

Myxococcus xanthus Viability Depends on GroEL Supplied by Either of Two Genes, but the Paralogs Have Different Functions during Heat Shock, Predation, and Development[∇]

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Myxococcus xanthus DK1622 contains two paralogous *groEL* gene loci that possess both different sequences and different organizations within the genome. Deletion of either one of these two genes alone does not affect cell viability. However, deletion of both *groEL* genes results in cell death unless a complemented *groEL1* or *groEL2* gene is present. The *groEL1* gene was determined to be essential for cell survival under heat shock conditions; a strain with mutant *groEL2* caused cells to be more sensitive than the wild-type strain to higher temperatures. Mutants with a single deletion of either *groEL1* (MXAN_4895) or *groEL2* (MXAN_4467) had a growth curve similar to that of the wild-type strain DK1622 in medium containing hydrolyzed proteins as the substrate. However, when cells were cultured on medium containing either *Escherichia coli* cells or casein as the substrate, deletion of *groEL2*, but not *groEL1*, led to a deficiency in cell predation and macromolecular feeding. Furthermore, *groEL1* was found to play an indispensable role in the development and sporulation of cells, but deletion of *groEL2* had no visible effects. Our results suggest that, although alternatively required for cell viability, the products of the two *groEL* genes have divergent functions in the multicellular social life cycle of *M. xanthus* DK1622.

Myxobacteria are characterized among the prokaryotes by their unique social behavior (4, 30, 36). The social behavior of myxobacterial cells is present in each stage of the life cycle; cells glide on solid surfaces in swarms, feed on macromolecules and other microbial cells in groups, and develop multicellular resting structures called fruiting bodies that contain myxospores when food is exhausted (32). Many genes are required to conduct this complicated social lifestyle. Genome sequencing revealed that myxobacteria have the largest bacterial genome and possess many multicopy genes (8, 31). A major duplication occurs with chaperone genes, which are essential for cell functions by assisting protein folding, assembly, transport, and degradation (21, 26), not only in normal cellular processes but also in response to nonpermissive temperatures (their products thus belong to the heat shock protein family) (5, 23). There are several reports related to the functions of chaperones in the sociality of *Myxococcus*. For example, in *Myxococcus xanthus*, there appear to be several duplicated genes encoding the chaperone HSP70 protein (DnaK) (8), of which only one, *sglK* (MXAN_6671), has been studied. *SglK* was shown to be essential for social motility and multicellular development in *M. xanthus* DK1622, but it did not respond to temperature changes or heat shock (35, 37).

GroEL protein, a major type of chaperonin, is ubiquitously distributed in bacteria (11). Bacterial cells usually contain one copy of the *groEL* gene within the genome. However, approx-

imately 20% of sequenced bacterial genomes were found to have duplicate or multiple copies of the *groEL* gene (10, 11). The products of duplicated *groEL* genes have been reported to play divergent roles in various bacteria. For example, in *Mycobacterium smegmatis*, which possess duplicate *groEL* genes, the GroEL2 protein was shown to be essential for cell growth. In contrast, inactivation of the *groEL1* gene did not affect the normal growth of cells but prevented the formation of mature biofilms and the biosynthesis of mycolic acid (24). There are three copies of the *groEL* gene in *Rhizobium leguminosarum*. The *cpn60.1* gene (*groEL1*) has been shown to be indispensable for the growth of this organism, while the other two paralogous genes, *cpn60.2* and *cpn60.3* (*groEL2* and *groEL3*), can be deleted without affecting cell viability (28). The three GroEL homologues of *R. leguminosarum* display distinct properties *in vitro* (7) and preferentially self-assemble rather than form mixed hetero-oligomeric proteins when co-expressed in *E. coli* cells (10). Furthermore, *Sinorhizobium meliloti* has five copies of the *groEL* gene in its genome. However, only the product encoded by *groEL1*, which is required for symbiosis, is required for viability (2).

