The **groEL2** gene, but not **groEL1**, is required for biosynthesis of the secondary metabolite myxovirescin in *Myxococcus xanthus* DK1622

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*Myxococcus xanthus* DK1622 possesses two copies of the **groEL** gene: **groEL1**, which participates in development, and **groEL2**, which is involved in the predatory ability of cells. In this study, we determined that the **groEL2** gene is required for the biosynthesis of the secondary metabolite myxovirescin (TA), which plays essential roles in predation. The **groEL2**-knockout mutant strain was defective in producing a zone of inhibition and displayed decreased killing ability against *Escherichia coli*, while the **groEL1**-knockout mutant strain exhibited little difference from the wild-type strain DK1622. HPLC revealed that deletion of the **groEL2** gene blocked the production of TA, which was present in the **groEL1**-knockout mutant. The addition of exogenous TA rescued the inhibition and killing abilities of the **groEL2**-knockout mutant against *E. coli*. Analysis of GroEL domain-swapping mutants indicated that the C-terminal equatorial domain of GroEL2 was essential for TA production, while the N-terminal equatorial or apical domains of GroEL2 were not sufficient to rescue TA production of the **groEL2** knockout.

**INTRODUCTION**

GroEL is a type of chaperone that is essential for cell function by assisting protein folding, transport, degradation and assembly (Fayet *et al.*, 1989; Lin & Rye, 2006; Lund, 2001, 2009; Radford, 2006; Ranson *et al.*, 1998; VanBogelen *et al.*, 1987). Because of these important roles, GroEL is ubiquitously distributed in bacteria (Goyal *et al.*, 2006). Although most species possess a single **groEL** gene, nearly 30% of sequenced bacteria contain two or more copies of **groEL** (Gould *et al.*, 2007; Goyal *et al.*, 2006; Lund, 2009). These duplicate **groEL** genes normally have distinct physiological functions. For example, in *Rhizobium leguminosarum*, which possesses three copies of the **groEL** gene, the cpn60.1 gene (**groEL1**) has been shown to be indispensable for cell growth, whereas the other two **groEL** genes can be inactivated without affecting cell viability (George *et al.*, 2004; Gould *et al.*, 2007; Rodriguez-Quinnamon *et al.*, 2005). *Sinorhizobium meliloti* contains five copies of the **groEL** gene, yet only the product encoded by **groEL1** is required for viability (Bittner *et al.*, 2007). Furthermore, there are duplicate **groEL** genes in *Mycobacterium smegmatis*; only the GroEL2 protein is necessary for cell growth, while deletion of the **groEL1** gene does not affect the normal growth of cells but prevents the biosynthesis of mycolic acid and biofilm formation (Ojha *et al.*, 2005). Similarly, in *Mycobacterium tuberculosis*, **groEL2** is essential for cell survival, while **groEL1**-knockout mutants are viable but fail to induce an inflammatory response in animal models of infection (Hu *et al.*, 2008).

Myxobacteria are Gram-negative bacteria that exhibit complex social behaviours at each stage of the life cycle (Dworkin & Kaiser, 1993; Rosenberg, 1984; Whitworth, 2007). Under starvation conditions, vegetative cells gather together to build fruiting bodies in which cells differentiate into spores (Shimkets, 1990). Myxobacterial cells feed on macromolecules and other microbial cells in groups by secreting antibiotics and hydrolytic enzymes (Anscombe & Singh, 1948; Berleman & Kirby, 2009; McBride & Zusman, 1996; Varon *et al.*, 1984). In addition, myxobacteria are a promising source of bactericidal products, as they can produce many kinds of secondary metabolites (Weissman & Müller, 2009, 2010; Wenzel & Müller, 2009). For example, the sequenced genome of *Myxococcus xanthus* DK1622 reveals 18 polyketide synthase/non-ribosomal peptide synthase (PKS/NRPS) gene clusters for secondary metabolite synthesis, at least five of which could produce antibiotic products possessing bactericidal activities besides the major secondary metabolite myxovirescin (TA) (Wenzel & Müller, 2009).

