devI is an Evolutionarily Young Negative Regulator of *Myxococcus xanthus* Development

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ABSTRACT

During starvation-induced development of *Myxococcus xanthus*, thousands of rod-shaped cells form mounds in which they differentiate into spores. The *dev* locus includes eight genes followed by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), comprising a CRISPR-Cas system (Cas stands for CRISPR-associated) typically involved in RNA interference. Mutations in *devS* or *devR* of a lab reference strain permit mound formation but impair sporulation. We report that natural isolates of *M. xanthus* capable of normal development are highly polymorphic in the promoter region of the *dev* operon. We show that the *dev* promoter is predicted to be nonfunctional in most natural isolates and is dispensable for development of a laboratory reference strain. Moreover, deletion of the *dev* promoter or the small gene immediately downstream of it, herein designated *devI* (development inhibitor), suppressed the sporulation defect of *devS* or *devR* mutants in the lab strain. Complementation experiments and the result of introducing a premature stop codon in *devI* support a model in which DevRS proteins negatively autoregulate expression of *devI*, whose 40-residue protein product DevI inhibits sporulation if overexpressed. DevI appears to act in a cell-autonomous manner since experiments with conditioned medium and with cell mixtures gave no indication of extracellular effects. Strikingly, we report that *devI* is entirely absent from most *M. xanthus* natural isolates and was only recently integrated into the developmental programs of some lineages. These results provide important new insights into both the evolutionary history of the *dev* operon and its mechanistic role in *M. xanthus* sporulation.
IMPORTANCE

Certain mutations in the dev CRISPR-Cas system of *M. xanthus* impair sporulation. The link between development and a CRISPR-Cas system has been a mystery. Surprisingly, DNA sequencing of natural isolates revealed that many appear to lack a functional *dev* promoter, yet these strains sporulate normally. Deletion of the *dev* promoter or the small gene downstream of it suppressed the sporulation defect of a lab strain with mutations in *dev* genes encoding Cas proteins. The results support a model in which the Cas proteins DevRS prevent overexpression of the small gene *devI*, which codes for an inhibitor of sporulation. Phylogenetic analysis of natural isolates suggests that *devI* and the *dev* promoter were only recently acquired in some lineages.
**INTRODUCTION**

*Myxococcus xanthus* is a Gram-negative bacterium of the δ-proteobacteria group. Typically found in the soil, *M. xanthus* exhibits social behaviors (1). The rod-shaped cells move in groups on solid surfaces, feeding on prey bacteria and organic matter. When starved, cells modify their movements and build mounds containing thousands of individuals that differentiate into ovoid spores. The resulting mound of dormant and insult-resistant spores is called a fruiting body.

Spores within the fruiting body germinate when conditions favorable for growth are sensed, producing a swarm of vegetative cells.

The process of fruiting body development provides an excellent model to study signaling and gene regulation (2-4). It also involves cell fate determination, since not all the starving cells become spores. Some cells persist outside fruiting bodies as peripheral rods, while a majority of the population undergoes cell lysis (5). How cells adopt one of the three possible developmental fates is a major unanswered question.

Recently, the period of development during which some cells become committed to form a spore has been defined (6). Under submerged culture conditions (i.e., growing cells are sedimented, resuspended in starvation buffer, placed in a plastic container, and allowed to settle out of solution so they adhere to the bottom and form fruiting bodies) (7), cells become committed to form sonication-resistant spores at 24-30 h poststarvation (6). During this period, an increasing percentage of the cells will differentiate into sonication-resistant spores despite replacement of the overlaying starvation buffer with nutrient medium. The molecular events responsible for the observed commitment to sonication-resistant spore formation are unknown.

Expression of the *dev* operon has been believed to be a key event leading to spore formation.

The *dev* locus was identified by transposon insertion mutations that impair development at a late
stage, permitting mound formation but reducing sporulation dramatically (8-10). The mutations were in devR, which was shown to be co-transcribed with the downstream devS gene, and the operon appeared to be negatively autoregulated by devR and/or devS (10). Subsequently, the dev operon was reported to include at least three additional genes upstream of devR, and a small in-frame deletion in devT (immediately upstream of devR) delayed mound formation and reduced sporulation (11). Further work found that a small in-frame deletion in devS reduced sporulation, but allowed normal mound formation (12). Also, the mutant was defective in negative autoregulation of the dev operon, which was shown to include three more genes, for a total of eight, plus at least two repeats of a downstream CRISPR. It was recognized that the devTRS genes are embedded in a CRISPR-Cas system since the two genes upstream are similar to cas6 and cas3, while the two genes downstream are similar to cas4-cas1 (i.e., the normally separate cas4 and cas1 genes are fused) and cas2, as depicted in Figure 1A. CRISPR-Cas systems typically form small interfering RNAs to inhibit expression of plasmid and bacteriophage genes (13). The function of devTRS and the surrounding cas genes is a mystery, although it was noted that the first spacer of the CRISPR matches the bacteriophage Mx8 integrase gene, suggesting that one function of this CRISPR-Cas system might be to protect developing M. xanthus cells from lysogenization by Mx8 (12). Importantly for the work presented here, the first gene of the dev operon, formerly identified as MXAN_7266 and herein designated devI for development inhibitor, was shown to be translated and the predicted 40-residue protein does not exhibit significant similarity to any protein in the database. How mutations in devTRS impair sporulation is not completely understood, but the mutants fail to express some developmental genes normally. The devR transposon insertion mutant fails to express genes at the Ω7536 locus, where the nine-gene exo operon is located (14, 15).
Exo proteins appear to be involved in synthesis and export of spore coat polysaccharides that maintain the spore shape (15). Also involved in this step are Nfs proteins (15), and expression of the eight-gene nfs operon is reduced in the devR mutant after 18 h poststarvation (16).

Interestingly, the devT mutant mentioned above exhibits reduced transcription of fruA (11), which codes for a transcriptional activator of the dev operon (17). These observations suggest that DevT is part of a positive feedback loop involving FruA-mediated activation of dev transcription during development. Aberrant expression of developmental operons such as exo and nfs, which govern completion of spore morphogenesis, or of fruA, which codes for a key transcription factor (see below), may explain the sporulation defects of dev mutants.