In different myxobacteria, there are also two copies of the *groEL* gene (13), of which little is known with regard to their specific cellular roles, especially in the social life of myxobacterial cells. In this study, the functions of duplicate *groEL* genes in cell growth, predation feeding, development, and heat shock response were investigated. Our results demonstrate that either copy of the duplicate *groEL* genes is indispensable for the survival of *Myxococcus xanthus* DK1622. Furthermore, these studies also suggest that the products of the two *groEL* genes have played divergent roles in the social life cycle of myxobacterial cells.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
<i>M. xanthus</i>		
DK1622	Wild-type strain	15; D. Kaiser, University of Stanford
YL0301	DK1622 Δ <i>MXAN_4895</i> (<i>groEL1</i> deletion)	This study
YL0302	DK1622 Δ <i>MXAN_4467</i> (<i>groEL2</i> deletion)	This study
YL0305	DK1622::pZC4895 (<i>lacZ</i> fused to <i>groEL1</i>)	This study
YL0306	DK1622::pZC4467 (<i>lacZ</i> fused to <i>groEL2</i>)	This study
YL0307	YL0301::pSWU4895 (<i>groEL1</i> integrated at <i>attB</i> site)	This study
YL0308	YL0307 Δ <i>MXAN_4467</i> (<i>groEL2</i> deletion)	This study
YL0309	YL0302::pSW4467 (<i>groEL2</i> integrated at <i>attB</i> site)	This study
YL0310	YL0309 Δ <i>MXAN_4895</i> (<i>groEL1</i> deletion)	This study
<i>E. coli</i>		
DH5 α (λ pir)	ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacU169</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> <i>supE44</i> <i>thi-1</i> <i>gyrA</i> <i>relA1</i> λ pir	H. B. Kaplan, University of Texas
XL1-Blue MR	Δ (<i>mcrA</i>)183 Δ (<i>mcrCB</i> - <i>hsdSMR</i> - <i>mrr</i>)173 <i>endA1</i> <i>supE44</i> <i>thi-1</i> <i>recA1</i> <i>gyrA96</i> <i>relA1</i> <i>lac</i>	Stratagene
Plasmids		
pBJ113	Gene replacement vector with KG cassette, Km ^r	14; Z. M. Yang, Virginia Tech
pMiniHimar1- <i>lacZ</i>	Km ^r <i>lacZ</i>	H.B. Kaplan, University of Texas
pSWU30	Site-specific integration vector with Mx8 <i>attP</i> integration site, Tet ^r	Mignot Târn, CNRS (Centre national de la recherche scientifique)
pSL1180	Cloning vector, Amp ^r	Amersham
pSL1180-Km	1.25-kb fragment of <i>kan</i> from pColE1 inserted into SmaI of pSL1180, Amp ^r Km ^r	This study
pSW4467	2.14-kb fragment of <i>MXAN_4467</i> , with its upstream 495-bp promoter sequence, inserted into XbaI/EcoRI sites of pSWU30, Tet ^r	This study
pSW4895	2.93-kb fragment of <i>MXAN_4895</i> , with its upstream 1.28-kb promoter sequence, inserted into XbaI/EcoRI sites of pSWU30, Tet ^r	This study
pZCY11	1.56-kb HindIII/EcoRI fragment from pSL1180-Km, blunted with Klenow fragment and ligated with 4.57-kb T4 polymerase-blunted KpnI fragment from pMiniHimar1- <i>lacZ</i> , Km ^r <i>lacZ</i>	This study
pBJ4467	Upstream and downstream homologous arms of DK1622 <i>MXAN_4467</i> inserted into SmaI site of pBJ113, Km ^r	This study
pBJ4895	Upstream and downstream homologous arms of DK1622 <i>MXAN_4895</i> inserted into SmaI site of pBJ113, Km ^r	This study
pZC4467	601-bp fragment of DK1622 <i>MXAN_4467</i> , containing a stop codon, inserted into SpeI/KpnI sites of pZCY11, Km ^r <i>lacZ</i>	This study
pZC4895	685-bp fragment of DK1622 <i>MXAN_4895</i> , containing a stop codon, inserted into SpeI/KpnI sites of pZCY11, Km ^r <i>lacZ</i>	This study

MATERIALS AND METHODS

Cultures, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The *M. xanthus* strains were cultivated in Casitone-based rich-nutrient medium CTT (12) for growth assays and on TPM agar (17) for developmental assays. *E. coli* strains were routinely grown on Luria-Bertani (LB) agar or in LB broth. *Myxococcus* strains were incubated at 30°C, and *E. coli* was grown at 37°C. When required, a final concentration of 40 μ g/ml or 20 μ g/ml of kanamycin (Km) was added to the solid or liquid media.

In-frame deletion of *groEL* genes. In-frame deletion of *MXAN_4467* (*groEL2*) and *MXAN_4895* (*groEL1*) in *M. xanthus* was performed using the positive-negative KG cassettes described by Ueki et al. (34). In brief, genomic DNA from DK1622 served as the template for PCR amplification of the upstream and downstream homologous arms using *Pfu* DNA polymerase (Stratagene). The arms were fused at the BamHI site for both *MXAN_4467* and *MXAN_4895* to form the internal deletion fragments. The fragments were cloned into SmaI-digested pBJ113 to construct the deletion plasmids pBJ4467 and pBJ4895, which were then transferred by electroporation into *M. xanthus* DK1622 cells, as previously described (16). Individual Km-resistant colonies were transferred onto CTT agar plates supplemented with 1% galactose (Sigma) for the second round of screening. Deletion mutants were identified by the phenotypes of galactose resistance and kanamycin sensitivity, as well as verified by PCR. The primers for verification are listed in Table 2.