*Myxococcus xanthus* DK1622 contains two paralogous **groEL** genes, which are functionally divergent (Goldman *et al.*, 2006; Li *et al.*, 2010). The **groEL1** gene is essential for the developmental process, and its deletion causes an
obvious defect in the development and sporulation of cells. By contrast, the groEL2 gene is necessary for cell predation. Deletion of groEL2 leads to a deficiency in cell predation (Li et al., 2010). In Myxococcus xanthus DK1622, secondary metabolites and several hydrolytic enzymes were suggested as candidate tools for cell predation (Goldman et al., 2006). Recently, Xiao et al. (2011) demonstrated that TA is a major factor for predation (Xiao et al., 2011). Although the two copies of the groEL gene were previously shown to perform divergent functions in the social activities of cells, such as development and predation (Li et al., 2010), if and how the two groEL genes function in the production of secondary metabolites and bactericidal activity remain unclear. In this study, by analysing the bactericidal activity and secondary metabolites of groEL-related mutants, we demonstrated that groEL2 is required for the biosynthesis of TA, which may represent a link between groEL2 and the predation ability of Myxococcus xanthus DK1622.

**METHODS**

**Cultures, bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Myxococcus xanthus DK1622 and mutants were cultured in casitone-based, rich-nutrition medium (CTT) (Hodgkin & Kaiser, 1977) for the growth assay and on CTT agar for the predation and E. coli killing assays (Li et al., 2010). E. coli strains were routinely grown on Luria–Bertani (LB) agar or in LB broth. E. coli was grown at 37 °C, and M. xanthus DK1622 and mutants were incubated at 30 °C. For kanamycin (Km)-resistant strain cultivation, a final concentration of 20 μg Km ml⁻¹ was added to the liquid or solid medium.

**Strain construction.** Strains YL0301 (groEL1 knockout) and YL0302 (groEL2 knockout) were constructed previously (Li et al., 2010). The complementation mutants were constructed using the site-specific integration plasmid pSWU30 (Wu & Kaiser, 1996). Briefly, the groEL1 gene with a 500 bp upstream sequence was amplified, digested with XbaI and BamHI, and ligated with pSWU30 digested with XbaI and BamHI to obtain pSWU–groEL1. Similarly, the groEL2 gene with a 500 bp upstream sequence was amplified, digested with XbaI and BamHI, and ligated with pSWU30 digested with XbaI and BamHI to obtain pSWU–groEL2. The constructs pSWU–groEL1 and pSWU–groEL2 were transferred by electroporation into YL0301 and YL0302, respectively. Individual Tet colonies were screened.

To construct the TA-knockout mutant, approximately 1 kb fragments on each side of the targeted deletion areas ACP–KS–KR–ACP (acyl-CoA carrier protein–ketosynthase–ketoreductase–acyl-CoA carrier protein) coding regions of tal and ACP–KS–ACP coding regions of tal. In DK1622 were amplified with Pfu DNA polymerase. The purified upstream and downstream homologous arms were fused by overlap-PCR and cloned into SmaI-digested pBJ113, which carries the positive/negative KG cassettes (Ueki et al., 1996), to construct the deletion plasmids pBJ3938 and pBJ3936. The two plasmids were confirmed by DNA sequencing and then transferred by electroporation into Myxococcus xanthus DK1622 as described by Kashefi & Hartzell (1995). Individual Km-resistant colonies were transferred onto CTT agar plates supplemented with 1 % galactose for the second round of screening.

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<tr>
<th>Strain/plasmid</th>
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<tr>
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<tr>
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Table 1. Bacterial strains and plasmids used in this study
The deletion mutants were identified by the presence of galactose-resistant and Km-sensitive phenotypes and verified by PCR with primers flanking the deletion site (primer details are given in Table S1, available in the online Supplementary Material). Using the same protocol, the plasmid pBJ3938 was electroporated into *Myxococcus xanthus* YL0911 (AtaL) to obtain the double Δ*ataL* mutant YL0912.

