A Dual-Functional Orphan Response Regulator Negatively Controls the Differential Transcription of Duplicate groELs and Plays a Global Regulatory Role in Myxococcus

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ABSTRACT Differential transcription of functionally divergent duplicate genes is critical for bacterial cells to properly and competitively function in the environment, but the transcriptional regulation mechanisms remain in mystery. Myxococcus xanthus DK1622 possesses two duplicate groELs with divergent functions. Here, we report that MXAN_4468, an orphan gene located upstream of groEL2, encodes a response regulator (RR) and is responsible for the differential expression regulation of duplicate groELs. This RR protein realizes its negative regulatory role via a novel dual-mode functioning manner: binding to the transcription repressor HrcA to enhance its transcriptional inhibition of duplicate groELs and binding to the 3’ end of the MXAN_4468 sequence to specifically decrease the transcription of the following groEL2. Phosphorylation at the conserved 61st aspartic acid is required to trigger the regulatory functions of MXAN_4468. Pull-down experiment and mutation demonstrated that two noncognate CheA proteins, respectively belonging to the Che8 and Che7 chemosensory pathways, are involved in the protein phosphorylation. A transcriptome analysis, as well as the pull-down experiment, suggested that MXAN_4468 plays a global negative regulatory role in M. xanthus. This study elucidates, for the first time, the regulatory mechanism of differential transcription of bacterial duplicate groELs and suggests a global regulatory role of a dual-functional orphan RR.

IMPORTANCE Multiply copied groELs require precise regulation of transcriptions for their divergent cellular functions. Here, we reported that an orphan response regulator (RR) tunes the transcriptional discrepancy of the duplicate groELs in Myxococcus xanthus DK1622 in a dual-functional mode. This RR protein has a conserved phosphorylation site, and the phosphorylation is required for the regulatory functions. Transcriptomic analysis, as well as a pull-down experiment, suggests that the RR plays a global regulatory role in M. xanthus. This study highlights that the dual-functional orphan RR might be involved in conducting the transcriptional symphony to stabilize the complex biological functions in cells.

KEYWORDS two-component system, orphan response regulator, duplicate groELs, differential transcription, Myxococcus

Two-component systems (TCSs) serve as the basic stimulus-response coupling mechanism allowing bacterial cells to sense and respond to diverse changes in environment (1, 2). Typically, a TCS consists of a histidine kinase (HK) and a cognate response regulator (RR). HK undergoes an ATP-dependent autophosphorylation of a conserved histidine (His) residue upon stimulation, creating a high-energy phosphoryl group that is subsequently transferred to the aspartate (Asp) residue of the downstream RR. Then the phosphorylated RR activates or represses transcription of target genes (1, 3). Although paired HK and RR may function efficiently, orphan RR is universal in bacteria in a broad range of numbers and ratios. For instance, 5 of the 32 RRs in...
**Escherichia coli** are orphan (4), 2 of the 20 RRs in Streptococcus sobrinus are orphan (5), and 2 of the 16 RRs in Acinetobacter baumannii are orphan (6). In comparison, Myxococcus xanthus possesses a total of 119 RRs, and more than 50% of them are orphan (7). During the past decade, functions of orphan RRs have been investigated in some bacteria, for example, in Streptomyces (8). However, little is known about the regulatory mechanisms of orphan RRs.

GroEL is an important molecular chaperone that belongs to the Hsp60 family of heat shock proteins, and it is involved in diverse biological functions in bacterial cells by participating in folding, maturation, and transport of many proteins under normal growth or heat shock conditions (9, 10). Most bacteria have a single copy of groEL, but the presence of two or more copies has been identified in 19.5% of the sequenced bacterial genomes (11). Duplicate groELs are strictly transcribed at different levels, to fit their divergent cellular functions. For example, *M. xanthus* DK1622 has duplicate groELs: groEL1 plays an essential role in development and sporulation, while groEL2 is required for cell predation and biosynthesis of the secondary metabolite myxovirescin (12–14). The transcriptional levels of these two groELs are significantly different: the transcriptional level of groEL1 is normally four times that of groEL2, and the transcriptional level of single groES, which is required for the functions of duplicate groELs, is almost the sum of the groEL1 and groEL2 transcriptional levels (11). We previously reported that the global positive regulator $\sigma^{32}$ and the local negative regulator HrcA are involved in the transcriptional regulation; these two regulators respectively target the $-10/-35$ region and the CIRCE element, which are separate in the promoter of groEL1 but overlapped in that of groEL2 (15). However, the regulation of $\sigma^{32}$ and HrcA does not explain the strictly differential transcription of duplicate groELs.

In *M. xanthus* DK1622, the groEL1 and groEL2 genes have distinct compositions and locations on the genome; groEL1 (*MXAN_4895*) is located in a groESL operon, while groEL2 (*MXAN_4467*) has no neighboring groES and is downstream of *MXAN_4468*, which encodes an orphan RR. In this study, we found that *MXAN_4468* deletion eliminated the negative transcriptional control of both groELs and increased the expression of groEL2 to almost the same level as that of groEL1. The RR has a conserved phosphorylation site, and phosphorylation of this Asp residue is required to trigger the RR regulatory functions. Pull-down assay and mutation indicated that two CheA proteins might be the noncognate HKs of this orphan RR. We determined that the regulator plays a central role for differential expression of duplicate groELs in a dual-functioning mode: it enhances the ability of HrcA to bind to the CIRCE sequences to regulate the transcription of groEL1 and groEL2, and it binds to the 3’ end of its own gene sequence to specifically negatively control the transcription of the downstream groEL2 gene. A network analysis based on the transcriptome data, as well as the pull-down experiment, suggested that the RR gene plays a global negative regulatory role and primarily affects the transcription of stress regulatory proteins. This study highlights that this orphan RR in *M. xanthus* cells negatively regulates the transcription of duplicate groELs and is involved in the control of complex biological functions.