Complementary inactivation. Due to several failed attempts to delete both copies of the *groEL* gene, complemented deletion strains were constructed (20). The

groEL1 gene and its upstream 1,280-bp sequence was PCR amplified, digested with XbaI and EcoRI, whose recognition sites were incorporated into the primers, and ligated with the XbaI-EcoRI-digested site-specific integration plasmid pSWU30 to give pSW4895. After electroporation, pSW4895 was integrated at the Mx8 *attB* site in the genome of YL0301 (Δ *groEL1*) mutant, producing a *groEL1* complemented mutant, designated YL0307. The mutant was selected with 10 μ g/ml tetracycline and verified by PCR using four primers specific for the 5' and 3' ends of the *attP* and *attB* regions. Then, the mutant YL0307 was used to delete the *groEL2* gene by electroporation of pBJ4467 as described above, producing the final complemented deletion mutant YL0308. Complementary inactivation of *groEL2* was performed using a similar procedure.

Construction of the *groEL-lacZ* reporter genes. The promoterless *lacZ* gene (4.57 kb) was obtained from pMiniHimar1-*lacZ* using KpnI digestion. T4 polymerase was used to blunt the end of the fragment. Separately, the Klenow fragment (MBI Inc.) was used to blunt the ends of a HindIII-EcoRI-digested fragment (1.56 kb) from pSL1180-Km. The fragments were ligated together to produce pZCY11. A 685-bp DNA sequence including the stop codon of *groEL1* was amplified from the genome of DK1622 using KpnI- and SpeI-incorporated primers. The PCR fragments and pZCY11 were digested with KpnI and SpeI. Then, the fragments were ligated together, producing pZC4895, in which the *lacZ* gene was fused to the *groEL1* gene. A similar strategy was used to construct the *groEL2-lacZ* fusion, producing pZC4467. The *groEL-lacZ* fusion constructions were separately transferred by electroporation into *M. xanthus* DK1622, and individual kanamycin-resistant clones were selected. The mutants of the *groEL-lacZ* transcription fusion were verified by PCR amplification (primers are listed in Table 2). The expression levels

TABLE 2. PCR primers

Primer	Sequence (5'-3') ^a	Use	Expected size (bp)
4467-KO-1-for	GGCTGTGGCGGGTGGACAAGGC	Amplification of the upstream homologous arm for deletion of <i>groEL2</i>	810
4467-KO-1-rev	<u>CGCGGATCCG</u> CCTCCGAGGGCTCGGGA		
4467-KO-2-for	<u>CGCGGATCCG</u> CAACCTCTGAATCACTGGG	Amplification of the downstream homologous arm for deletion of <i>groEL2</i>	895
4467-KO-2-rev	CAGATGAGGCAGTCCGCGGTTC		
4895-KO-1-for	CGCAGGTGAACAGCTTGTCG	Amplification of the upstream homologous arm for deletion of <i>groEL1</i>	979
4895-KO-1-rev	<u>CGCGGATCC</u> ACCGCGTCGCGCAGGATGTT		
4895-KO-2-for	<u>CGCGGATCC</u> GCATCGACCCGCAAGGT	Amplification of the downstream homologous arm for deletion of <i>groEL1</i>	1,072
4895-KO-2-rev	GTCGCTTGCGGGAAAGGTCT		
4895-CIs-F	<u>CTAGTCTAG</u> AGTGGGACCCACGCCTCATA	PCR amplification of <i>groEL1</i> with its promoter	2,931
4895-CIs-R	<u>CGGAATTC</u> GGGGTGAGGGCGGACTACAT		
4467-CIs-F	<u>CTAGTCTAG</u> AACTCTGCACGATGCTCTCTC	PCR amplification of <i>groEL2</i> with its promoter	2,139
4467-CIs-R	<u>CGGGATTCT</u> CAGTAGTCCATGTCTCGCCG		
Km-for	GTGCTGACCCCGGATGAATGTCAG	PCR amplification of <i>kan</i>	1,251
Km-rev	ATCGAGCCCGGGTGGGCGAAGAA		
4467-Rp-for	<u>GGGGTACCC</u> ATCCGCACGCAGATTGACTCC	<i>lacZ</i> transcription fusion of <i>groEL2</i>	601
4467-Rp-rev	<u>GGACTAGTT</u> CAGTAGTCCATGTCTCGCCG		
4895-Rp-for	<u>GGGGTACCC</u> GACAAGGACAACACCACCATC	<i>lacZ</i> transcription fusion of <i>groEL1</i>	685
4895-Rp-rev	<u>GGACTAGT</u> GGCGGACTACATGCCCATACC		
PV-4895-Rp	GCCGAGGAGAACAAGACCAAG	Reverse primer for verification of proper integration of YL0305	2,216
PV-4467-Rp	GTCCGTCACCGAGCCGTCCT	Reverse primer for verification of proper integration of YL0306	2,182
PV-Rp-pZCY11	TTCTTCTGAGCGGGACTCTGGG	Forward primer for verification of proper integration of YL0305 and YL0306	

^a Restriction sites that were incorporated into the primers are underlined, and the added bases needed for the restriction enzyme digestion of PCR products are in italics.

of *lacZ* were visualized using X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; Sigma) as a substrate.

