading to upregulation of the QS systems. Our results revealed a novel regulatory mechanism of the QS systems in response to a host-generated metabolite.

IMPORTANCE Bacterial infectious diseases impose a severe threat to human health. The ability to orchestrate virulence determinant in response to the host environment is critical for the pathogenesis of bacterial pathogens. Pseudomonas aeruginosa is a leading pathogen that causes various infections in humans. In P. aeruginosa, the quorum-sensing (QS) systems play an important role in regulating the production of virulence factors. In this study, we find that a small RNA binding protein, CspC, regulates the QS systems by repressing the expression of a QS negative regulator. We further demonstrate that CspC is acetylated in response to a host-derived metabolite, itaconate, which alters the function of CspC in regulating the QS system. The importance of this work is in elucidation of a novel regulatory pathway that regulates virulence determinants in P. aeruginosa in response to a host signal.

KEYWORDS acetylation, CspC, itaconate, Pseudomonas aeruginosa, quorum sensing

Pseudomonas aeruginosa is an opportunistic pathogen that predominates as a major cause of pulmonary infections in patients with cystic fibrosis and chronic obstructive pulmonary disease (COPD) (1, 2). The successful colonization of P. aeruginosa depends on its ability to evade host immune clearance and efficiently acquire and utilize available nutrients by orchestrating global gene expression in response to the host environment.

P. aeruginosa possesses a variety of mechanisms to sense and respond to host signals, such as two-component regulatory systems and extracytoplasmic function (ECF) sigma factors (3–6). Surface structural proteins are also involved in signal sensing. For instance, the type IV pilus component PilY1 is involved in surface sensing and regulates synthesis of the secondary messenger c-di-GMP and expression of various virulence
CspC is required for pyocyanin production. To examine whether the CspA family proteins are involved in bacterial response to cell density and extracellular nutrients, we monitored the growth curves of the strains with mutations in each of the cspA family genes. All of the mutants grew normally in LB medium (see Fig. S1a in the supplemental material). However, the color of the ΔcspC mutant culture remained yellow at an optical density at 600 nm (OD600) of 3.0, while the others’ turned blue-green, indicating a defect in pyocyanin production (Fig. S1b). Indeed, mutation of the cspC gene reduced the extracellular amount of pyocyanin (Fig. 1a and b). In PA14, genes phzM and phzS and two nearly identical operons, phzA1G1 and phzA2G2, are involved in pyocyanin synthesis (34). We determined the mRNA levels of phzM and overall phzA (phzA1 plus phzA2) by using quantitative reverse transcription-PCR (qRT-PCR) primers targeting phzM and the common sequences of phzA1 and phzA2 (Table S1). Mutation of cspC resulted in downregulation of the overall phzA and phzM (Fig. 1b). Complementation with a cspC gene restored the

REGULATION OF QS SYSTEMS BY CspC

P. aeruginosa harbors five CspA family proteins, namely, CspC (PA0456), PA0961, PA1159, CspD (PA2622), and CapB (PA3266) (33). Previously, we found that CspC regulates the translation of the type III secretion system (T3SS) master regulatory gene exsA by binding to the 5′ untranslated region (5′UTR) of its mRNA (33). We further demonstrated that the binding affinity of CspC to the exsA mRNA is affected by the acetylation at the K41 residue in response to temperature shift, indicating a role of CspC in bacterial response to environmental signals (33). In this study, we explored the roles of the P. aeruginosa CspA family proteins in response to nutrient availability and abundance and found that CspC regulates pyocyanin production without affecting bacterial growth in complete medium. We further found that CspC regulates the QS systems by binding to the 5′UTR of the rsaL mRNA. In addition, we demonstrated that carbon sources affect the acetylation level of CspC, which affects its regulatory effect on rsaL. Overall, our results reveal a novel regulatory mechanism of the QS systems in response to environmental nutrients.
expression of the phz genes and production of pyocyanin (Fig. 1a). These results reveal a role of CspC in regulating the production of pyocyanin.