In addition to FruA, transcription of the dev operon appears to require two other DNA-binding proteins that act directly. LadA binds to DNA about 350 bp downstream of the dev promoter (P_{dev}) and positively regulates transcription (17). MrpC2 appears to bind cooperatively with FruA to two sites upstream of P_{dev} (18). Binding of the two transcription factors to a site located immediately upstream of P_{dev} is necessary for expression. Binding to a site centered at about -240 relative to the transcriptional start site boosts dev expression after 24 h poststarvation, but deletion of this site does not impair sporulation under laboratory conditions.

Combinatorial regulation by MrpC2 and FruA has been documented for four other developmental genes or operons of M. xanthus (19-22) in addition to the dev operon (18). All of these genes and operons depend on C-signaling for expression during development (23-27). The mechanism of C-signaling is not completely understood, but considerable evidence suggests that the CsgA protein is cleaved at the cell surface and a 17-kDa fragment acts as the C-signal (28-34). A receptor has not been identified, but C-signaling requires cells to move into close proximity or contact (35-38), and FruA mediates the response to C-signal (39, 40). As cells...
move into nascent fruiting bodies, they come in contact (41, 42), and C-signaling is proposed to activate FruA (39). In this way, C-signaling and FruA may inform cells when they are close-packed in a nascent fruiting body. In agreement, expression of the dev operon and other C-signal-dependent genes is localized to nascent fruiting bodies (43, 44). If FruA informs cells of their location, what information does MrpC2 provide that would explain its cooperative binding with FruA? MrpC2 and a slightly longer form called MrpC are derived from the same gene by alternative translation or post-translational cleavage (45). The concentration of both proteins rises in developing cells and this is regulated by mechanisms that sense starvation (46, 47), so it was proposed that MrpC and MrpC2 inform developing cells whether they are still starving (20). In agreement, MrpC and MrpC2 are rapidly degraded if nutrients are added to developing cells (6). This halts expression of the dev operon. Hence, combinatorial regulation by MrpC, MrpC2, and FruA appears to ensure that the dev operon and other C-signal-dependent genes are fully expressed only if cells are starving and close-packed in nascent fruiting bodies. If these conditions are met, a simple model would be that full expression of the dev operon permits normal expression of the exo and nfs operons (and perhaps other genes) needed for sporulation. This simple model predicts that the dev operon plays an indispensable role in sporulation.

Here, we report the discovery that a large majority of M. xanthus natural isolates are not only highly polymorphic in P_{dev} relative to the lab strain DK1622, but also entirely lack devI. Yet the strains lacking devI develop normally (48). Since a devS mutant was known to overexpress the dev operon, we hypothesized that overexpression of devI inhibits sporulation. Our results support this notion and suggest that the small protein DevI functions in a cell autonomous fashion. We conclude that devS and devR are dispensable for sporulation, and we discuss the possibility that the entire dev operon is dispensable in light of our findings.
MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. Strains, plasmids, and primers used in this study are listed in Table S1. The construction of plasmids used to make deletions of $P_{dev}$ or $devI$, or to introduce a premature stop codon into $devI$ (designated $devI$-STOP), is described in detail below.

In each case, DNA fragments were initially generated using PCR with chromosomal DNA from *M. xanthus* strain DK1622 as template. Either the initial DNA fragments or plasmids derived from them served as template for sequence overlap extension (SOE) PCR. Desired SOE PCR fragments were cloned into pCR2.1-TOPO (Invitrogen) as described by the manufacturer, the DNA sequence was verified, and the fragment was subcloned into pBJ113. Alternatively, the SOE PCR fragment was cloned into pBJ113 and the sequence was verified. The resultant plasmids were transformed into the appropriate *M. xanthus* strains using electroporation (49), with outgrowth in CTT liquid prior to plating on CTT agar (see below for description of media).

The plasmids contain a positive selection marker for kanamycin (Km) resistance and a negative selection marker, *galK*, which confers galactose sensitivity (50). Transformants with a single crossover were obtained by plating on CTT agar supplemented with 40 μg/ml Km. These transformants were grown in CTTYE liquid without selection to allow a second crossover and then plated on CTT agar supplemented with 2.5% galactose to select *M. xanthus* that had lost the plasmid. Isolates that had successfully completed allelic exchange were identified by colony PCR in the case of deletion mutants or by colony PCR and sequencing in the case of $devI$-STOP mutants.

To delete $P_{dev}$ (i.e., from -38 to +19 relative to the transcriptional start site), primers PD-Eco-Fwd and PD-OL-Rev, and PD-OL-Fwd and PD-Bam-Rev, were used to generate initial PCR

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fragments, which were then used as template in SOE PCR with primers PD-Eco-Fwd and PD-
Bam-Rev. The resultant fragment was cloned into pCR2.1-TOPO to generate pRR08 and
subcloned into pBJ113 using restriction sites EcoRI and BamHI to generate pRR09. Isolates of
*M. xanthus* containing the deletion were identified by colony PCR using primers Dev c.p F 2 and
Dev c.p R 1, or Dev c.p F 3 and Dev c.p R 1.

To delete *devI*, primers SO-Eco-Fwd2 and SO-Int-Rev2, and SO-Int-Fwd2 and SO-Bam-
Rev2, were used to generate initial PCR fragments that were cloned into pCR2.1-TOPO to
generate pRR011 and pRR012, respectively. Primers SO-Eco-Fwd2 and SO-OL-Rev were used
with pRR011 as template, and primers SO-OL-Fwd and SO-Bam-Rev2 were with pRR012 as
template, to generate PCR fragments used as template in SOE PCR with primers SO-Eco-Fwd2
and SO-Bam-Rev2. The resultant fragment was cloned into pBJ113 using restriction sites EcoRI
and BamHI to generate plasmid pRR013. Isolates of *M. xanthus* containing the deletion were
identified by colony PCR using primers Dev c.p F 3 and Dev c.p R 2, or Dev c.p F 4 and Dev c.p
R 2.