Growth assays. *M. xanthus* cells were inoculated in liquid CTT medium to a final concentration of 1×10^7 cells/ml. The cultures were shaken at 30°C and monitored every 4 h by measuring culture turbidity using a Klett-Summerson photoelectric colorimeter equipped with a red filter (Unic Tech, Inc., China). The growth was reported as optical density at 600 nm (OD₆₀₀) values. Assays were performed in triplicate, and the results are presented with standard deviations.

Predation and feeding assays. A predation assay was conducted according to a previously published method (1). Briefly, cultures were harvested at mid-log phase and were washed three times in 10 mM MOPS (morpholinepropanesulfonic acid; pH 7.6) buffer. *M. xanthus* and *E. coli* cells were concentrated to a final cell density of 5×10^9 cells/ml and 1×10^{11} cells/ml, respectively. Fifty microliters of *E. coli* cells was pipetted onto the plate and allowed to dry, forming a 1-cm-diameter colony. A 2- μ l aliquot of *M. xanthus* cells was then spotted onto the center of the prey *E. coli* mat, with an inoculation size of 0.15 cm in diameter. The assay was repeated three times. During a 6-day incubation, the swarming size of *M. xanthus* cells was measured and monitored every 12 h with a dissection microscope. The swarming size of *M. xanthus* cells is reported as the predation ability. The liquid feeding assay was carried out by cultivating the mutants and the wild-type strain DK1622 in CT medium, for which casein was used as the only nutrient (29).

Developmental assays. *M. xanthus* cells were harvested at mid-log phase and resuspended in TPM buffer (17) at a concentration of 5×10^9 cells/ml. Aliquots of 10 μ l were deposited onto TPM agar. The cultures were incubated at 30°C and were detected every 24 h under a dissection microscope to monitor the formation of fruiting bodies. The sporulation was measured on 5-day TPM cultures as previously described (9). The sporulation rate was calculated as the number of colonies divided by the number of inoculated cells. Assays were performed at least three times.

Heat shock assays. Mid-log-phase cultures of *M. xanthus* were harvested as described above. After a heat shock of 30 min or 60 min at 42°C, the cells were immediately serially diluted and plated on the CTT agar. After 6 days, the numbers of CFU were calculated, with untreated cells as a control.

Viability staining. Following heat treatment, the cell viability of the wild-type strain DK1622 and the mutants lacking *groEL1* or *groEL2* were measured using the LIVE/DEAD BacLight bacterial viability kit (Invitrogen Co.; L7012), as described by Nariya and Inouye (22), with minor modifications. Briefly, the cells collected from cultures were incubated at 42°C for 30 min or 60 min, followed by two washes in TM buffer. The cells were then adjusted to 5×10^8 cells per

milliliter, stained according to the manufacturer's protocol, and observed and counted under a fluorescence microscope (Nikon-TE2000s).

Quantitative real-time PCR analysis. *M. xanthus* DK1622 cells were collected from a 36-h culture and inoculated in CTT medium to a final concentration of 1×10^7 cells/ml. The culture was harvested every 6 h, and the RNA was extracted immediately using a total RNA extraction kit (Promega) by following the manufacturer's instructions. Genomic DNA contamination was removed by using a DNA-free kit (ABI). The purified RNA extract was reverse transcribed to cDNA and stored in aliquots at -70°C. Quantitative real-time PCR was performed in a Bio-Rad sequence detection system with a total reaction volume of 20 μ l, containing 250 nM primers, 10 μ l of SYBR green PCR master mix (Bio-Rad Co.), 8.5 μ l of RNase-free water (Takara), and 0.5 μ l of a 10-fold-diluted cDNA template. The PCR was conducted in the following manner: 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 15 s at 72°C. The 16S rRNA was used as the normalization signal (3). Calibration curves of *groEL1*, *groEL2*, and 16S rRNA were generated from 10-fold dilutions of genomic DNA of *M. xanthus* DK1622. The primer pairs used for each gene were as follows (forward and reverse): for *groEL1*, 5'-GAGAAGGTGGGCAAGGAAGG-3' and 5'-TCACGAAGTACGGGGACA GG-3'; for *groEL2*, 5'-ACAGCCCGATGGACCTCA-3' and 5'-TGGTCTCATCC CCGTTGG-3'; and for 16S rRNA, 5'-CGGCGTGACAAGTCCGGGTGTGAAA G-3' and 5'-CGTCTCAGCGTCAAGTACCGTCCAG-3'.

Measurement of β -galactosidase activity. β -Galactosidase-specific activity was assayed as described by Kroos et al. (18), with minor modifications. In brief, the cells were broken with a mini-beadbeater (Biospec) at a speed of 3,000 rpm. Between each agitation, the samples were cooled with ice water. The enzymatic activity of β -galactosidase was determined by using ONPG (*o*-nitrophenyl- β -galactopyranoside; Sigma) as the substrate and read at 420 nm with a SpectraMax 190 plate reader (Molecular Devices). The amount of proteins in the extracts was determined by a bicinchoninic acid (BCA) protein assay (Pierce), and the specific activity was calculated using the following formula: specific activity = $213 \times A_{420}/(\text{sample volume [ml]} \times \text{protein concentration [mg/ml]} \times \text{reaction time [min]})$.