**Extraction of metabolites from *Myxococcus xanthus***. Wild-type DK1622 and *groEL* mutants were grown on solid CTT medium (1% Casitone, 10 mM Tris-HCl [pH 7.6], 1 mM KHPO4, 8 mM MgSO4) for 3 days; the strains were then transferred to 50 ml of CYE liquid medium (10 mM MOPS, 10 g/l Casitone, 5 g/l yeast extract, and 8 mM MgSO4, pH 7.6) at 30 °C with shaking at 200 r.p.m. for 1 day. Then, 1% XAD-16 resin was added to the cultures, and the strains were cultured for another 2 days. After harvesting, the metabolites absorbed on the resin were extracted with methanol, and the final volume was adjusted to 2 ml for further analysis. A 50 μl aliquot of each extract was analysed by HPLC (ProStar 210; Varian) with a reversed-phase column (C18HL, 4.6 mm × 250 mm; Alltima). Water/0.025% trifluoroacetic acid (solvent A) and acetonitrile/0.025% trifluoroacetic acid (solvent B) were used as the mobile phases. The HPLC programme was as follows: 5–60% B from 0 to 5 min, 60–100% B from 5 to 20 min, 100% B from 20 to 23 min, 100–5% B from 23 to 28 min, and 5% B from 28 to 30 min. The metabolites were detected at 239 nm with a UV detector (ProStar 310; Varian). Purified TA used as reference was extracted with the same protocol.

**Bactericidal activity test**. *E. coli* DH5α was used as the indicator strain to evaluate the bactericidal activity of various mutants of *Myxococcus xanthus* DK1622. *E. coli* was mixed with LB medium (20 ml) and poured into a plate. After the plate had solidified, sterilized stainless steel tubes were placed on the surface of the LB plate, and an aliquot (25 μl) of each extract was added to each tube. The plates were incubated at 37 °C overnight (Zhang et al., 2011).

**Survival rate of *E. coli***. The survival rate assay was performed according to a previously published method with some modifications (Xiao et al., 2011). Briefly, *Myxococcus xanthus* cultures were harvested at mid-exponential phase and resuspended in TPM buffer [10 mM Tris/HCl (pH 7.6), 1 mM KHPO4 (pH 7.6), 8 mM MgSO4]. The cells were concentrated to a final density of 5 × 10⁹ cells ml⁻¹. Ten microlitres of *Myxococcus xanthus* cells was spotted onto LB solid medium and pre-incubated at 30 °C. Then, aliquots (5 μl) of *E. coli* cells (1 × 10¹¹ cells ml⁻¹) were pipetted directly on the centre of the *Myxococcus xanthus* swarm. The plates were cultured at 30 °C, and the swarms were scraped over a series of time points. The cells were serially diluted and incubated on LB solid medium to count the survival of *E. coli* cells.

**Predation assay**. The predation assay was performed according to previously published methods with some modifications (Li et al., 2010). Cultures were harvested at mid-exponential phase and were washed in 10 mM MOPS (pH 7.6) buffer. The *Myxococcus xanthus* DK1622 and *E. coli* cells were concentrated to final densities of 5 × 10⁸ and 1 × 10¹¹ cells ml⁻¹, respectively. A 50 μl aliquot of *E. coli* cells was pipetted onto a TPM plate to form a 1-cm-diameter lawn. A 2 μl aliquot of *Myxococcus xanthus* cells was spotted on top of the *E. coli* lawn. The plate was incubated at 30 °C, and the diameters of the predation zones were measured every 12 h.