**CspC controls the production of QS signal molecules.** The synthesis of pyocyanin is regulated by the quorum-sensing systems. To understand the mechanism of CspC-mediated regulation on pyocyanin synthesis, we examined extracellular amounts of the QS signal molecules 3-oxo-C12-HSL and C4-HSL by utilizing *P. aeruginosa* reporter strains (35). Compared to wild-type PA14 and the complemented strain, the ΔcspC mutant produced smaller amounts of 3-oxo-C12-HSL and C4-HSL (Fig. 2a). The results were further confirmed by utilizing *Escherichia coli*-based reporter systems (Fig. 2b). In agreement with defective production of the QS signals, the mRNA levels of lasI, lasR, rhlI, and rhlR as well as the promoter activities of lasI and rhlI were decreased in the ΔcspC mutant and restored to wild-type levels by complementation with the cspC gene (Fig. 2c and d).

**CspC controls the QS systems through rsaL.** Previously, we demonstrated CspC regulates gene expression by binding to mRNA (33). To elucidate the mechanism of CspC-mediated regulation on the QS systems, we checked our previous RNA immunoprecipitation (RIP) sequencing result and found enrichment of the region upstream of the rsaL gene by CspC (33). By performing a RIP-coupled qRT-PCR assay, we verified the binding of CspC to the 5′UTR of the rsaL mRNA (Fig. 3a), whereas the neighboring 5′UTR of the lasI mRNA was not enriched by CspC (Fig. 3a), indicating possible post-transcriptional regulation of rsaL by CspC.

To examine whether CspC controls the translation of the rsaL mRNA, we constructed a
FIG 2 CspC controls the production of QS signal molecules. (a) The relative levels of 3-oxo-C12-HSL and C4-HSL of indicated strains. The indicated strains were grown overnight. The bacterial supernatants were mixed with the reporter strains at a volume ratio of 1:1. The GFP fluorescence and OD600 were measured every 30 min at 37°C for 12 h. The data were calculated as GFP fluorescence/OD600.

(b) 3-Oxo-C12-HSL and C4-HSL contents of indicated strains measured with *E. coli* reporter strains in LB. Miller units are used for the mean results from at least three independent experiments. ***, *P* < 0.001; **, *P* < 0.01 by Student’s *t* test.

(c) Relative mRNA levels of *lasI*, *lasR*, *rhlI*, and *rhlR*. Total RNA of indicated strains was isolated from bacteria grown overnight, and mRNA levels of *lasI*, *lasR*, *rhlI*, and *rhlR* were determined by qRT-PCR. Data represent the means from three independent experiments, and error bars indicate standard deviations. **, *P* < 0.01; *, *P* < 0.05 by Student’s *t* test.

(d) Bacteria carrying PA14-*lasI-lacZ* and PA14-*rhlI-lacZ* were grown in LB to an OD600 of 0.3. Miller units are used for mean results from three independent experiments. **, *P* < 0.01 by Student’s *t* test.
FIG 3 CspC binds to the 5’ UTR of the rsaL mRNA. (a) Fold enrichment of the indicated fragments tested by an RIP-coupled qRT-PCR assay. Locations of the primers used in the qRT-PCR are indicated by arrows. The exsA gene was used as a positive control, and the rpsL gene was used as an internal control. Data represent the means from three independent experiments, and error bars indicate standard deviations. *** P < 0.001 compared to the other samples by Student’s t test. (b) Schematic diagram of the rsaL-GST fusions driven by a tac promoter with indicated length of upstream regions. (c) Bacteria carrying the rsaL-GST with indicated upstream segments (P_tac-74/37/-rsaL-GST) were grown in LB containing 150 µg/mL carbenicillin. The RsaL-GST and RpoA levels were determined by Western blotting. (d) The purified CspC-GST was incubated with a 37-nt ssDNA that represents the 37-nt 5’-UTR of the rsaL mRNA and a complementary ssDNA for 30 min at 25°C. The samples were subjected to electrophoresis in a native gel, followed by staining with SYBR Gold nucleic acid gel stain.
C-terminal gst-tagged rsaL driven by a P_tac promoter. It has been demonstrated that the transcription of the rsaL mRNA initiates at 74 nucleotides (nt) upstream of its start codon (23). Thus, we included the 74-bp region in the construct, resulting in P_tac-74-rsaL-gst (Fig. 3b). The RsaL–glutathione-S-transferase (GST) level was higher in the ΔcspC mutant (Fig. 3c). To narrow down the region that is involved in the translational regulation, we reduced the 5′ UTR to 37 nt (designated P_tac-37-rsaL-gst), which also resulted in a higher level of RsaL-GST in the ΔcspC mutant (Fig. 3c). However, replacement of the native 5′ UTR sequence with an exogenous ribosome binding sequence (designated P_tac-RsaL-gst) resulted in similar levels of RsaL-GST in wild-type PA14 and the ΔcspC mutant (Fig. 3c), indicating an essential role of the 37-nt 5′ UTR in the CspC-mediated translational regulation of rsaL.