RESULTS

Deletions of *groEL* genes in *Myxococcus xanthus*. The two *groEL* genes in *Myxococcus xanthus* DK1622 are *MXAN_4467* (*groEL2*) and *MXAN_4895* (*groEL1*). The levels of similarity of the nucleotide and amino acid sequences of the duplicate

groELs are 83% and 79%, respectively. The most significant difference between the two paralogous genes is that *groEL1* is arranged with an upstream cochaperone gene, *groES*, forming a complete bicistronic *groESL* operon. In contrast, the second *groEL* gene (*groEL2*) does not have an accompanying *groES* gene. The genetic composition and arrangement differences between the two *groEL* genes suggest they may possess divergent functions in *M. xanthus* cells.

The *groEL1* and *groEL2* genes were deleted individually, producing the mutants YL0301 and YL0302. We tried to obtain, in addition to the single mutants, a double mutant with mutations in the two *groEL* genes by either insertion or deletion mutation of *groEL1* in YL0302 or *groEL2* in YL0301. However, attempts to produce a double *groEL* mutant were unsuccessful, suggesting that a double mutant is not viable. In order to confirm that the two *groELs* could not be inactivated at the same time, complemented double-deletion mutants were constructed by site-specific integration of *groEL1* or *groEL2* (including the upstream promoter region in each case) at the Mx8 *attB* site in the genome of YL0301 or YL0302, respectively. Following the complementation, the other *groEL* gene could be deleted from their native site in the genome.

Heat shock responses of *Myxococcus groEL* mutants. Using two-dimensional gel electrophoresis, Otani et al. showed that both of the GroEL proteins in *M. xanthus* were overexpressed following a heat shock treatment (25). However, their specific roles in the heat shock response remain unclear. Visualized by using the live/dead staining method (22), more than 80% of the YL0301 cells were most likely dead (dyed red) after 30 min of a 42°C heat shock. However, approximately 80% of the YL0302 cells were determined to be alive (dyed green). In comparison, about 90% of the wild-type DK1622 cells survived following the 42°C heat treatment. The survival rates of the *groEL* mutants were further assayed by calculation of the number of CFU. After a 30-min heat shock treatment at 42°C, relative to the wild-type DK1622 cells, only 2.1% of the YL0302 cells survived, while the YL0301 cells were unviable following the 30-min heat shock. These results suggest that *groEL1* is essential for cell survival under heat shock conditions; mutating *groEL2* caused cells to be more sensitive than the wild-type strain to higher temperatures.

Growth ability of the *groEL* mutants. When cultured in CTT medium, both of the *groEL* mutants YL0301 ($\Delta groEL1$) and YL0302 ($\Delta groEL2$) demonstrated a growth curve similar to that of the wild-type strain (Fig. 1A). The generation times during the exponential growth stage were 4.06 ± 0.1 , 4.61 ± 0.1 , and 4.34 ± 0.15 h for DK1622, YL0301, and YL0302, respectively. The generation times of the mutants showed that deletion of one of the two *groELs* had minor effects on cell growth. After the exponential growth stage, both of the mutants reached nearly the same cell density as the wild-type strain DK1622. Quantitative PCR showed that expression of the *groEL1* gene increased during the first 24-h incubation in CTT medium (Fig. 1B). After 30 h of incubation, the expression of the *groEL1* gene was sharply decreased and reached the lowest level at about 48 h of incubation. Then, the expression of *groEL1* increased again in the late stationary growth stage. On the other hand, compared to that of *groEL1*, the expression of *groEL2* was relatively stable but lower than that of *groEL1* throughout the growth stage. The quantitative PCR results