To create *devI*-STOP, primers 7266-Eco-Fwd and 7266-Int-N-Rev, and 7266-Int-N-Fwd and
PD-Bam-Rev, were used to generate initial PCR fragments, which were then used as template in
SOE PCR with primers 7266-Eco-Fwd and PD-Bam-Rev. The resultant fragment was cloned
into pCR2.1-TOPO to generate pRR020 and subcloned into pBJ113 using restriction sites EcoRI
and BamHI to generate plasmid pRR021. Isolates of *M. xanthus* containing *devI*-STOP were
identified by colony PCR using primers Dev c.p F 3 and Dev c.p R 2, and sequencing of the PCR
product.

Plasmids used for complementation analysis (pPVO1152 and pPVO833) were electroporated
into the appropriate *M. xanthus* strains and transformants were selected on CTT agar.
supplemented with 40 μg/ml Km. These plasmids integrate into the *M. xanthus* chromosome at the Mx8 phage attachment site.

**Genome sequencing and bioinformatics.** In brief, the whole genome sequences of 16 *M. xanthus* natural isolates were inferred using Illumina MiSeq-resequencing of 300-bp paired-ends at an average coverage of ~110-fold, with a minimum of 56-fold (Table S2). Read quality was assessed and inspected using FASTQC v0.11.2 (S. Andrews, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and quality and length trimmed accordingly using Trimmomatic v.0.32 (51). We refer to these 16 isolates as ‘WGS’ isolates. Only paired reads were retained and combined, and, along with an outgroup (*Myxococcus fulvus* HW-1, (52)) mapped against the fully sequenced reference genome DK1622 (53) using the program RealPhy v1.06 (54). We inferred the phylogeny based on ~4.5 Mbp of aligned orthologous sequence using RAxML v8 (55), assuming the General-Time-Reversal (GTR) model of sequence evolution, and branches were assessed for statistical support relying on 1000 bootstrap replicates.

Each isolate’s paired reads were assembled using SPADES v3.1.1 (56) and each raw assembly was corrected by mapping reads back to the assembly using bresseq v0.24rc5 (57). The quality of the assemblies was assessed and summarized using QUAST v2.3 (58). The region spanning the end of MXAN_7267, intergenic regions up- and downstream of devI, as well the start of *cas6* were extracted from the DK1622 genome and used in a BLAST search against these assembled genomes. We found that the region where *devI* is present in DK1622 is absent from all but two of the 16 WGS isolates. It is unlikely that the absence of this region in any isolate is due to misassembly, as both conserved regions next to *devI* in DK1622 were always found on the same contig, flanking a short unrelated sequence ~100 bp in length.
A 419 bp segment of MXAN_7267 was present in all but three of the fully sequenced natural isolates. Thus, we inferred the maximum likelihood phylogeny of this region assuming the GTR model of sequence evolution in MEGA v6.06 (59). The tree was statistically assessed based on 1000 bootstrap replicates.

**Growth and development.** *Escherichia coli* strains containing plasmids were grown at 37°C in Luria-Bertani (LB) medium that was supplemented with 50 μg/ml of Km sulfate as needed. *M. xanthus* strains were grown at 32°C in CTTYE liquid medium (1% Casitone, 0.2% yeast extract, 10 mM Tris-HCl [pH 8.0], 1 mM KH2PO4-K2HPO4, 8 mM MgSO4, [final pH 7.6]) with shaking, except outgrowth after electroporation was in CTT liquid medium (same as CTTYE except no yeast extract). CTT agar (1.5%) was used for growth on solid medium and was supplemented with 40 μg/ml of Km sulfate as required. Fruiting body development under submerged culture conditions was done using MC7 (10 mM morpholinepropanesulfonic acid [MOPS, pH 7.0], 1 mM CaCl2) as the starvation buffer in either 6-well plates for observation of developmental phenotypes and sporulation analysis, or in petri dishes for collection of samples to be used for RNA analysis as described previously (6, 7). Upon incubation at 32°C, cells adhere to the bottom of the plate and undergo development. For sample collection, cells were scraped from the plate bottom using a sterile 1-ml plastic pipette tip, placed in a 15-ml centrifuge tube, and mixed as described previously (6). The methods used to observe cellular shape change and measure sonication-resistant spores and mature spores have been described recently (6). Briefly, to observe cellular shape change, samples were left undisturbed for 5-10 min to allow cell aggregates to settle and cell aggregates were removed for microscopic observation. To measure sonication-resistant spores, samples were sonicated and ovoid spores were counted microscopically using a Neubauer counting chamber. To quantify mature spores, samples were
heat- and sonication-treated, then serially diluted and spread on CTT soft (1.0%) agar plates, and colonies were counted after incubation at 32°C for 5-7 days. For the experiment shown in Figure 6C, 200 colonies obtained on CTT soft agar plates were toothpicked onto CTT agar supplemented with 40 μg/ml of Km, to determine whether the colonies arose from mature spores produced by the DK1622 derivative (bearing a Km-resistance marker) upon co-development with the ΔdevS mutant.

**Microscopy.** Fruiting body images at low-resolution were obtained with a Leica Wild M8 microscope equipped with an Olympus E-620 digital camera. High-resolution images of cell aggregates were obtained with an Olympus BX51 microscope using a DIC filter and a 100X oil-immersion objective, and equipped with an Olympus DP30BW digital camera.

**RNA extraction and analysis.** Cells were harvested, and RNA was extracted and analyzed by quantitative reverse transcription-PCR (qRT-PCR) as described previously (6). Briefly, developing cells were treated with a solution to inhibit RNase activity, scraped from the bottom of the plate, aspirated into a 15-ml centrifuge tube, flash frozen in liquid nitrogen, and stored at -80°C. After thawing and centrifugation, RNA was extracted using the hot-phenol method and digested with DNase I. RNA was used to synthesize cDNA with reverse transcriptase and either random primers or a specific reverse primer. Control reactions omitted reverse transcriptase prior to the qPCR reaction. A standard curve for each set of qPCR reactions was generated using DK1622 chromosomal DNA. Gene expression was quantified using the relative standard curve method (user bulletin 2; Applied Biosystems). 16S rRNA was used as the internal standard. The primer sequences used for qPCR are listed in Table S1.