We then examined whether CspC directly binds to the 37-nt 5′ UTR by electrophoretic mobility shift assay (EMSA). It has been demonstrated that the CspA family proteins bind to single-stranded DNA (ssDNA) as efficiently as RNA (36, 37). Thus, we used ssDNAs in the EMSAs. The 37-nt ssDNA that represents the rsaL 5′ UTR was bound by CspC, whereas no obvious binding was observed between the complementary ssDNA and CspC (Fig. 3d). In combination, these results demonstrate that CspC represses the translation of the rsaL mRNA by binding to its 37-nt 5′ UTR.

Since RsaL represses the transcription of lasI, the defective production of pyocyanin by the ΔcspC mutant might be due to the derepression of rsaL. Indeed, deletion of the rsaL gene in the ΔcspC mutant increased the expression of the lasI and lasR genes (Fig. 4a) as well as the production of 3-oxo-C12-HSL and pyocyanin (Fig. 4b and c).

**The K41 residue of CspC is involved in the translational regulation of rsaL.** Previously we demonstrated that acetylation at K41 of CspC reduces its affinity to the exsA mRNA, resulting in derepression of the translation (33). To examine whether K41 acetylation is involved in the CspC-mediated regulation of rsaL, we performed EMSA with CspC with K41R and K41Q mutations, which mimic the unacetylated and acetylated states, respectively (38). The K41Q mutation significantly reduced the binding affinity between CspC and the 37-nt ssDNA (Fig. 5a). In the ΔcspC mutant carrying P_tac-37-rsaL-gst, overexpression of the K41Q cspC mutant did not affect the expression of RsaL-GST, whereas overexpression of the K41R cspC mutant repressed the expression (Fig. 5b). In addition, overexpression of K41R cspC but not K41Q cspC in the ΔcspC mutant restored the expression of the lasI, lasR, and phzA genes as well as the production of pyocyanin (Fig. 5c and d). These results indicate a role of K41 acetylation in the CspC-mediated regulation of rsaL and the Las QS system.

**Acetylation of CspC modulates the expression of rsaL and the Las QS system in response to itaconate.** A recent study demonstrated that during lung infection, the host-derived itaconate promotes biofilm formation by *P. aeruginosa* (39). Gaviard et al. demonstrated alternation of the acetylome of *P. aeruginosa* by carbon sources (40). Since the QS systems are required for biofilm formation, we suspected that the acetylation of CspC was affected by itaconate, which subsequently regulates the Las QS system through RsaL. Thus, we grew wild-type PA14 in a minimal medium with itaconate or glucose as the sole carbon source. Compared to glucose, itaconate decreased the expression of RsaL-GST (Fig. 6a and b). Deletion of cspC resulted in an equally higher level of RsaL-GST in the presence of itaconate and glucose (Fig. 6e). Complementation with K41R cspC reduced the expression levels of RsaL-GST in the presence of itaconate and glucose. However, K41Q cspC was not able to reduce the expression of RsaL-GST in either of the carbon sources (Fig. 6e). In combination, these results demonstrate that acetylation at K41 modulates the activity of CspC in the regulation of rsaL in response to itaconate.