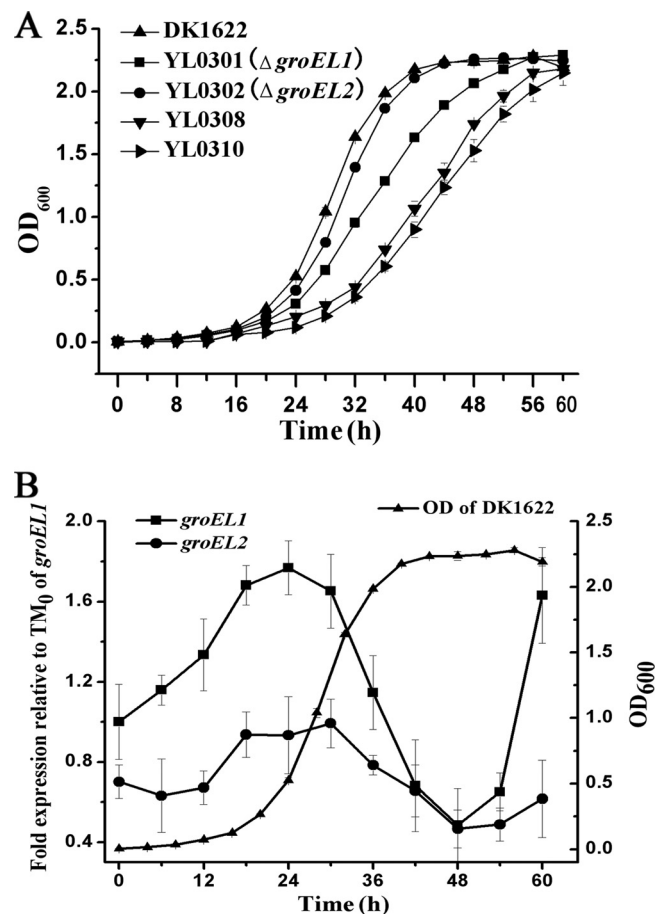


FIG. 1. (A) Growth curves of the mutants with single deletions of either *groEL1* or *groEL2* or a double deletion of *groEL1* and *groEL2* and complementation with *groEL1* (YL0308) or *groEL2* (YL0310) and of the wild-type strain DK1622. (B) Quantitative PCR analysis of the expression levels of *groEL1* and *groEL2* of the wild-type strain DK1622 in CTT medium with an overlaid representative growth curve of the wild-type strain DK1622. The values are shown as levels relative to the expression of *groEL1* at the onset of inoculation from a 36-h culture; the error bars represent standard deviations from three independent biological replicates. TM₀, time zero.

also demonstrated that, after deletion of *groEL1* (in YL0301), the expression of the *groEL2* gene increased 3.1- and 2.5-fold compared to that in wild-type strain DK1622 at 24 h and 28 h, respectively, during which the expression of *groEL2* was relatively stable in the wild-type strain (see Fig. 1B). However, expression of *groEL1* in YL0302 was similar to that in the wild-type strain DK1622.

Interestingly, after the introduction of *groEL1* or *groEL2* (with their respective promoters) into the single *groEL* deletion mutant, both strains (containing one original *groEL1* or *groEL2* gene and one transposed *groEL2* or *groEL1* gene) exhibited growth characteristics similar to those of the wild-type strain in the CTT medium. However, both complemented double-deletion mutants exhibited a significant delay in cell growth (Fig. 1A). The generation time of the *groEL1*-complemented double-deletion mutant YL0308 was 6.92 ± 0.29 h and that of the *groEL2*-complemented mutant YL0310 was 7.05 ± 0.23 h in CTT medium, respectively. Several studies have re-

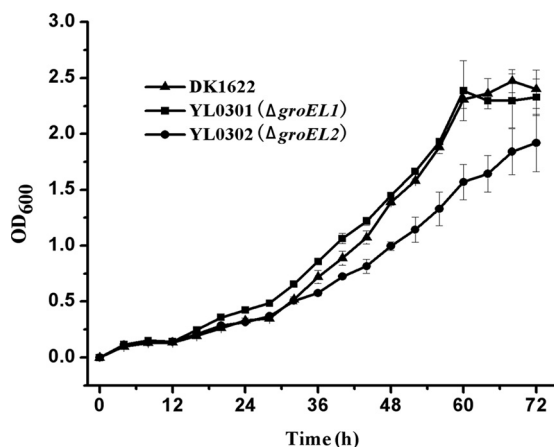


FIG. 2. Growth curves in CT medium (29) for mutants with the *groEL1* or *groEL2* deletion and for the wild-type strain DK1622.

ported reduced expression of genes at the Mx8 *attB* site (6, 19, 33). The quantitative-PCR results indicated that the levels of expression of the transposed *groEL1* and *groEL2* genes were approximately 30% to 50% and 75% to 90%, respectively, of that in the wild-type strain DK1622 at 24 h and 28 h in CTT cultures. Thus, both *groEL* genes are involved in the growth of *M. xanthus* DK1622.

Predation feeding ability of the *groEL* mutants. The presence of a single *groEL1* and *groEL2* gene was able to support the growth of *M. xanthus* cells and gave a growth curve similar to that of the wild-type strain DK1622, with hydrolyzed proteins as the substrates (CTT medium). However, it was interesting to observe a growth difference among the mutants when casein was used as the substrate (Fig. 2). The growth of both mutants and the wild-type strain was significantly delayed when casein was substituted for hydrolyzed proteins in the CTT growth medium. Unlike with growth in the hydrolyzed-protein-containing medium, YL0301 demonstrated nearly the same growth curve as the wild-type strain DK1622. The generation times of YL0301 and the wild-type strains were 7.63 ± 0.51 h and 7.43 ± 0.32 h, respectively. However, the YL0302 cells showed a growth delay under these culture conditions, exhibiting a generation time of 8.68 ± 0.44 h during the exponential growth stage, and the final cell density of YL0302 at 72 h was lower than those of both the wild-type strain and YL0301.