**RESULTS**

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A large segment of the DK1622 dev promoter region is absent from most developmentally proficient natural isolates. To characterize potential diversity in the cis-regulatory region of the dev operon, a region encompassing the dev promoter was sequenced in 26 natural isolates of M. xanthus and one isolate of M. fulvus, either by whole genome sequencing (WGS, Fig. 1B, Table S2) or targeted Sanger sequencing (Fig. S1). Surprisingly, most isolates were found to lack an ~400 bp region near the DK1622 dev promoter beginning just downstream of the MXAN_7267 stop codon. Instead, the majority of strains carry a shorter, unrelated sequence (<100 bp). The polymorphic region includes the promoter of the DK1622 dev operon (12), the sequence of which is absent from the majority of strains, as well as the devI gene (formerly identified as MXAN_7266) and the 5’ end of cas6 (MXAN_7265), both of which are also absent from the majority of strains. Of 26 M. xanthus natural isolates examined, only five were found to carry a dev promoter region similar to that of DK1622 (two WGS isolates (Fig. 1B) and three Sanger-sequences isolates (Fig. S1)). The M. fulvus isolate also lacked the DK1622-version of the highly polymorphic region. Three WGS isolates appear to lack the entire sequenced region including all of MXAN_7267 (Fig. 1B).

Twelve of the natural isolates examined here were previously shown to develop normally and produce large numbers of spores upon starvation (DK801, DK816, DK836, DK897, Mxx15, Mxx41, Mxx104, Mxx144 (48), Mxx38 and Mxx143 (60), and KF328c11 and MC359c15 (61)). For example, in experiments performed in submerged starvation culture (7) as part of this study, DK897 forms dark fruiting bodies and produces nearly as many spores as does DK1622 (Fig. S2). Surprisingly, of the twelve developmentally proficient strains, only three (Mxx15, Mxx38 and Mxx143) were found to carry the longer DK1622-version of the dev promoter region that includes devI (although the region sequenced for strain Mxx38 did not extend to the end of devI.
The high variability of the dev promoter region and the absence of devI in developmentally proficient strains was surprising since null mutations in devT (11), devR (10), or devS (12) have been reported to impair sporulation.

**The DK1622 version of the polymorphic region is evolutionarily derived.** The long DK1622 dev-region sequence is absent from i) the M. fulvus outgroup, ii) the most deeply rooted M. xanthus isolate (Indiana MC359c15) and iii) a majority of sub-clades within the whole-genome based phylogeny of WGS isolates (Fig. 1B). We therefore infer that the common ancestor of all WGS strains carried a short variant of this region lacking devI and part of cas6 and that the long DK1622-like version of this region is an evolutionarily derived insertion.

However, because DK1622 and the two WGS isolates carrying a DK1622-like long version do not cluster in a monophyletic sub-clade (Fig. 1B), we infer that this segment has migrated across genomic lineages via recombination. More generally, recombination events around the dev region are reflected by the topological incongruities between the whole-genome based phylogeny of the WGS strains and a phylogeny based on only a conserved segment of MXAN_7267.

**dev genes under control of the DK1622 dev promoter are required for normal development in submerged culture.** As noted above, mutations in the dev operon have been reported to impair sporulation when development was performed under a variety of conditions (10-12). We examined dev mutants under our submerged culture conditions. Aggregates were formed by the ∆devS and ∆devR mutants, but not by the ∆devT mutant (Fig. 2A). However, the aggregates formed by the ∆devS and ∆devR mutants failed to darken. To determine whether cells were changing shape from rods to ovoid spores, samples were collected at 24, 48, 72, and 96 h poststarvation, gently dispersed, and observed microscopically. The ∆devS and ∆devR mutants exhibited no change in cellular shape at 24 or 48 h, but at 72 and 96 h some cells...
exhibited shape change (Fig. 2B). In contrast, the parent strain DK1622 from which the mutants were derived exhibited shape change as early as 24 h, and many cells formed ovoid spores by 48 h. On the other hand, cellular shape change was not detectable in the ΔdevT mutant even at 96 h, consistent with its failure to form fruiting bodies. In agreement with our observations of gently dispersed samples, all three dev mutants failed to exhibit spores upon microscopic examination of developmental samples collected at 24, 30, or 36 h poststarvation and subjected to sonication (data not shown), whereas DK1622 formed many sonication-resistant spores by 30 h and the number increased about 10-fold by 36 h as shown previously (6). Quantification of mature spores (i.e., heat- and sonication-resistant, and able to germinate and form a colony) at 72 h poststarvation revealed that the dev mutants form far fewer spores than DK1622 during submerged culture development (Fig. 2C). We conclude that the dev mutants exhibit severe developmental defects under our conditions.

Deletion of the dev promoter in DK1622 does not impair sporulation. How do natural isolates of M. xanthus develop normally despite mutations in the dev promoter region that presumably eliminate expression of downstream genes? One possible explanation is that natural isolates contain compensatory mutations. Therefore, we examined the effect of deleting the dev promoter in DK1622. DNA spanning from -38 to +19 of the dev promoter region was deleted by double recombination. The resulting ΔPdev mutant was tested for development under submerged culture conditions. By 72 h, fruiting bodies had darkened normally, similar to DK1622 (Fig. 3), and the number of mature spores was comparable to that observed for DK1622 (Fig. 2C).

We next considered the possibility that another promoter is responsible for transcription of the dev genes in the natural isolates and in the ΔPdev mutant. We performed qRT-PCR on RNA extracted from the ΔPdev mutant and from DK1622 at 24 h poststarvation using primers that...
amplify a region from within devT near its 3' end to within devR near its 5' end. Very little dev
transcript was detected in the ΔP_{dev} mutant compared with DK1622 (Fig. 4A).

Taken together, our results show that the known promoter of the DK1622 dev operon is
dispensable for sporulation and this does not appear to be due to another promoter directing
transcription of dev genes.