**DISCUSSION**

In this study, we demonstrated that the CspA family protein CspC regulates the QS...
systems by repressing the expression of rsaL. The CspC protein directly binds to the 37-nt fragment of the 5′ UTR of the rsaL mRNA, which might alter the secondary structure of the mRNA or block the access of ribosome, thus inhibiting the translation.

CspA family proteins are conserved RNA chaperones in bacteria that are involved in bacterial response to environmental signals (41–45). The CspA family proteins usually contain five anti-parallel β-strands and fold into a β-barrel structure (46, 47). In *E. coli*, CspA and another CspA family protein, CspE, are involved in bacterial cold adaptation by altering mRNA secondary structures, which may affect transcription and mRNA translation (48–50). In *Staphylococcus aureus*, CspA binds to the 5′ UTR of its own mRNA, which disrupts the stem-loop in the 5′ UTR and subsequently blocks the processing by RNase III, leading to repression of translation (36). In *Salmonella enterica*...
serovar Typhimurium, CspC and CspE play important roles in bacterial response to membrane stress, motility, biofilm formation, and virulence in a mouse systemic infection model (51). One of the targets of CspC and CspE is the **ecnB** mRNA, which encodes a protein involved in bacterial response to starvation. The binding of CspC and CspE to the ecnB mRNA protects it from degradation by RNase E (51).

We previously found that in *P. aeruginosa*, CspC regulates the T3SS by directly
binding to the 5′UTR of the exsA mRNA, which results in translational repression (33). The binding affinity between CspC and the exsA mRNA is reduced by acetylation at K41 of CspC (33). The reversible lysine acetylation is one of the most common post-translational modifications (PTMs) that play critical roles in bacterial metabolism, response to environmental stresses, and virulence (52–57). For nucleic acid binding proteins, acetylation of lysine residues decreases the positive charge of the protein, thereby affecting the folding of the protein and its affinity to nucleic acids (57). Our previous study demonstrated that the acetylation of CspC is increased when the
culture temperature is switched from 25°C to 37°C, indicating a CspC-mediated regulatory mechanism in sensing mammalian host body temperature (33).

Carbon sources play important roles in affecting PTMs in bacteria (58–61). Through proteomic analyses, Gaviard et al. demonstrated that the global protein acetylation of PA14 was altered by different carbon sources (40). When glucose was the sole carbon source, 320 acetylated proteins were identified in PA14, which are mainly involved in carbon metabolism, lipopolysaccharide (LPS) biosynthesis, oxidative stress response, and virulence factor production (62, 63).

During infection by Gram-negative pathogens, aerobic glycolysis is enhanced in macrophages and monocytes upon sensing LPS, leading to succinate accumulation and generation of reactive oxygen species (ROS) (64). The bactericidal ROS also causes damage to host cells and local tissue. In response to oxidative stress and inflammation, macrophages and monocytes generate and release itaconate, an electrophilic α,β-unsaturated carboxylic acid that suppresses glycolysis, production of proinflammatory cytokines, including interleukin 1β (IL-1β) (65) and IL-6 (66), and reactive oxygen species (65, 67). During lung infection with P. aeruginosa, itaconate is released into airways (68, 69). P. aeruginosa isolates from CF patients have been shown to become adapted to utilize itaconate as a carbon source (39). Iaconate represses LPS synthesis while inducing the production of extracellular polysaccharides in P. aeruginosa, which promotes biofilm formation and chronic infection (39). Here, we found that growth with itaconate as the sole carbon source resulted in lower levels of acetylation of CspC in wild-type PA14 than that when glucose was the sole carbon source. By using the CspC K41R and K41Q mutants, we found that deacetylation of CspC might increase the affinity of CspC to the rsal mRNA, resulting in repression of its translation (Fig. 7). Rsal was identified as a negative regulator of the lasl gene (70). The rsal gene and the lasl gene are located next to each other and transcribed in opposite directions (Fig. 7) (34). A previous study demonstrated that LasR and Rsal can simultaneously bind to the intergenic region between rsal and lasl (23, 24). After binding to LasI-generated 3-oxo-C12-HSL, LasR activates the transcription of both rsal and lasl (13). On the contrary, Rsal represses the transcription of the two genes (Fig. 7) (23, 24). Besides lasl, Rsal has been found to directly repress the transcription of phzA1, phzM, and hcnA, which are involved in the synthesis of pyocyanin and hydrogen cyanide, respectively (23). A chromatin immunoprecipitation sequencing analysis further identified that Rsal binds to the promoter regions of pqsH and cdpR and activates their expression (25). pqsH encodes the monooxygenase that converts HHQ to PQS (15). CdpR is a transcriptional regulator that directly activates the transcription of pqsH (25). These results demonstrate a complex role of Rsal in regulating the QS circuits and genes regulated by the QS systems.