In a natural environment, myxobacteria usually live on solid surfaces by feeding in groups on microbial cells or their debris (27, 32). Based on bioinformatic analysis of the *Myxococcus* genome sequence, Goldman et al. speculated that the duplication of chaperone proteins may be responsible for the predatory-feeding lifestyle of *M. xanthus* cells (8). A predation feeding experiment was performed using live *E. coli* cells on an agar plate (Fig. 3). After the myxobacterial strains were spotted on the *E. coli* mat containing 1×10^{11} cells/cm², *M. xanthus* DK1622 and YL0301 cells formed visible degradation regions as early as 12 h after inoculation. In contrast, the degradation region produced by the YL0302 cells was not visible until 48 h after inoculation. These results suggest that the GroEL2 protein plays an important role in predation feeding on macromolecule substrates and *E. coli* cells. However, the GroEL1 protein appears not to exhibit the same function. Following

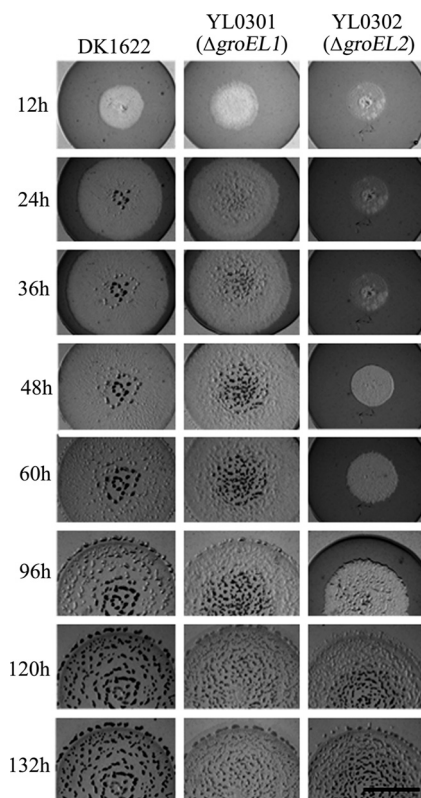


FIG. 3. Predation feeding of *M. xanthus* DK1622, YL0301 (Δ *groEL1*), and YL0302 (Δ *groEL2*) on an *E. coli* prey mat. The bar is equal to 5 mm.

exhaustion of *E. coli* cells, the developmental behaviors of YL0301, YL0302, and the wild-type strain DK1622 were similar to those on TPM medium (see below).

Development ability of the *groEL* mutants. Upon starvation, myxobacterial cells are able to form multicellular fruiting body structures, in which myxospores develop in the late stage of morphogenesis (32). When cultured on the developmental medium TPM, the *groEL1* deletion mutant YL0301 formed irregular aggregates (Fig. 4A). The sporulation ability of the *groEL1* mutant was only $3.0\% \pm 2.1\%$ of that of DK1622 and therefore was also determined to be significantly defective. Conversely, the *groEL2* deletion mutant YL0302 exhibited a phenotype similar to that of fruiting body structures of the wild-type strain. The sporulation capacity of the *groEL2* mutant was also observed to be similar to that of strain DK1622 ($98.3\% \pm 16.5\%$). In the case of predation on *E. coli* cells, after the exhaustion of food, the YL0302 cells were also able to develop normal fruiting body structures, although the developmental process was significantly delayed due to the belated predation behavior (Fig. 3). Upon exhaustion of *E. coli* cells, YL0301 was also able to form irregular aggregates, but they did not mature. In parallel to the developmental behaviors of the *groEL* mutants, expression of the *lacZ* gene transcriptionally fused after the *groEL1* gene in the wild-type strain DK1622 (YL0305) was found to be increased in the early stage (Fig. 4B). Peak levels of *lacZ* product were observed after approximately 8 h of starvation. The enzymatic activity of the *groEL1*-fused *lacZ* product sharply decreased during the next 20 h of incubation.