**RESULTS**

The RR gene upstream of groEL2 is a negative regulator of the transcription of duplicate groELs in *Myxococcus xanthus*. *M. xanthus* DK1622 has two groELs and one groES; groEL1 forms a complete operon with the single groES, and groEL2 exists alone. *MXAN_4468* lies upstream of groEL2 and is annotated by NCBI to encode a response regulator (RR) belonging to the cdd388505 protein family. *MXAN_4468* is an orphan RR. We found that the RR gene is located adjacent to groEL2, upstream and/or downstream, in a phylogenetically specific manner in different myxobacteria but does not occur adjacent to groEL1 (Fig. 1A; see also Table S1 in the supplemental material). Based on the sequenced myxobacterial genomes, the *MXAN_4468* homologues locate upstream of groEL2 in the same direction in *Myxococcus* genomes, downstream of groEL2 in the opposite direction in *Anaeromyxobacter* genomes, and upstream and downstream of groEL2 in the genomes of the genera Corallococcus, Stigmatella, and Cystobacter. However, there is no RR gene...
adjacent to groEL2 in the genomes of the Sorangineae suborder (Fig. 1A). The RR proteins encoded by the upstream genes are highly conserved, the second RR proteins encoded by the downstream RR genes are closely grouped in a separate branch, and the single downstream RRs in the Anaeromyxobacter genomes form the third branch (Fig. 1B). These phylogenetically conserved RR genes might have similar cellular functions critical for myxobacteria.

The MXAN_4468 and groEL2 genes are separated by a 169-bp sequence, and the two genes were confirmed to be transcribed by their own promoters (Fig. 1C and Fig. S1A). This interval sequence contains the core promoter region of groEL2, which includes a CIRCE sequence for binding the negative regulator HrcA (15). We found that deletion of the RR gene greatly increased the transcriptional levels of groES, groEL1, and groEL2 in DK1622 (Fig. 1D; t test, groES and groEL2, P value < 0.001; groEL1, P value < 0.01). The increases in the transcriptional levels of groES and groELs were completely abolished by the in situ complementation of MXAN_4468. However, in situ overexpression of MXAN_4468 had no effect on the transcription of groES and groEL1 but significantly decreased groEL2 expression (Fig. 1D; t test, P value < 0.05).

The RR gene deletion, complementation, and overexpression were confirmed by quantitative PCR (qPCR) analysis (Fig. S1B). The above-described results indicate that MXAN_4468 is a strong negative regulator of the expression of groES and groELs and has an additional inhibitory effect specifically on groEL2. The transcriptional curves of groES and groELs in the MXAN_4468 knockout strain indicated that compared with wild-type DK1622, the negative regulation of groES and groELs by the MXAN_4468 gene occurred mainly in the exponential growth phase, when the chaperonin genes were expressed under either the normal temperature condition (30°C) or after heat shock at 42°C for 1 h (Fig. S1C). Notably, according to the transcriptome data, the
transcription of MXAN_4468 was significantly changed at 24 h and 36 h under the normal and heat shock conditions (Fig. S1D). The orphan MXAN_4468 is phosphorylated by two noncognate CheAs belonging to the Che8 and Che7 chemosensory pathways. MXAN_4468 is a single-domain RR, which is represented by the chemotaxis-associated protein CheY (16). This protein has the highest sequence identity with CheY4 of Vibrio cholerae (42.48%), and the two proteins are also highly similar in structure based on the modeling analysis (Fig. S2A). CheY4 is involved in chemotaxis-related motility to control the direction of flagellar movement (17, 18). Active CheY4 is a phosphorylated protein, and the phosphorylated amino acid is located in the domain that receives signals transmitted from the upstream component (19). Based on the amino acid sequence alignment with CheY4, MXAN_4468 may contain a similar phosphorylation site at the 61st aspartic acid (Fig. 2A). Notably, M. xanthus DK1622 has 20 single-domain RR homologues belonging to the cdd388505 protein family (Table S1), and some of them have been annotated in different chemosensory pathways (20, 21). These 20 RR homologues in M. xanthus DK1622 (Fig. S2B), as well as the 21 groEL2-adjacent RRs in different myxobacterial genomes (Fig. S2C), were all conserved at the 61st aspartic acid.
To explore the phosphorylation and its functional effects, we mutated the \textit{MXAN\_4468} gene by altering the 61\textsuperscript{st} aspartic acid to valine and phenylalanine in the DK1622 genome according to references 22 and 23), producing the $\Delta4468:\textit{4468}$-D61V and $\Delta4468:\textit{4468}$-D61F mutant strains, respectively. This amino acid swapping led to almost the same transcriptional levels of \textit{groES} and \textit{groEL}s as those in the \textit{MXAN\_4468} knockout mutant under either normal temperature or heat shock conditions (Fig. 2B and C). To facilitate the phosphorylation check, we further linked a 6×His-encoding sequence to the C terminus of \textit{MXAN\_4468} in the wild-type DK1622 and the two mutants. The Phos-tag PAGE analysis showed that only purified \textit{MXAN\_4468} protein with no addition of HK was not phosphorylated (Fig. 2D, lane 1). However, the \textit{MXAN\_4468} protein in the supernatant of DK1622, which contains HKs, was phosphorylated (lane 2), but both the the 61\textsuperscript{st} mutant proteins in mutant strains, which also contain HKs, were not (lanes 3 and 4) (Fig. 2D). The results indicate that the 61\textsuperscript{st} aspartic acid is the phosphorylation site of \textit{MXAN\_4468}, and phosphorylation of this amino acid is essential for \textit{MXAN\_4468} to regulate the transcription of \textit{groES} and \textit{groEL}s.