**RESULTS**

*groEL2* is indispensable for bactericidal activity toward *E. coli*

To explore the killing ability of myxobacterial cells toward *E. coli*, a killing assay was performed using a series of *groEL* mutants of *Myxococcus xanthus* DK1622 (Fig. 1). The killing ability of YL0302 (groEL2 knockout) was weak compared with that of wild-type DK1622 and mutant YL0301 (groEL1 knockout). DK1622 killed nearly half of the *E. coli* cells within 6 h of co-cultivation, and only one-sixth of the *E. coli* cells were still alive after 12 h. After 12 h of co-cultivation, the survival rate was stable. If we added an extra dose of fresh myxobacterial cells, 20 μl of the inoculum (5 × 10⁹ cells ml⁻¹), at the 12 h time point, the survival rate decreased to approximately 1–2% after an extra 6 h of co-cultivation. For the mutant YL0302, the survival rate decreased steeply in the first 3 h, similar to wild-type DK1622. However, after the initial 3 h, the killing ability of YL0302 was obviously weak compared with that of DK1622. At 6 and 12 h, 80 and 60% of the *E. coli* cells were typically alive. Approximately 50% of the *E. coli* cells were dead after 18 h of co-cultivation with YL0302. While YL0302 exhibited decreased bacterial killing activity, the killing ability of YL0301 was similar to that of DK1622 (Fig. 1). We thus speculated that the chaperone *groEL2* is indispensable for bactericidal activity. To test this hypothesis, using the site-specific integration vector pSWU30, we complemented one copy of *groEL1* (named YL0906) or *groEL2* (named YL0907) into YL0302 (Wang et al., 2013). The two resulting mutants exhibited very different bactericidal activities toward *E. coli* cells. The bactericidal activity of YL0906, which contains two copies of the *groEL1* gene, was similar to that of YL0302, while the bactericidal activity of YL0907, which contains an original *groEL1* gene and a complemented *groEL2* gene, was obviously higher than that of YL0302 and YL0906 and similar to that of wild-type DK1622 (Fig. 1). Thus, *groEL2*, but not *groEL1*, is indispensable for bactericidal activity.

Fig. 1. *E. coli* survival. DK1622, wild-type strain of *Myxococcus xanthus*; YL0301, *groEL1*-knockout mutant; YL0302, *groEL2*-knockout mutant; YL0906, YL0302 complemented with *groEL1*; YL0907, YL0302 complemented with *groEL2*. The experiment was repeated three times. Error bars represent SD.
**groEL2 is essential for TA production**

To study the effect of the inactivation of *groEL* on secondary metabolite production and inhibition of Gram-negative bacteria, the secondary metabolites of different mutants were extracted and evaluated by a bactericidal assay. As shown in Fig. 2, metabolites from the wild-type strain DK1622 formed an obvious inhibition zone. In comparison with the wild-type strain, there was no obvious difference in the bactericidal activity of YL0301. Interestingly, YL0302 was associated with a greatly decreased inhibition zone. Furthermore, the zone of inhibition was recovered considerably if YL0302 was complemented with the *groEL2* gene (YL0907) but not the *groEL1* gene (YL0906). These results suggest that *groEL2*, but not *groEL1*, is responsible for the anti-Gram-negative bacterial activity of *Myxococcus xanthus* DK1622.

The total extracts of wild-type DK1622 and a series of mutants were further analysed by HPLC. One major peak in DK1622 and YL0301 with a retention time of approximately 15.9 min was not visible in YL0302. To verify that this peak was absent due to the inactivation of *groEL2*, we further analysed the extracted components of YL0906 and YL0907. The HPLC analysis revealed that the target peak was restored in YL0907, while no corresponding peak appeared in YL0906 (Fig. 3a). MS analysis revealed two obvious peaks at 624.60 and 646.60 m/z (Fig. 3b). Based on the MS results, the putative molecular mass of the compound is 623.60 ([M + H] 624.60, [M + Na] 646.60), which corresponds well with the exact molecular mass of TA, one of the main secondary compounds of *Myxococcus xanthus* DK1622 (Gaspari et al., 2005; Gerth et al., 1982, 2003). To further confirm that the compound was TA, we knocked out the TA biosynthesis genes MXAN3936 and MXAN3938 to construct the mutant YL0912. The same peak (TA) was absent in both YL0912 and YL0302 (Fig. 3a). Thus, we concluded that *groEL2* is necessary for TA production.