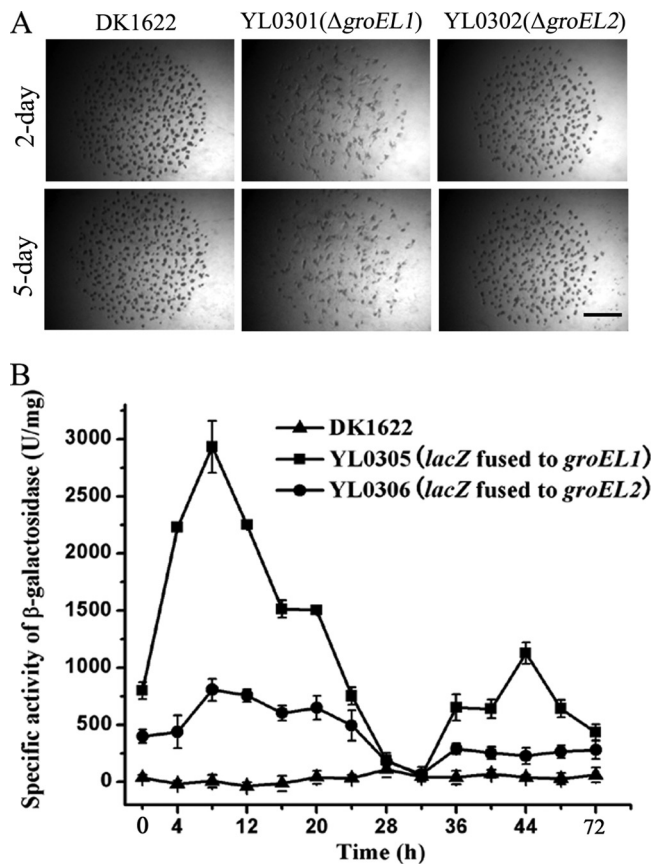


FIG. 4. (A) Development of fruiting bodies of the different *groEL* mutants and the wild-type strain DK1622 on TPM plates. The bar is equal to 1.5 mm for each panel. (B) β -Galactosidase activity in the wild-type strain DK1622, the fused *groEL1-lacZ* DK1622 mutant (YL0305), and the fused *groEL2-lacZ* DK1622 mutant (YL0306) on TPM agar. The datum at each point represents the mean of independent triplicate determinations with standard deviations.

Afterwards, expression of the *groEL1* gene significantly increased again, forming the second peak. Highly regulated expression of *groEL1* during development was correlated with our observations that *groEL1* plays important roles in the formation of fruiting bodies and myxospores. On the other hand, assayed by the β -galactosidase activity of the *groEL2-lacZ* product (YL0306), *groEL2* was expressed at much lower levels throughout development, consistent with our observation that *groEL2* is not required for the formation of fruiting bodies or myxospores.

DISCUSSION

Genome sequencing has revealed many duplicated genes within the myxobacterial genome. Many of these duplicated genes have been suggested to have evolved to accommodate the social life cycle of myxobacteria (8). The studies in this paper investigate the duplicate *groEL* genes of myxobacteria and explore how they may be involved in the social lifestyle of *Myxococcus*. The GroEL proteins typically participate in growth and in responses to various environmental stresses and many other cellular physiological processes by assisting in the

proper folding of many proteins (5). In some bacterial species, duplicate or multiple copies of the *groEL* gene have evolved, along with divergence of their functions. In most cases, one GroEL is essential, while the others are specialized in their roles to meet the requirements of different bacterial lifestyles (2, 24, 28). In *M. xanthus* DK1622, there are two *groEL* gene loci. The sequences of the duplicate *groEL* genes and their organization in the genome are significantly different, suggesting that they possess divergent cellular roles. The *groEL* deletion experiments described in this paper have indicated that either one of the duplicate *groEL* genes could support survival of the cell but that mutation of both *groEL* genes was lethal for the cell unless one *groEL* gene was complemented. These results indicate that the two GroEL proteins are partially redundant in function during growth.

Single deletions of *groEL* genes resulted in almost no observable effects on the growth of *Myxococcus* cells in CTT medium containing hydrolyzed proteins as the substrates. However, the two *groEL* genes showed divergent roles for the social life cycle of *Myxococcus*. GroEL2, but not GroEL1, was found to participate in the predation and feeding processes of the bacteria, while GroEL1, but not GroEL2, was strictly required for the fruiting body development and sporulation of *Myxococcus*. Previous studies have reported that chaperone HSP70 proteins (DnaK and its homologue) are involved in the control of S motility (35, 37). However, the deletion of a single *groEL* gene had no apparent effect on S motility (unpublished data). Thus, it appears that different chaperones are involved in different physiological processes in the social life of *Myxococcus*. Interestingly, whether or not *groEL1* and *groEL2* functioned, both genes were expressed in the growth and development stages. GroEL1 was almost always expressed at a higher level than GroEL2. However, if *groEL1* was deleted, the expression of the *groEL2* gene was increased. Thus, there is a balanced expression of the duplicate *groEL* genes in *Myxococcus* cells. There are several possibilities for the imbalance of functions and expression levels of the duplicate *groEL* genes. For instance, our experiments have suggested that deletion of the *groEL1* gene led to a higher expression level of the *groEL2* gene, possibly as compensation to support the normal growth of cells. However, expression of the *groEL1* gene was not affected by deletion of *groEL2*. It is also possible that other chaperone proteins may be able to partially share the tasks of the GroEL proteins during cell growth, especially when one of the duplicated *groEL* genes is deleted. Higher expression of GroEL1 might allow GroEL1 to participate in some more physiological processes than GroEL2 during the growth stage. However, in the development stage, no matter whether or not the expression of *groEL2* was upregulated by the deletion of *groEL1*, *groEL1* deletion led to severe developmental defects. We thus speculate that the function divergences of the duplicate *groELs* not only are probably due to the different expression levels but also are the results of their substrate specificity. In order to clearly determine the functions and collaborations of the two *groEL* genes, further investigations, such as coimmunoprecipitation studies for identification of the proteins that are folded by GroEL1 and GroEL2, are required and are currently being undertaken in our laboratory.

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