We performed a pull-down assay of the \textit{MXAN\_4468} wild-type protein, as well as the D61V and D61F mutant proteins, with the supernatant of disrupted DK1622 cells to screen its potential interacting proteins. The expression and purification of these proteins are shown in Fig. S2D. We noticed that the proteins specifically bound by MBP-MXAN\_4468 included two CheA proteins, MXAN\_4758 and MXAN\_6964, which, however, were not among the binding proteins of the two \textit{MXAN\_4468} mutants (Table S2). CheA proteins are HKs that are responsible for transferring the phosphoryl group to the RRs in the corresponding TCSs (24, 25). MXAN\_4468 is an orphan RR, and the two CheA proteins may be the noncognate HKs for the phosphorylation of MXAN\_4468. Interestingly, the MXAN\_4758 and MXAN\_6964 genes belong to the Che8 and Che7 chemosensory pathways, respectively (21). The Che7 system has been reported to be associated with a HEAT repeat domain-containing protein and required for the appropriate coupling of aggregation and sporulation, while the functions of the Che8 system have not yet been identified (21, 26). We knocked out each of the two cheA genes from the DK1622 strain. The two deletions led to similar transcriptional levels of \textit{groESL} genes as that resulting from \textit{MXAN\_4468} deletion (Fig. 2E). Comparably, the \textit{MXAN\_4758} (cheA8) deletion mutant had a closer transcriptional level of \textit{groESL} to that of the \textit{MXAN\_4468} deletion mutant than the \textit{MXAN\_6964} (cheA7) deletion mutant. Notably, in the eight chemosensory pathways (21), while five CheA proteins are hybrid HKs (CheA-RR fusion protein) responsible for autophosphorylation, three are typical HKs (single-domain CheA), including the above-mentioned two CheAs and MXAN\_6692. The latter is in the Dif chemosensory pathway and was not bound by MXAN\_4468 in the pull-down assay. We similarly deleted the MXAN\_6692 gene, which, however, did not change the transcription of \textit{groESL}s (Fig. 2E). The above-described results strongly suggest that \textit{MXAN\_4758} and \textit{MXAN\_6964} are both responsible for the phosphorylation of \textit{MXAN\_4468} protein and that \textit{MXAN\_4758} is probably more important for this process.

\textbf{Phosphorylated \textit{MXAN\_4468} binds to HrcA to inhibit \textit{groESL} transcription.}

HrcA (MXAN\_6726) is a negative transcription regulator of duplicate \textit{groEL}s (15). This protein was also pulled down by MXAN\_4468 but was not by the two MXAN\_4468 mutants. Compared to the $\Delta\text{hrcA}$ mutant, the $\Delta4468$ mutant showed almost unchanged \textit{groES} and \textit{groEL1} transcriptional levels, but its \textit{groEL2} transcriptional level was approximately three times higher (t test, $\textit{groEL2}$, $P$ value $<$ 0.001). Further deletion of \textit{hrcA} in the $\Delta4468$ mutant caused no more changes in the \textit{groES} and \textit{groEL}s transcriptional levels (Fig. 2F; t test, $\textit{groES}$, $P$ value = 0.122; \textit{groEL1}, $P$ value = 0.141; \textit{groEL2}, $P$ value = 0.088). Thus, compared to the double deletion mutant of \textit{MXAN\_4468} and \textit{hrcA}, only \textit{hrcA} without \textit{MXAN\_4468} ($\Delta4468$) had almost no regulatory effect on the transcription of \textit{groES} and \textit{groEL}s, only \textit{MXAN\_4468} without \textit{hrcA} ($\Delta\text{hrcA}$) negatively and specifically regulated the transcription of \textit{groEL2}, and the presence of both \textit{hrcA} and \textit{MXAN\_4468} (DK1622) led to negative regulation of the transcription of \textit{groES} and \textit{groEL}s. The results suggest that
MXAN_4468 not only cooperates with HrcA on the transcriptional regulation of groES and groELs but also alone specifically inhibits the transcription of groEL2.

To investigate the binding ability between MXAN_4468 and HrcA, we constructed two recombinant expression vectors, 4468-pET32a and hrcA-pMAL-c5x, and labeled the C termini of MXAN_4468 and HrcA with His and maltose binding protein (MBP) tags, respectively (the purification of HrcA protein is shown in Fig. S2E). In vitro pull-down experiments showed that although there existed some excess proteins in penetrating liquid (Fig. 2G, lane 3), 4468-His and HrcA-MBP were both absorbed on beads for His proteins in the elution of imidazole washing buffer (lane 4), while MBP, mixed with 4468-His, was not absorbed on beads (lane 2). This result further confirmed the presence of a binding interaction between the MXAN_4468 and HrcA proteins.

Through electrophoretic mobility shift assay (EMSA) and isothermal titration calorimetry (ITC) binding experiments, we previously proved that HrcA has the capacity to bind CIRCE1_groEL1 or CIRCE_groEL2 but no capacity to interact with CIRCE2_groESL1 in M. xanthus DK1622 (15). To investigate whether MXAN_4468 affected the binding reaction of HrcA and CIRCE, we added MXAN_4468 and its mutants of D61V and D61F to the reaction mixtures. As shown in Fig. 2H, HrcA alone formed a weak blocking band with CIRCE1_groEL1 or CIRCE_groEL2, and the addition of MXAN_4468, but not the MXAN_4468 mutants, significantly enhanced the brightness of the blocking band and completely weakened the brightness of DNA band (lanes 7 and 8); MXAN_4468 itself was unable to bind to the CIRCE elements (lanes 9 and 10). The weak binding ability of HrcA alone to CIRCE sequences suggests its regulatory effect on the transcription of groES and groELs, which, however, is inconsistent with the phenotypic comparison results of the Δ4468 and Δ4468 ΔhrcA mutants (Fig. 2F). In M. xanthus DK1622, CIRCE1_groEL1 and CIRCE_groEL2 sequences overlap the transcription start site (TSS) and 35 regions of the promoters of groEL1 and groEL2, respectively, and these regions are the binding targets of many essential factors for transcription, such as σ72 and HrcA regulators (15). We suggest that the binding of MXAN_4468 to HrcA is necessary for the regulatory role of HrcA in the transcription of groES and groELs, probably by enhancing the binding competition ability of HrcA to the CIRCE sequences.