The absence of TA in the *groEL2* mutant causes a decreased inhibition zone and defective killing ability toward *E. coli*

Bactericidal and killing assays demonstrated that *groEL2* is important for the bacterial inhibition ability of *Myxococcus xanthus* DK1622. In addition, secondary metabolite analysis suggested that deletion of *groEL2* affected TA production. We hypothesized that the absence of TA was responsible for the decreased bactericidal activity and bactericidal ability of YL0302. To test this hypothesis, we determined if the bactericidal deficiency of YL0302 could be rescued by the addition of exogenous TA antibiotic. We used 1, 5, 10 and 20 µg TA ml⁻¹ to attempt to complement the impaired bactericidal ability of YL0302. To clearly reveal the zone of inhibition, we decreased the concentration of indicator *E. coli* cells in the TA complementation assay, which formed a larger circular zone. As shown in Fig. 4, the extract of wild-type DK1622 produced an obvious inhibition zone surrounding the stainless steel tube, whereas no inhibition zone appeared in the presence of the extract of the mutant YL0302. When 1 µg TA ml⁻¹ (final concentration) was added to the YL0302 extract, a small transparent zone appeared. As the concentration of TA increased, the size of the inhibition zone increased. The YL0302 (*groEL2* knockout) extract produced a similar zone of inhibition as DK1622 if at concentrations of exogenous TA up to 20 µg ml⁻¹. Thus, the addition of exogenous TA to the YL0302 extract restored the production of an inhibition zone, and the extent of restoration was dependent upon the amount of TA. Correspondingly, to verify the role of TA in *E. coli* killing ability, we added exogenous pure TA to CTT agar plates at a final concentration of 20 µg ml⁻¹ and conducted killing assays using YL0302 with the same protocol described previously. As shown in Fig. 1, in the presence of exogenous TA, the bactericidal ability of YL0302 was improved. These results suggest that the absence of TA is one of the key reasons for the decreased bactericidal activity and bactericidal ability of YL0302.

**Exogenous addition of TA could rescue the predation defect of YL0302**

A previous study demonstrated that *groEL2* is necessary for the predation process of *Myxococcus xanthus* DK1622 (Li et al., 2010). Several secondary and primary metabolites were suggested as candidate factors for the predation progress, such as the antibiotic TA, which was identified as a secondary metabolite with bactericidal activity, as well as numerous hydrolytic enzymes (Goldman et al., 2006). Recently, Xiao et al. (2011) found that TA could be the
factor responsible for predation in Myxococcus xanthus DK1622. Based on their work, as well as the above inhibition assays, we hypothesized that the absence of TA affected the predation ability of YL0302. To verify our hypothesis, we added pure TA to a resuspended solution of Myxococcus xanthus (5 x 10^9 cells ml^-1) to a final concentration of 20 µg ml^-1 and performed the predation assay. DK1622 completed the predation process in 48 h.

Fig. 3. (a) HPLC analysis of secondary metabolites of groEL mutants. DK1622, wild-type strain of Myxococcus xanthus; YL0301, groEL1-knockout mutant; YL0302, groEL2-knockout mutant; YL0906, YL0302 complemented with groEL1; YL0907, YL0302 complemented with groEL2; YL0912, TA biosynthesis gene-knockout mutant. (b) MS analysis of secondary metabolites; the left part is MS data: the putative molecular mass of the compound is 623.60 ([M+H] 624.60, [M+Na] 646.60); the right part is structure and chemical formula of TA.
whereas YL0302 and the TA-knockout mutants showed weak predation ability, requiring 84 h to complete predation of the 10 mm *E. coli* lawn. When 20 μg TA ml⁻¹ was added, the predation speed of YL0302 was obviously improved. Similar to DK1622, it took only 48 h for the YL0302 mutant to reach the edge of the *E. coli* lawn in the presence of exogenous TA (Fig. 5). Given that YL0302 has predation ability similar to that of the TA-knockout mutant and that exogenous TA could complement the predation speed of YL0302, the absence of the secondary metabolite TA is probably one major reason for the predation defect of YL0302.