**Deletion of P_{dev} or devI from DK1622 restores sporulation of ΔdevS and ΔdevR mutants.**
The large DK1622 dev promoter-region sequence absent in most natural isolates spans
downstream DNA so that devI is also missing (Fig. 1B and S1). This gene is expressed during
development (12) and is predicted to code for a 40-residue peptide with no significant similarity
to known proteins. We hypothesized that devI codes for an inhibitor of development, which is
overexpressed in dev mutants, since DevS has been shown to negatively autoregulate
transcription from P_{dev} (12). This model predicts that deletion of P_{dev} or devI would suppress the
developmental defect of a devS mutant. To begin testing the model, the ΔP_{dev} mutation described
above was introduced into the ΔdevS, ΔdevR, and ΔdevT mutants. As predicted, the ΔP_{dev} ΔdevS
double mutant appeared to develop normally, forming darkened fruiting bodies (Fig. 3) and a
similar number of mature spores as DK1622 (Fig. 2C) at 72 h poststarvation. The ΔP_{dev} ΔdevR
double mutant also appeared to develop normally (Fig. 3 and 2C), suggesting that DevR, like
DevS, negatively autoregulates transcription from P_{dev}. Indeed, we found that dev transcripts are
about 10-fold more abundant in both the ΔdevR mutant and the ΔdevS mutant as compared with
DK1622 at 24 h poststarvation (Fig. 4B). Interestingly, the ΔP_{dev} ΔdevT double mutant failed to
form fruiting bodies (Fig. 3) and formed very few mature spores (Fig. 2C), similar to the ΔdevT
single mutant. This suggests that DevT plays a different role in development than DevS and
DevR.
To test whether deletion of \textit{devI} would exhibit a similar pattern of suppression of \textit{dev} mutants, we first made an in-frame deletion of the coding sequence by double recombination in \textit{M. xanthus} DK1622. The resulting \textit{ΔdevI} mutant appeared to develop normally, indicating that \textit{devI} is not required for formation of darkened fruiting bodies (Fig. 3) or mature spores (Fig. 2C).

The \textit{ΔdevI} mutation was likewise introduced into the \textit{ΔdevS}, \textit{ΔdevR}, and \textit{ΔdevT} mutants. As predicted by the model, the \textit{ΔdevI ΔdevS} double mutant appeared to develop normally (Fig. 3 and 2C). As expected since DevR appears to play a similar role in development as DevS, the \textit{ΔdevI ΔdevR} double mutant also appeared to develop normally. Consistent with DevT playing a different role in development, the \textit{ΔdevI ΔdevT} double mutant failed to develop normally.

Taken together, our results support a model in which DevS and DevR negatively autoregulate transcription from \textit{P\textsubscript{dev}}, preventing overexpression of \textit{devI} that would inhibit sporulation. Also, DevT appears to play a different role in development than DevS and DevR.

\textbf{Complementation of the \textit{ΔdevI ΔdevS} double mutant requires a large segment of DNA.}

To further explore the role of \textit{devI} in sporulation, we focused on the \textit{ΔdevI ΔdevS} double mutant in which sporulation was restored. We performed a complementation test in order to determine whether loss of \textit{devI} expression was responsible for restoring sporulation of the double mutant, rather than an unanticipated effect of the \textit{ΔdevI} in-frame deletion on expression of other genes in the \textit{dev} operon, or the effect of a mutation(s) elsewhere in the chromosome that might have occurred during construction of the double mutant (although we considered this possibility unlikely since several isolates of the double mutant exhibited restored sporulation). If loss of \textit{devI} expression was responsible for restoring sporulation of the double mutant, then our model predicts that overexpression of \textit{devI} in the double mutant (due to loss of DevS-mediated negative autoregulation of transcription from \textit{P\textsubscript{dev}}) would inhibit sporulation. Previous work showed that...
high-level expression from $P_{dev}$ in a $\Delta devS$ mutant requires considerable upstream and downstream DNA (12). Figure 5A shows a map of the $dev$ promoter region and two different DNA segments that were fused transcriptionally to $lacZ$ and tested for expression during development after ectopic integration in the chromosome of the $\Delta devS$ mutant (12). Each segment contains the $devI$ gene and has the same 5’ end, but the 3’ ends differ. The fusion with the 3’ end farther downstream exhibited higher expression, suggesting that downstream $cis$-acting DNA elements enhance transcription from $P_{dev}$. Each fusion was integrated ectopically at the same site in the chromosome of the $\Delta devI \Delta devS$ double mutant. The two resulting strains were induced to undergo development under submerged culture conditions. The strain with the larger segment (-934 to +833) exhibited complementation, failing to form darkened fruiting bodies and forming very few mature spores, similar to the $\Delta devS$ single mutant (Fig. 5B and 5C). The strain with the smaller segment (-934 to +219) formed darkened fruiting bodies and a large number of mature spores, although not as many spores as DK1622, suggesting partial complementation. We conclude that full complementation of the $\Delta devI \Delta devS$ double mutant requires a large segment of DNA, perhaps to allow overexpression of $devI$ from $P_{dev}$.

To quantify $devI$ expression from the ectopically-integrated segments, RNA was isolated at 24 h poststarvation from the strains described above and qRT-PCR was performed using primers designed to amplify the region from +36 to +159 relative to the transcriptional start site of the $dev$ operon. These primers were chosen because the amplicon overlaps the region deleted by the $\Delta devI$ mutation. As expected, the $\Delta devI \Delta devS$ double mutant lacking an ectopically-integrated segment produced only a low background signal with these primers (Fig. 5D). In support of the hypothesis that $devI$ overexpression inhibits sporulation, the double mutant with the larger segment (-934 to +833) integrated ectopically exhibited a signal nearly 6-fold higher than
also the double mutant with the smaller segment (-934 to +219), which formed a large number of mature spores (Fig. 5C), exhibited a signal similar to DK1622 (Fig. 5D). Interestingly, the ΔdevS single mutant exhibited a signal about 3-fold higher than DK1622 with these primers (Fig. 5D), whereas the mutant signal was about 10-fold higher than DK1622 with primers farther downstream (+237 to +440) (Fig. 4B). Although we do not understand this difference (see Discussion), both results indicate a higher level of dev transcripts in the ΔdevS mutant, consistent with our model that devI overexpression in the absence of DevS negative autoregulation inhibits sporulation.