Phosphorylated MXAN_4468 binds to its own gene sequence to specifically inhibit the transcription of the downstream groEL2. The interval sequence between MXAN_4468 and groEL2 is 169 bp long and contains the promoter region of groEL2. Because MXAN_4468 deletion had an additional specific effect on the transcription of groEL2, we inferred that the deletion fragment of the Δ4468 mutant probably contained a regulatory sequence to which MXAN_4468 binds to negatively and specifically regulate the transcription of groEL2. To investigate whether there is such a regulatory sequence, we further constructed four mutants at different positions to retain the 3′ end of MXAN_4468 (Fig. 3A). Briefly, while the 3′-terminal regions were retained in the four partial mutants, the deleted regions were different: in Ki68-1, only the 5′-terminal region of MXAN_4468 was deleted; in Ki68-2 and Ki68-3, the deleted regions included the promoter region and different 5′-terminal regions (retaining the 61st phosphorylation site or not) of MXAN_4468; and in Ki68-4, the deletion included the 3′ terminus of the upstream MXAN_4469, the promoter region, and a 5′-terminal region of MXAN_4468. We did not additionally express the MXAN_4468 gene in other places of the genome. We found that the transcriptional levels of groES and groEL1 in these four incomplete deletion mutants of MXAN_4468 were the same as those in the complete deletion mutant, but the transcriptional level of groEL2 was much lower (Fig. 3B) and was almost the same as that in the ΔhrcA mutant (refer to Fig. 2F). Compared to the double deletion mutant of MXAN_4468 and hrcA, the existence of the 3′-end region of MXAN_4468 and complete hrcA (Ki68 mutants) had a more negative regulatory effect on the groEL2 transcription, similar to that in the mutant with MXAN_4468 but not hrcA (ΔhrcA). In other words, the 3′-end region of MXAN_4468 would be essential to maintain the lower expression of groEL2, regardless of the existence of hrcA. The results indicate that the 3′-end region of MXAN_4468, which was retained in the MXAN_4468-
incompletely deleted mutants, contains a sequence that is for the specific negative regulation of groEL2 transcription.

To confirm this unusual regulatory mechanism, we performed an EMSA of the MXAN_4468 proteins with the upstream 370-bp sequence of groEL2 (Fig. S3A) or the inner 200-bp 3’-end sequence of MXAN_4468 (Fig. 3C). The results showed that there were obvious blocking bands between the MXAN_4468 protein and the two DNA sequences, and the brightness of the DNA-protein complexes was enhanced with an increase in the concentration of MXAN_4468 protein. These blocking bands were determined by mass spectrometry to be MXAN_4468 (Fig. S3B to D) and were presumed to be the monomeric and polymeric forms of MXAN_4468. The band intensity showed that the MXAN_4468 proteins preferred to bind to their own DNA sequence in polymeric forms. Besides MXAN_4468, there exist other 19 single-domain RR homologues in M. xanthus DK1622 (Table S1), which were probably able to bind to the 3’-end region of MXAN_4468 to decrease the groEL2 transcription. Compared to the MXAN_4468 complete knockout mutant, the four incomplete deletion mutants (KI68 mutants) had lower groEL2 transcription (Fig. 3B).

FIG 3 The transcriptional relationship between MXAN_4468 and its own coding region. (A) Representations of the MXAN_4468 knockout mutants. (B) qPCR analysis of the transcriptional levels of groES and groEL in MXAN_4468 knockout mutants and the wild-type strain DK1622. The transcriptional level of groEL2 in DK1622 was set to 1. For statistical analysis, **** and ***** mean P values of <0.001 and <0.01, respectively. (C) Analysis of binding activity between the MXAN_4468 protein and the inner 200-bp 3’-end sequence of MXAN_4468 using EMSA (presented by native PAGE). The monomers of the MXAN_4468 protein are marked in the diagram. Other protein bands with high concentrations were proven to be the polymers of MXAN_4468 (Fig. S3B to D).
but the two mutant strains exhibited almost the same groES and groEL transcriptional levels as those exhibited by the Δ4468 mutant, especially for groEL2 transcription. Due to the distance advantage of the MXAN_4468 gene, nonphosphorylated MXAN_4468 proteins could combine with the 3’-end region of MXAN_4468, thus preventing the binding of other MXAN_4468 homologues on the location. However, the interaction would not lead to negative regulation on the groEL2 transcription because of nonphosphorylation of the mutated MXAN_4468 proteins. We conclude that the phosphorylated MXAN_4468 protein specifically inhibits the transcription of groEL2 by binding to its own sequence, thus interfering with the binding of transcriptional factors to the promoter of the downstream groEL2 gene.