**C-terminal equatorial domain is essential for TA production**

The TA biosynthesis genes are tightly organized in gene cluster (Simunovic *et al.*, 2006). Our previous studies demonstrated that GroEL1 and GroEL2 function divergently for substrate specificity; some secondary metabolite biosynthesis proteins, e.g. MXAN3935, coded for by a core gene for TA biosynthesis (Simunovic *et al.*, 2006), were found to be bound by GroEL2 (Wang *et al.*, 2013). The substrate specificity was determined by the apical domain and the C-terminal apical domain of the duplicated chaperonins (Wang *et al.*, 2013). We then studied TA production in domain-swapping mutants to assay the domain of GroEL2 responsible for TA production. The swapped regions included the N-terminal equatorial, apical and C-terminal equatorial regions between GroEL1 and GroEL2 (Wang *et al.*, 2013). The results showed that TA production in the *groEL2*-deletion mutant YL0302 was not reserved by GroEL1–equatorial–C<sub>GroEL2</sub> (strain YL0908, containing an original *groEL1* gene and a complemented *groEL1* hybrid with the N-terminal equatorial region of *groEL2*) and GroEL1–apical<sub>GroEL2</sub> (YL0909, *groEL1* plus *groEL1* hybrid with *groEL2* apical region). However, TA production in the strain complemented with GroEL1–equatorial–N<sub>GroEL2</sub> (YL0910, *groEL1* plus *groEL1* hybrid with *groEL2* N-terminal equatorial region) was 31.6% compared with wild-type DK1622 (Fig. 6).
Recovery efficiency is similar to the predation phenotype of the YL0910 strain (Wang et al., 2013). This indicated that the C-terminal equatorial domain of GroEL2 is specifically essential for correct folding of some TA-related proteins.

**DISCUSSION**

*Myxococcus xanthus* DK1622 has a large genome, in which there are many duplicated genes, including two copies of *groEL* (Goldman et al., 2006). The two copies of *groEL* play divergent functions in the social activities of *Myxococcus xanthus* DK1622: *groEL1* is necessary for development and sporulation, while *groEL2* is essential for the predation process (Li et al., 2010). Xiao et al. (2011) found that TA is a major factor for predation. In this study, we demonstrated that *groEL2* is necessary for the production of TA, while *groEL1* is dispensable for the production of this bactericidal antibiotic. The most likely mechanism is that some of the proteins for TA biosynthesis, such as MXAN3935 (Wang et al., 2013), are among the specific substrates of the GroEL2 chaperonin. Deletion of *groEL2* caused incorrect folding of some TA-related proteins, and thus absence of TA. Consistently, a weak bactericidal activity and a decreased *E. coli* killing ability of YL0302 (*groEL2*-deletion mutant) were verified.

The GroEL proteins have been divided into five regions based on structural characteristics and sequence conservation (Brocchieri & Karlin, 2000). Our previous studies have shown that the apical domain and the C-terminal equatorial domain are both important for their substrate specificity and functional divergency (Wang et al., 2013). In this study, we found that the C-terminal equatorial domain, which is at the bottom of the GroEL central cavity (Azem et al., 1994), is essential for TA production. However, compared with the *groEL2*-deletion mutant YL0302, the complemented strains containing *groEL1* plus *groEL1* hybrid with the *groEL2* apical or N-terminal equatorial region (YL0909 and YL0910) both improved their predation abilities (Wang et al., 2013). That is, although the C-terminal equatorial domain and the apical domain are both essential for the specific functions of GroEL2 in predation, they worked on different proteins that are involved in predation. Accordingly, we suggested that, while the C-terminal equatorial domain of GroEL2 is specifically essential for correct folding of TA-related proteins, the apical domain of GroEL2, which also functions in predation, is involved in folding of some other unidentified proteins in the predation process.

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