We found that mutation of cspC in wild-type PA14 and the ΔRSAI mutant decreased the mRNA level of lasR compared to the corresponding parental strains (Fig. 2C and 4A), indicating that CspC positively regulates lasR independent of RsalL. The transcription of lasR is directly activated by Vfr independent of cyclic AMP (cAMP) (71, 72). However, cAMP is required for the positive autoregulation of Vfr, thus rendering cAMP indirectly involved in the regulation of lasR (71). Further studies are needed to examine whether CspC influences the expression of vfr or the adenylate cyclase genes cyaA and cyaB. Another possibility is that CspC affects the stability of the lasR mRNA. Binding of CspC to the lasR mRNA might confer protection against RNases. Combining the results from lasR-lacZ transcriptional and translational fusions might clarify the regulatory mechanism.

Collectively, our results reveal a regulatory pathway in P. aeruginosa that modulates the QS systems in response to host metabolite. In the presence of macrophage-generated itaconate, the acetylation level of CspC is low, which represses the translation of rsal, leading to activation of the Las QS system that promotes biofilm formation. In combination with our previous report, we postulate that acetylation of CspC in P. aeruginosa acts as a switch between acute and chronic infection. Upon entering the host, CspC is acetylated due to temperature upshift, which results in upregulation of exsA and subsequent T3SS genes. Meanwhile, Rsal contributes to the homeostasis of the
Las QS system. When macrophage-generated itaconate is accumulated at the infection site, CspC is deacetylated, resulting in downregulation of rsaL and upregulation of the QS systems, which might promote chronic infection. Further studies are warranted to examine the expression of the T3SS genes in response to itaconate at body temperature and elucidate the mechanism that controls the acetylation of CspC in response to environmental signals.

MATERIALS AND METHODS

Bacterial strains and plasmids. Primers, plasmids, and bacterial strains used in this study are listed in Table S1 in the supplemental material. The detailed methods are provided in Text S1. Bacteria were grown in LB or the M9 minimal medium at 37°C with agitation. For the M9 minimal medium with a sole carbon source, 22.2 mM glucose (C₆H₁₂O₆) or 26.7 mM itaconic acid (C₅H₆O₄) was used in the medium to achieve the same molar carbon atoms, and the pH of the medium was adjusted to 7.4. Antibiotics were purchased from BBI Life Sciences, Shanghai, China, and used at the following concentrations: tetracycline, 50 μg/ml for P. aeruginosa and 10 μg/ml for E. coli; carbenicillin, 150 μg/mL for P. aeruginosa; and ampicillin, 100 μg/mL for E. coli.

Pyocyanin production assay. The pyocyanin levels were determined as previously described (73). Bacteria were grown in indicated medium at 37°C overnight. The supernatant was collected by centrifugation at 13,000 × g for 2 min; 1 mL of the supernatant was mixed with 600 μL chloroform. The lower chloroform layer was mixed with 300 μL 0.2 M HCl, followed by centrifugation at 13,000 × g for 5 min. The upper layer was taken for the measurement of OD₅₂₀ by a microplate reader (Bio-Rad, USA).