**Global effects of MXAN_4468 deletion in M. xanthus DK1622 cells.** Our above-mentioned results demonstrated that the phosphorylated MXAN_4468 protein plays a critical role in regulation of the expression of groES and groELs, leading to their differential transcriptional levels. GroEL is a type I chaperonin and an essential component in different bacterial species (27, 28). It is required for correct in vivo folding of more than 10% of the total proteins in *E. coli* (10). Our previous studies indicate that the protein clients of the *M. xanthus* GroELs, although consistent with those of the single *E. coli* GroEL in their secondary structural features, vary significantly in the substrate spectra, and GroEL1 and GroEL2 have their own exclusive protein clients in addition to the shared clients (13). Moreover, the proteins that were specifically pulled down by MXAN_4468 included not only the CheA and HrcA proteins but also some other regulatory proteins, such as an RNA polymerase sigma 70 factor (MXAN_7454), a transcriptional regulator (MXAN_1757), a sigma 54 transcriptional regulator (MXAN_0907), which are related to stress and transcriptional regulation (Table S2) (the energies for binding of these three proteins to MXAN_4468 were estimated by the JSmol website to be $-28.47$, $-21.32$, and $-10.97$, respectively). These results suggested that MXAN_4468 probably plays a global transcriptional regulator role, not only via the control of transcriptional levels of groESLs but also via other regulatory proteins. To explore potential global effects, we performed a transcriptome analysis on the MXAN_4468 knockout strain and the wild-type strain DK1622 using cells grown for 24 h and 36 h under the normal temperature or heat shock condition. We BLAST searched the reads of transcriptome data against the reference genome of DK1622 using the Bowtie2 program (29) and found that the total mapped value of all samples exceeded 99% (Table S3), which indicates that the samples were not contaminated and that the transcriptome data were reliable.

We found that the transcriptomes of the ΔMXAN_4468 strain and DK1622 had significant differences (Fig. S4; for details, see Data Set S1 [sheets 1 to 4]). Functional annotation of the differentially expressed genes in the cells grown for 24 h under the normal temperature condition indicated that the genes that were significantly upregulated by MXAN_4468 deletion were mainly related to the two-component system and metabolism, while those that were significantly downregulated were mainly related to ribosomes and binding proteins (Fig. 4A). After heat shock, the most significantly upregulated genes were involved in the pathways of metabolism and phosphotransferase system, and the most significantly downregulated genes were related to partial amino acid degradation and oxidative phosphorylation. Under the 36-h normal temperature and heat shock conditions, the significantly upregulated genes were related to amino acid and RNA degradation, and the significantly downregulated genes were related to metabolism and amino acid biosynthesis.

To highlight the transcriptional impacts of the MXAN_4468 deletion, we performed a network analysis of the top 200 differentially expressed genes in all transcriptome samples. These top 200 genes were classified into four classes according to their annotation: stress regulatory proteins (40), metabolites (66), ribosomal proteins (39), and unknown proteins (55) (Data Set S1 [sheets 5 to 8]). Notably, the genes encoding stress regulatory proteins were mostly upregulated (36 of 40; the gene names in white with a purple background in Fig. 4B), while the genes encoding metabolites and ribosomal proteins were either upregulated or downregulated (colored in purple or green). These
FIG 4 Transcriptomic analysis of the MXAN_4468 knockout mutants and the wild-type strain DK1622. (A) KEGG enrichment analysis of differentially expressed genes under different conditions. (B) Network analysis of the top 200 differentially expressed genes with high expression in all transcriptomic samples.
results suggest that the transcription of the genes encoding stress regulatory proteins was regulated in a similar manner by MXAN_4468 deletion; i.e., the orphan RR probably played a global negative regulatory role in the transcription of these genes.

**MXAN_4468 deletion decreases cell competition ability in M. xanthus.** M. xanthus cells have complex social behavior (30) and the ability to produce diverse secondary metabolites (31). We previously determined that the two *groEL* genes have divergent roles in development, predation, and biosynthesis of secondary metabolites (12–14). To efficiently achieve their cellular functions, the expression level of *groEL1* is about four times higher than that of *groEL2*, and their total expression level is approximately equal to the expression level of *groES*, the cochaperone gene of the duplicate *groELs* (11). However,
deletion of the MXAN_4468 gene led to not only high expressions of the duplicate groEL genes but also unbalanced transcription of groES and groELs. We found that the MXAN_4468 deletion had almost no effects on development, sporulation, and predation abilities of M. xanthus (Fig. 5A to C) but markedly increased the biosynthesis of myxovirescin (Fig. 5D). In contrast, the in situ overexpression of MXAN_4468, which had almost no effect on the transcription of groES and groEL1 but significantly decreased the transcription of groEL2 (Fig. 1D), had no effects on development and sporulation but caused a weak deficiency in predation and led to near-undetectable myxovirescin biosynthesis. Thus, overexpression of groEL1 or groEL2 allowed or improved their related cellular functions but caused no effect on their unrelated functions; i.e., groEL1 plays an essential role in fruiting body development and sporulation, while groEL2 is required in predation and myxovirescin biosynthesis (12–14).

Although the high and unbalanced transcription of groES and groELs had no significant effects on social behavior, it should be a metabolic burden to the host (32–34) and probably led to weaker competitive ability than that exhibited by the wild-type strain. To assay their growth competitive abilities, we constructed kanamycin-resistant strains of M. xanthus DK1622 and the Δ4468 mutant and cocultured wild-type and mutant strains to form a competitive environment following the previous protocol (35). When the kanamycin-resistant and -sensitive DK1622 or Δ4468 strains were cocultured on a casitone-based rich-nutrient (CTT) plate for 0, 2, and 4 days, the two types of cells in the harvested mixtures had the same number of CFU. However, when kanamycin-resistant Δ4468 and -sensitive DK1622, or kanamycin-resistant Δ4468 and -sensitive Δ4468, were mixed for cocultivation, the growth of the Δ4468 mutant was significantly slower than that of the wild-type DK1622 (Fig. 5E). These results indicate that MXAN_4468 deletion weakened the competitive ability of cells, probably due to the metabolic burden of high and unbalanced transcription of groES and groELs.