Introduction of a premature stop codon into devI restores sporulation of the ΔdevS mutant. Previous work showed that devI is translated (12), suggesting that its predicted 40-residue small protein product may be functional. However, we could not rule out the possibility that the ΔdevI mutation removes a structural feature in the mRNA that accounts for our results described above. To distinguish between these possibilities, we introduced a single-bp insertion that creates an in-frame stop codon near the 5' end of devI. This mutation, designated devI-STOP, was expected to prevent synthesis of the small protein but have little effect on the mRNA structure. The devI-STOP mutation was introduced by double recombination into DK1622 and the ΔdevS mutant. Under submerged culture conditions, both the devI-STOP single mutant and the devI-STOP ΔdevS double mutant appeared to develop normally, forming darkened fruiting bodies (Fig. 6A) and a similar number of mature spores as DK1622 (Fig. 6B) at 72 h poststarvation. The results were similar to those for the ΔdevI single mutant and the ΔdevI ΔdevS double mutant, for which the number of mature spores was measured in parallel as a control (Fig. 6B). We conclude that introduction of a premature stop codon into devI restores
sporulation of the \( \Delta \text{devS} \) mutant. This result strongly suggests that the small protein DevI must be made in order for sporulation to be inhibited in the \( \Delta \text{devS} \) mutant.

**DevI appears to act in a cell-autonomous manner.** Many small proteins are proteolytically cleaved and a resulting peptide is secreted to act as an extracellular signal (62). To test whether overexpression of devI in the \( \Delta \text{devS} \) mutant produces an extracellular peptide that inhibits development, the \( \Delta \text{devS} \) mutant was induced to undergo submerged culture development and the overlaying conditioned starvation buffer after 18, 24, or 30 h was used to replace the overlaying buffer of comparably-treated DK1622 at 18 or 24 h poststarvation. At 30 and 36 h poststarvation, the DK1622 cultures were photographed and then samples were collected to measure sonication-resistant spore formation. Conditioned buffer from the \( \Delta \text{devS} \) mutant did not prevent formation or darkening of fruiting bodies by DK1622 (Fig. S3), and spores formed normally (Fig. 7A). Hence, we could not detect an inhibitory peptide in the conditioned buffer.

It remained possible that DevI or a peptide derived from it acts extracellularly, but is not released from the cell surface. To test this possibility, we co-developed a derivative of DK1622 containing a kanamycin resistance marker (that has no effect on development) with the \( \Delta \text{devS} \) mutant at ratios of 1:1 or 1:10. In both cases, darkened fruiting bodies appeared to form normally (Fig. 7B). The number of mature spores was measured at 96 h poststarvation, with serial dilutions plated as usual on nonselective growth medium. The number of spores was reduced 2-fold in the 1:1 mixture and 10-fold in the 1:10 mixture (Fig. 7C), suggesting that the kanamycin-resistant DK1622 derivative formed spores normally in the mixtures. In agreement, when colonies that had grown on nonselective medium in the sporulation assays were tested, all 200 from each mixture grew on medium containing kanamycin. Hence, only the DK1622 derivative formed spores in the mixtures. The \( \Delta \text{devS} \) mutant neither formed spores nor inhibited
sporulation of the DK1622 derivative. We conclude that DevI likely acts cell-autonomously, since it does not appear to act extracellularly.

DISCUSSION

Our results dramatically change the view of the role of the dev operon in M. xanthus development. The simple notion that DevRS proteins are indispensable for sporulation must be revised. Our findings support a model in which DevRS proteins negatively autoregulate expression of devI, whose small protein product inhibits sporulation if overexpressed, at least in the minority of strains similar to DK1622 that carry devI. The predicted 40-residue DevI protein appears to act in a cell-autonomous manner. In the absence of devI or the dev promoter, the DevRS proteins are dispensable for sporulation. This new view of the role of the dev operon raises many questions for further study. Foremost, it raises the question of why negative regulation of development by DevI – and concomitant negative regulation of devI by the DevRS proteins – first evolved and spread among a minority of natural M. xanthus lineages.

Dispensability of dev. Is the entire dev operon dispensable for development? Two of our results suggest that it is, at least under certain circumstances. First, several natural isolates have multiple mutations that presumably inactivate the dev promoter (Fig. 1B and S1), yet they sporulate normally (48, 60, 61), and this was confirmed in the case of strain DK897 under our submerged culture conditions (Fig. S2). Second, deletion of the dev promoter in the laboratory strain DK1622 did not appear to impair development (Fig. 2C and 3), and we showed that very little dev transcript is produced in this strain (Fig. 4A). Paradoxically, our results suggest that devT is indispensable. Unlike the ΔdevS and ΔdevR mutants, the ΔdevT mutant failed to form nascent fruiting bodies in submerged culture (Fig. 2A), and its developmental defect was not

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suppressed by deletion of the dev promoter or devI (Fig. 2C and 3). This suggests that DevT plays a different role in development than DevS and DevR, and that DevT is indispensable. However, the ΔdevT mutant has a small in-frame deletion of codons 408-502 out of 570 total (11), so it is possible that the altered DevT protein interferes with development. We think this is unlikely since very little of the altered protein would be expressed in the ΔP_dev ΔdevT double mutant, which exhibited a similar developmental defect as the ΔdevT single mutant (Fig. 2C and 3). We considered the possibility that the ΔdevT mutant we obtained had acquired a second-site mutation that affects development, so we obtained the same mutant from a different source. Its developmental defect was indistinguishable from the original mutant. This does not rule out the possibility of a second-site mutation at the time the mutant was created or prior to its distribution to different sources. To further address the role of DevT, we plan to reconstruct the mutant with the small in-frame deletion and to construct a mutant with an in-frame deletion of nearly the entire gene. If both mutants exhibit the same developmental defect as the ΔdevT mutant examined herein, it would strengthen the suggestion that DevT is indispensable for development. Also, it would imply that very little dev transcript is needed to make sufficient DevT for development, since the ΔP_dev mutant appeared to develop normally (Fig. 2C and 3) despite producing very little dev transcript (Fig. 4A).

Our results clearly show that DevS and DevR are dispensable for formation of spore-filled fruiting bodies under submerged culture conditions in the laboratory, provided that the dev promoter or devI is deleted (Fig. 2C and 3). Why, then, are devI and the dev promoter present in strain DK1622 and some natural isolates but not in most isolates (Fig. 1B and S1)? Phylogenetically, it seems clear that the Myxococcus developmental program first evolved without devI, which was recently incorporated into the developmental pathways of a minority of
lineages by mechanisms and causes that remain to be determined. (However, it is plausible that
the spread of devI across lineages is phage-mediated.) If devI was first incorporated into the M. xanthus developmental program because it was adaptive, perhaps the original benefit of dev operon regulation was niche-dependent. Our results do not rule out the possibility that DevS and DevR, as well as other products of the dev operon such as DevI and Cas proteins, exert effects on the timing or magnitude of developmental events that affect fitness in some environments, since we only measured the number of mature spores at 72 or 96 h poststarvation.