RNA isolation and qRT-PCR. Total bacterial RNA was isolated with a bacterial total RNA kit (Zomanbio, Beijing, China). cDNA synthesis and qRT-PCR were performed as previously described (33). The ribosomal protein gene rplL was used as the internal control.

QS signal molecule reporter assay. The QS signal molecule reporter assay was performed as previously described, with minor modifications (74). A PA01 lasI rhlI double mutant containing a lasB-gfp (ASV) or an rhlA-gfp (ASV) translational fusion was used as the reporter strain to determine the relative levels of 3OC12-HSL or C4-HSL (35). Wild-type PA14, the ΔcspC mutant, and the complemented strain were grown in the ABTG medium [2.2 g/liter glucose, 2 g/liter Casamino Acids, 1.827 g/liter MgCl₂·6H₂O, 0.3816 g/liter Na₂HPO₄, 0.2933 g/liter KH₂PO₄, 0.1995 g/liter (NH₄)₂SO₄, 0.3816 g/liter NaCl, 9.981 mg/liter CaCl₂, 1.4598 mg/liter FeCl₃, 0.292 mg/liter NaCl] overnight. Supernatants of the bacterial cultures were
collected by centrifugation. The reporter strains were grown in ABTGC medium, followed by mixing with the same volume of the collected bacterial supernatant to achieve an OD_{600} of 0.01 in each well of a 96-well plate. The plate was incubated at 37°C, and the green fluorescent protein (GFP) fluorescence and OD_{600} were measured every 30 min for 12 h.

For the *E. coli* QS signal reporter strains, measurement of the signal molecule 3-oxo-C12-HSL or C4-HSL was performed as described previously (75). The *P. aeruginosa* strains were grown overnight and the supernatants were collected by centrifugation; 1 mL of the supernatant was mixed with 4 mL DHSA (OD_{600} 0.1) containing pECP64 and a labB-lacZ translational fusion or pECP61.5 and an rhlA-lacZ translational fusion (75). When the OD_{600} reached 0.3, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. After 3 h, the β-galactosidase activities were measured as previously described (33).

**Electrophoretic mobility shift assay.** A total of 40 ng ssDNA was incubated with the purified GST-CspC protein at indicated concentrations for 30 min at 16°C as previously described (33). The electrophoresis was performed at 120 V on ice for 1 h on an 8% native polyacrylamide gel that had been prerun for 1 h in Tris-borate-EDTA (TBE). The DNA bands were visualized after staining with the SYBR Gold nucleic acid gel stain (ThermoFisher Scientific).

**Western blot.** Proteins from the same amount of bacteria were separated by SDS-PAGE and then transferred to a PVDF (polyvinylidene difluoride) membrane. The membrane was blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBST) for 1 h at room temperature, followed by probing with antibodies against glutathione-S-transferase (GST) (Sigma, USA), RNA polymerase α antibody (BioLegend), or an anti-ace-tyllysine antibody (Jingjie PTM Biolab, China) at room temperature for 2 h. After washing with PBST four times, the membrane was incubated with corresponding secondary antibodies at room temperature for 2 h. The membrane was washed four times with PBST, and the signals on the membrane were detected with the Immobilon Western kit (Millipore).

**RIP–qRT-PCR.** RIP was performed as previously described (33). The CspC-GST recombiant protein was purified with a GST tag protein purification kit (Beyotime, China) in the presence of 1 mM dithiothreitol and an RNase inhibitor (Beyotime, China). The eluted protein samples were subjected to RNA isolation with a bacterial total RNA kit (Zomanbio, Beijing, China). After cDNA synthesis, qRT-PCR was performed with primers targeting different parts of the *lasI*, *rsaL*, and *exsA* mRNA. The ribosomal protein gene *rpsL* was used as the internal control.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**TEXT S1**, DOCX file, 0.02 MB.

**FIG S1**, TIF file, 0.2 MB.

**TABLE S1**, RTF file, 0.3 MB.

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We declare no conflict of interest.

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Regulation of QS Systems by CspC

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May/June 2022 Volume 13 Issue 3

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