**DISCUSSION**

There are multiple copies in the DK1622 genome of the genes that encode single-domain RR proteins (Table S1). Seven of them are located in different chemosensory
pathways (21) and the rest are orphan ones, like MXAN.4468. Previous studies have revealed that MXAN.6693 (difD) in the Dif system (the Che2 system) is related to the chemotaxis of lipids (36, 37), MXAN.2684 and MXAN.6956, belonging to the Che4 and Che6 systems, respectively, are both responsible for aggregation and sporulation (38, 39), and MXAN.6965 in the Che7 system is important for resistance to temperature stress and for the production of viable spores (26). Little is known as yet about the function of the Che8 chemosensory pathway, as well as the substantial orphan RRs. In this study, we found that an orphan RR (MXAN.4468) plays a central role in tuning the transcriptional differences of groESLs via a novel functioning mode. First, the RR binds to HrcA and thereby enhances its ability to bind to the CIRCE elements, which enables it to achieve the transcriptional inhibition of the duplicate groELs. Second, the RR binds to the 3’ end of its own gene sequence, thereby specifically decreasing the transcription of the following groEL2 gene. These functions require phosphorylation of the 61st
aspartic acid residue of the RR. In addition to these specific functions, MXAN_4468 is probably involved in global regulation of cellular functions, indicated by significant transcriptomic changes caused by MXAN_4468 deletion. The genes specifically regulated by MXAN_4468 are the duplicate groELs, whose encoded proteins play essential roles in protein folding, assembly, and transport. In addition, pull-down results showed that MXAN_4468 could bind to some other transcriptional regulators and kinases (Table S2). It is thus unclear whether the transcriptomic changes were caused by MXAN_4468 directly, by GroELs indirectly, or by both.

In Bacillus subtilis cells, the HrcA protein is usually present in an inactive monomer form (40, 41), which is dimerized into an active form for binding to the CIRCE elements (42). Structural modeling indicated that dimerized MXAN_4468 is much more stable than dimerized HrcA (the dimerization energy of HrcA is 21.32, but that of the MXAN_4468 dimer is 295.77 [Fig. S5A]; the binding energy between MXAN_4468 and HrcA is 26.73 [Fig. S5B]). We posit that the MXAN_4468 dimer may recruit and bind to HrcA, consequently improving the formation of an active HrcA dimer for binding to the CIRCE elements in the promoter region of the duplicate groELs. We propose a functioning model for the negative regulatory role of MXAN_4468 in the transcription of groELs in M. xanthus cells (Fig. 6). The regulation of groESL1 by MXAN_4468 via the recruitment of HrcA leads to the formation of a more active dimer to play a negative regulatory role in combination with CIRCE. This protein-protein interaction may promote the binding of this negative regulator to its target sequence, further enhancing its regulatory effect. In addition, to regulate groEL2, MXAN_4468, in polymeric forms, bound to the internal sequence of its own gene to further inhibit the transcription of groEL2. The binding of the MXAN_4468 protein to the MXAN_4468 3'-end DNA sequence was analyzed using nucleic acid-protein dock (NPdock) software (43), which showed that the C-terminal region of MXAN_4468 was likely responsible for binding to its own gene sequence on a 12-bp reverse complementary palindromic sequence (Fig. S5C).

There are 119 RRs in M. xanthus, and more than 50% of them are orphan. MXAN_4468 is a typical orphan RR; i.e., its function requires phosphorylation but without an HK gene adjacent on the genome (8). Here, we report that two noncognate HKs are the upstream components of MXAN_4468. The two corresponding cheA genes, i.e., MXAN_6964 and MXAN_4758, belong to the Che7 and Che8 systems, respectively (21). Many organisms carry a substantial fraction of their two-component genes as orphans probably from duplication or loss of one component, which might be a mechanism for generating cross-regulation systems (44). The cross talk between noncognate HKs and an orphan RR (45) suggests complex and global regulation of MXAN_4468. Our network analysis suggested that among the multiple RR copies in M. xanthus DK1622, the special location of MXAN_4468 may have extensive effects on the expression of stress regulatory proteins and yet have no correlation with other RR genes involved in the regulation of transcription. The global transcriptional effects of MXAN_4468 may be directly or indirectly related to the functions of groELs. We speculate that in bacteria possessing multiple copies of orphan RR genes, these RR genes are involved in regulating the transcriptional symmetry in cells to ensure that complex biological functions can occur.

MATERIALS AND METHODS

Cultures, plasmids, and growth conditions. The strains, plasmids, and primers that were used in this study are shown in Table S4. The M. xanthus strains were cultivated in CTT medium (46) for growth assays. E. coli strains were routinely grown on LB agar or in LB broth. E. coli strains were grown at 37°C, while Myxococcus strains were incubated at 30°C. The temperature for heat shock treatment was 42°C. When required, kanamycin, ampicillin, and chloramphenicol were added to the media at final concentrations of 40, 100, and 34 μg/mL, respectively.

Evolutionary relationship analysis. The analysis of evolutionary relationships was completed using MEGA6 software (47). Briefly, protein sequences were constructed by the neighbor-joining method (NJ model) and pairwise deleted (pairwise deletion of gaps), and different evolutionary tree models were selected according to the type of protein. All tree self-expansion tests were set at 1,000 repeats to evaluate the stability of the nodes.
Deletion of MXAN_4758, MXAN_6964, MXAN_6692, MXAN_4468, or MXAN_4468 and hrcA. An in-frame deletion in \textit{M. xanthus} was performed using positive-negative KG cassettes described by Ueki et al. (48). Genomic DNA from DK1622 served as the template for the PCR amplification of the upstream and downstream homologous arms using Phanta Super-Fidelity DNA polymerase (Vazyme). The fragments were cloned into SmaI-digested pB113 to construct the deletion plasmid pB1-4758, pB1-6964, pB1-6692, or pB1-4468, which was then transferred via electroporation into \textit{M. xanthus} DK1622 cells. Individual kanamycin-resistant colonies were selected and then inoculated onto CTT agar plates supplemented with 1% galactose for the second round of screening. Deletion mutants were identified by the phenotypes of galactose resistance and kanamycin sensitivity, as well as by PCR verification. The MXAN_4468 and hrcA double-knockout mutant was based on the MXAN_4468 knockout mutant, in which the hrcA gene was deleted.