Related to the issue of the dispensability of the dev operon in M. xanthus, it is worth noting that the DevTRS proteins are not conserved in most other myxobacteria for which a genome sequence is available. Within the order Myxococcales, M. xanthus is in the suborder Cystobacterineae, based on phylogenetic analysis of 16S rRNA sequences (63, 64). Within this suborder, Stigmatella aurantiaca and the non-fruitering Anaeromyxobacter dehalogens lack devTRS genes (63), as do Myxococcus fulvus and Corallococcus coralloides (65). Interestingly, in the Sorangiineae suborder of the Myxococcales, Sorangium cellulosum has devTRS genes in the context of a CRISPR-Cas system, similar to M. xanthus (63). Even so, S. cellulosum lacks most genes in signal transduction pathways important for M. xanthus fruiting body formation (63, 65). We searched the NCBI database for orthologs of the M. xanthus DevTRS proteins using blastp. As expected, we found many orthologs (especially for DevR) since these proteins have been found in CRISPR-Cas systems in diverse bacteria (see below). However, among the myxobacteria, we found only the S. cellulosom orthologs described previously (63) and a DevR ortholog in Myxococcus stipitatus (NCBI Reference Sequence: WP_015353452.1). Using these orthologs and blastp to search “The Myxobacteria (taxid:29)”, we found a DevS ortholog in Chondromyces apiculatus (GenBank: EYF07493.1) with significant similarity (3e^{-16}) to S.
cellulosum DevS. The database contained full genome sequence representation of seven other myxobacteria in addition to those mentioned above, yet no other proteins with an E value $<e^{-4}$ were identified. Hence, most myxobacteria for which a genome sequence is available do not code for DevTRS orthologs.

The lack of dev genes in most myxobacteria and the great heterogeneity of the dev promoter region among natural isolates of M. xanthus revealed here (Fig. 1B and S1) echo a broader theme of plasticity in the genetic elements that underlie proficient myxobacterial development. This theme emerges from analyses that span in breadth from inter-specific comparative genomic analysis (63, 65) to fine-scale comparison of laboratory-evolved conspecifics that differ by only a few mutations (66, 67). Several other loci important for development in M. xanthus strain DK1622 are also absent from other fruiting myxobacteria, suggesting that there are many genetically viable pathways to proficient fruiting body development (63, 65). More broadly, the intraspecific variability in the functional gene content of the dev region speaks to the general question regarding the degree to which conserved morphological themes can be achieved by variable developmental pathways (68).

**Function of the products of the dev operon.** DevS and DevR are involved in negative autoregulation of the dev operon, but the mechanism is unknown. Analysis of transposon insertion mutations in devR (that could have a polar effect on expression of the downstream devS gene) provided an early indication that devRS are required for negative autoregulation of dev expression (10). Subsequently, a devS mutant with an in-frame deletion was shown to overexpress (relative to DK1622) lacZ reporter fusions to the dev promoter (12). The overexpression ranged from 1.4- to 6.4-fold, depending on the upstream and downstream boundaries of the promoter region tested, suggesting that DevS-mediated negative autoregulation...
depends on long-range interactions between upstream and downstream cis-regulatory elements. Here, we found that dev transcripts overaccumulated 3- to 10-fold in the devS mutant compared with DK1622 (Fig. 4B and 5D), depending on the position of the primers used for qRT-PCR. This suggests that DevS-mediated negative autoregulation depends in part on premature transcription termination and/or differential stability of parts of the long dev transcript. Importantly, we also found that dev transcripts overaccumulated in a devR mutant with an in-frame deletion (Fig. 4B). Moreover, the devR and devS mutants were indistinguishable with respect to suppression by mutations in the dev promoter or devI (Fig. 2C and 3). We conclude that both DevS and DevR are required for negative autoregulation of dev expression.

As mentioned above, the M. xanthus DevTRS proteins are similar to proteins found in CRISPR-Cas systems in diverse bacteria. Understanding of these systems has progressed rapidly in recent years owing to interest in their function as RNA-based adaptive immune systems and their application as tools for genetic engineering (69). Because the dev operon includes a cas3 gene, it is classified as a Type I CRISPR-Cas system (70). Our blastp analysis of the M. xanthus DevT protein revealed similarity to Cas8a1 proteins, which are signature proteins of subtype I-A systems (70). In these systems, Cas8a1 is the large subunit of the CRISPR-associated complex for antiviral defense (Cascade). The Cascade of subtype I-A systems typically include three repeat-associated mysterious proteins (RAMPs) with an RNA recognition motif (RRM). The three RAMPs are Cas5, Cas6, and Cas7 (71). M. xanthus DevS and DevR are orthologs of Cas5 and Cas7 proteins, respectively (70). Hence, the M. xanthus DevTRS proteins likely are part of the Cascade of this system, which also is expected to include the Cas6 ortholog (Fig. 1A) based on analogy with better-characterized Cascades of other Type I systems (69). In these systems, Cas6 is an endoribonuclease that cleaves pre-CRISPR RNA to produce short, mature CRISPR...
RNA (crRNA). The crRNA and Cas6 assemble with other Cas proteins (e.g., DevTRS in this case), forming the Cascade. Typically, the crRNA targets the Cascade to viral or plasmid DNA, and the Cas3 ortholog (Fig. 1A) is recruited and activated to cleave the target DNA. Since the first unique insert between repeats in the CRISPR downstream of the Cas2 ortholog is transcribed as part of the dev operon and matches the 3’ end of the bacteriophage Mx8 intP gene, which codes for the viral integrase, it was proposed that a function of the dev CRISPR-Cas system is to protect developing M. xanthus from lysogenization by Mx8 (12). Further, it was proposed that the dev CRISPR-Cas system might autoregulate and/or regulate other developmental genes by targeting mRNA. The unique insert between the 23rd and 24th repeats of the CRISPR was found to be complementary (with a few mismatches) over a stretch of nearly 80 nucleotides to the sense strand of MXAN_7283, which codes for a Cas protein of a different CRISPR-Cas system. This raised the possibility of “crosstalk” regulation between different CRISPR-Cas systems. At the time, there was no precedent for involvement of CRISPR-Cas systems in gene regulation. Since then, several examples have emerged, although details of the molecular mechanisms remain unclear (72, 73). Of potential relevance is the finding that subtype III-B CRISPR-Cas systems have been shown to target RNA rather than DNA (74, 75). The Cas protein MXAN_7283 mentioned above appears to be part of an incomplete subtype III-B CRISPR-Cas system that includes eight genes from MXAN_7283 to MXAN_7276, based on our blastp analysis of the predicted protein sequences. The system is incomplete since it lacks Cas1 and Cas2 orthologs. These proteins are necessary to add unique inserts to the CRISPR in a process called “spacer acquisition” (69, 70). Nevertheless, the CRISPR downstream of MXAN_7276 has 52 unique inserts or spacers. How were these acquired? Using blastn, we found that the repeats of this CRISPR are very similar to the repeats of the dev CRISPR (Fig.
S4A). Also, the sequences downstream of the last cas gene of each operon are very similar (Fig. S4B). These sequences precede the first repeat of each CRISPR. The similarities suggest that the CRISPR downstream of MXAN_7276 may derive from the dev CRISPR, since the dev operon includes Cas1 and Cas2 orthologs (Fig. 1A). We performed a blastn search with each unique insert of the CRISPR downstream of MXAN_7276, but we found no significant matches in the M. xanthus genome that would suggest a role in gene regulation. Neither did we find significant matches to bacteriophage or plasmid DNA. Hence, the target(s) of this subtype III-B CRISPR-Cas system are unknown.