Construction of MXAN_4468 in situ complement or overexpression mutant. An MXAN_4468 in situ complement or overexpression mutant was constructed on the basis of the obtained MXAN_4468 knockout mutant, and then a 4468 or P\_hrcA plus 4468 fragment was inserted in situ by the secondary homologous recombination method to construct the in situ complement or overexpression mutant.

qPCR analysis. \textit{M. xanthus} DK1622 and mutants were inoculated into CTT medium at an initial concentration of 1 \times 10^7 cells/mL and collected after 24 h of incubation. The cultures were harvested at the testing time points, and the RNA was extracted immediately using a bacterial total RNA isolation kit (Sangon Biotech) according to the manufacturer’s instructions. The purified RNA extracts were reverse transcribed to cDNA. Quantitative real-time PCR was performed with a total reaction volume of 20 \mu L containing 1 \mu L of 250 nM primers, 10 \mu L of SYBR green PCR master mix, 8.5 \mu L of RNase-free water, and 0.5 \mu L of a 10-fold-diluted cDNA template. The gapA gene was used as the reference. The primers for real-time PCR are listed in Table S4.

Transcriptome analysis. Differential expression analysis of \textit{M. xanthus} DK1622 and mutants subjected to two temperature conditions (normal and heat shock) was performed using the DESeq R package (1.18.0) (49). DESeq provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting \( \hat{P} \) values were adjusted using Benjamini and Hochberg’s approach (50) for controlling the false-discovery rate. Genes found by DESeq and with an adjusted \( \hat{P} \) value of less than 0.05 were assigned as differentially expressed.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource for understanding high-level functions and utilities of a biological system, such as a cell, an organism, and an ecosystem, from molecular-level information, especially large-scale molecular data sets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/) (51). We used KEGG Orthology-Based Annotation System software to test the statistical enrichment of differentially expressed genes in KEGG pathways.

Network analysis. The network was based on the transcriptomic data, such that each node (or vertex) in the network represents a gene (or a protein), and each edge indicates a strong and significant transcriptional correlation. All analyses were performed in R version 3.5.0. The degree of centrality of each node was measured to determine the importance of nodes in the network. Accordingly, all of the nodes were classified into three categories based on the abundance of links with other members in the network: key nodes (vertices with the top 20% centrality), peripheral nodes (vertices with the lowest 20% centrality), and moderate nodes (the remainder of the vertices). All analyses were performed using psych package version 1.8.4 (52).

Protein structure modeling analysis. Based on the amino acid sequence of the individual wild-type and mutant protein, the protein structures were modeled using the I-TASSER tool (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) (53). Prediction of protein-protein interactions was performed using the Prism 2.0 tool (http://cosbi.ku.edu.tr/prism/) (54).

Cell phenotypic analysis. (i) Analysis of developmental ability and calculation of sporulation rate. Briefly, each strain was cultured in CTT/CTT-resistant liquid medium for 20 to 24 h, and then the bacterial concentrations of the growing cultures were adjusted to 5 \times 10^9 cells/mL (optical density at 600 nm [OD\_600] = 33). Subsequently, 10-\mu L volumes of bacterial solutions were added onto TPM (Tris-HCl phosphate medium) plates, and the plates were incubated at 30°C. The development of fruiting bodies was observed and recorded using a stereoscopic microscope at 12-h intervals. After 5 days of culture, five fruiting bodies of each strain were scraped and cultured in the same centrifuged tube, 100 \mu L of TPM buffer was added to the tubes, and the bacterial suspensions were subjected to three treatments of low-power ultrasound (<200 W, 4 s) to evenly disperse the cells. The suspensions were then incubated in a bath at 50°C for 2 h. The treated spore suspensions were diluted 10 times with TPM buffer. Subsequently, 50-\mu L bacterial suspensions of appropriate dilutions were mixed evenly with melted CTT soft agar (0.3% agar) and spread onto CTT/CTT-resistant plates. This process was repeated three times for every strain suspension. The plates were cultured at 30°C for 5 days, and then the number of colonies on the plates was counted and the sporulation rate of each strain was calculated.

(ii) Analysis of predation ability. \textit{E. coli} DH5\_x was cultured in LB liquid medium for 12 h. Based on the expected OD\_600 value, the bacterial concentration was adjusted to an OD\_600 of 100 using TPM buffer, and 30 \mu L was inoculated onto a water (WAT) plate (containing only CaCl\(_2\)). The mutants and wild-type strain of Myxococcus were cultured in CTT liquid medium for 20 to 24 h. The bacterial concentrations of these growing bacterial cultures were adjusted to 5 \times 10^6 cells/mL (OD\_600 = 35) using TPM buffer, and 2 \mu L of each bacterial suspension was inoculated onto the middle of an \textit{E. coli}-inoculated WAT plate. This process was repeated three times for every strain suspension. After drying, the colonies were
incubated at 30°C. The predation activity was observed and photographed using a stereo micro- 
scope at 12-h intervals.

**Determination of the fermentation level of myxovirescin (TA).** The wild-type DK1622 and mutants were activated on CTT/CTT-resistant solid plates and inoculated into CYE liquid medium for 20 to 24 h. Subsequently, 1% ion-exchange resin was added to each medium, and the culturing was con- 
ducted for approximately 2 days until the later stage of growth stability. The supernatant was removed to 
collect the bacteria and resin, and the bacteria were gently washed with double-distilled water (ddH₂O). 
Each sample was extracted with approximately 8 mL of methanol and shaken overnight at a uniform 
temperature. This step was repeated two more times. The combined extracts were concentrated to approxi- 
mately 3 mL using a vacuum concentration dryer. The concentrated solution was centrifuged at 
12,000 rpm for 15 min at 4°C, and the supernatant was used for the next step. The production of TA in 
each strain was detected by HPLC with a Mycobacterium (TA) standard as a control.

**MXAN_4468 protein purification.** The heterogeneously expressed MXAN_4468 proteins were puri- 
fied with some modifications. BL21 cells were grown at 37°C in LB medium with 100 μg/mL of ampicillin 
to an OD₆₀₀ of 0.6 to 0.8 and were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The 
cells were resuspended in lysis buffer (25 mM Tris-HCl [pH 8.0], 300 mM NaCl, 2 mM dithiothreitol) and 
sonicated. After gentle ultrasonic treatment, the samples were centrifuged at 12,000 rpm for 30 min at 
4°C. The supernatant of His-tagged MXAN_4468 was applied on Ni²⁺-nitrilotriacetic acid (NTA) and then 
purified by ion-exchange chromatography using a Sephadex 200 gel filtration column. The protein concen- 	ration was determined using a microconcentration detector.

**EMSA.** The purified MXAN_4468 proteins with different concentrations were incubated with DNA 
at 30°C for 30 min and then subjected to nondenatured polypropylene gel electrophoresis (90 V, 1 h). 
The gel was visualized by detecting chemiluminescence after blocking it with the solution and antibody 
(streptavidin-horse radish peroxidase [HRP]) in the electrophoretic mobility shift assay (EMSA) kit 
(Thermo Fisher).

**Construction of key site mutation expression vector and mutant strains.** The aspartic acid at posi- 
tion 61 (GAC) of MXAN_4468 was mutated to valine (GTC) or phenylalanine (TTC). The expression vectors 
pET32a, with a His tag, and pMAL-cS, with an MBP tag, were selected. The mutation-harboring recombinant 
vector was constructed using the ClonExpress II one-step cloning kit (Vazyme). The same homologous 
recombination method was used to construct a pBluescript plasmid containing the MXAN_4468 gene with a 
key site mutation, together with its upstream and downstream homologous arms.

**Phos-tag PAGE.** A His tag was attached to the C terminus of MXAN_4468 and the mutant proteins. 
To ensure correct formation of the spatial structure of the fusion protein, a linker sequence (TCTG 
CGACGGCTGAGCGCGCCGGCGG) was added between the fusion parts. Phos-tag composite electrophore- 
sis adhesive was prepared using the Phos-tag acrylamide AAL-107 kit. When electrophoresis was per- 
formed in the Phos-tag composite electrophoresis gel, the specific binding of Phos-tag to the phosphate 
group of the phosphorylated protein hindered its migration, resulting in lower mobility of the phospho- 
rylated protein than of the unphosphorylated protein (55). During the experiment, a sufficient amount of 
commercialized histidine protein kinase (L-histidine K1) was added to activate the phosphorylation of 
MXAN_4468.

**Pull-down analysis for potential binding proteins.** The mixtures of 4468-pMAL, 4468-D61V-pMAL, 
and 4468-D61F-pMAL were induced and purified, and pMAL (MBP-tagged protein) was established as 
the negative control. Strain DK1622 cultured on solid medium was transferred to CTT liquid medium 
and cultured for 20 to 24 h. The cells were collected by centrifugation, washed three times with TPM 
buffer, then suspended in a heavy suspension buffer, and lysed by ultrasound. After centrifugation, 
appropriate amounts of supernatant and 4468-MBP plus MBP filler, 4468-D61V-MBP plus filler, 4468- 
D61F-MBP plus filler, MBP plus filler, or only filler were mixed and incubated at 4°C for 4 h. The incubated 
mixture was washed through the column with buffer, and then 10 mM maltose was added to elute 
MXAN_4468 and the protein bound to the filler. The eluted-protein-containing solutions obtained from 
either the experimental and control groups were sent to Shanghai Zhong Ke Xin Sheng Ming Company for 
identification by mass spectrometry.

**Co-culture of strains.** The nonresistant and resistant strains were cultured in CTT/CTT-km liquid 
medium at 30°C for 20 to 24 h. The cells were collected, and their concentrations were adjusted to 5 × 10⁸ 
cells/mL. The nonresistant and resistant strains were mixed at a 1:1 (vol/vol) ratio, and 5-μL volumes of 
the obtained bacterial solutions were inoculated onto CTT plates. After culturing at 30°C for 0, 2, and 
4 days, the whole colonies were scraped and resuspended in 500 μL of TPM buffer. The resulting bacte- 
riral suspensions were diluted 10 times, and the suitable four dilutions of three repeated 50-μL suspensions 
were mixed evenly with 2.5 mL of semisolid CTT (0.3% agar) and then spread onto the CTT/CTT-km 
plates. After 5 days of culture, the number of colonies on the plate was counted and the CFU of each 
strain were calculated.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**DATA SET S1**, XLSX file, 0.5 MB.
**FIG S1**, TIF file, 1.6 MB.
**FIG S2**, TIF file, 2.9 MB.
**FIG S3**, TIF file, 2.2 MB.
**FIG S4**, TIF file, 0.7 MB.
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