In addition to possibly targeting bacteriophage Mx8 DNA and possibly regulating MXAN_7283 of the subtype III-B CRISPR-Cas system as mentioned above, DevS and DevR negatively autoregulate dev expression (12) (Fig. 4B), but the mechanism is unknown. RAMPs like DevS and DevR may have endoribonuclease activity, or they may recruit such an enzyme, leading to degradation of a target mRNA (71-73). Alternatively or in addition, binding of Cascade to mRNA might block its translation (72, 73). In any case, negative autoregulation mediated by DevS and DevR (and possibly DevT, see above) is crucial to prevent overexpression of the small gene devI in strains similar to DK1622 that carry devI.

Our findings support a model in which the small protein DevI acts cell-autonomously to inhibit sporulation if overexpressed. Introduction of a single-bp insertion to create a premature stop codon in devI suppressed the sporulation defect of a ∆devS mutant (Fig. 6). This strongly suggests that the small protein DevI inhibits sporulation if overexpressed. We cannot rule out the possibility that the single-bp insertion we made in devI changes the mRNA structure in a way that leads to suppression, but we think it is unlikely. We tested the possibility that a peptide derived from DevI acts extracellularly to inhibit sporulation, but obtained no evidence for such a
model. If the predicted 40-residue DevI protein acts cell-autonomously, how might it inhibit sporulation? Small proteins have been shown to affect diverse processes including spore formation, cell division, enzyme activity, transport of ions and macromolecules across membranes, and signal transduction (76). In *Bacillus subtilis*, two small proteins affect endospore formation. SpoVM is a 26-residue amphipathic protein that associates with the convex membrane at the forespore surface and is required for assembly of the spore coat (77-80). CmpA is a 37-residue protein that also localizes to the forespore surface and appears to inhibit cortex formation (81). Overproduction of CmpA delays sporulation until the small protein is removed by a post-translational mechanism. Cortex is a modified peptidoglycan whose synthesis is proposed to be coupled to the initiation of spore coat assembly by CmpA.

Overproduction of DevI in *M. xanthus ΔdevS and ΔdevR* mutants could likewise inhibit a step in peptidoglycan modification that must occur during morphogenesis of rod-shaped cells into oval spores. DevI does not exhibit significant similarity to CmpA or to any predicted protein using tblastn. The central portion of DevI could form an amphipathic α-helix (Fig. S5) that mediates membrane association. Most small proteins function at a membrane (76). Whether DevI localizes to the membrane, whether it over-accumulates in ΔdevS and ΔdevR mutants as we propose, and how DevI inhibits sporulation are interesting questions for future research.

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REFERENCES


**FIGURE LEGENDS**

**FIG 1** Map of the *M. xanthus* laboratory strain DK1622 dev operon and structural evolution of the dev promoter region among natural lineages. (A) Map of the DK1622 dev operon and expanded view of the dev promoter region. The top part depicts the eight genes of the dev operon, which also includes at least two repeats of the downstream CRISPR. The expanded view shows the region of some natural isolates that was subjected to Sanger sequencing (sequences are shown in Fig. S1). This region includes the dev promoter and the small gene devI. Numbers are relative to the transcriptional start site (+1) and a bent arrow indicates the direction of transcription. (B) Structure and evolution of the dev promoter region. The leftward part shows phylogenetic relationships among natural isolates inferred from ~4.5 Mbp of orthologous sequence. *M. fulvus* HW-1 (52) served as the outgroup. Bootstrap values supporting branch inferences are shown near each branch. The center part shows the structure of the dev promoter region. Black boxes highlight orthologous regions shared among natural isolates and the reference strain DK1622. Thin lines depict DK1622 sequence regions that are absent from the respective genomes, either because there is no sequence at all in the corresponding region or the sequence that is present is non-orthologous to that of DK1622. The rightward part shows a phylogenetic tree of a conserved partial segment of MXAN_7267 highlighting recent evolutionary history of the orthologous coding region upstream of the dev promoter. Bootstrap values larger than 60% are shown at the respective nodes. Filled circles indicate strains identified by the horizontally corresponding strain in the leftward phylogeny, whereas open circles identify strains found at a different horizontal level in the leftward phylogeny. Note that the topological structure of MXAN_7267- and whole genome-based trees are divergent.
Branches of the MXAN_7267 tree were rotated around nodes to maximize horizontal alignment to the genome-based phylogeny, but these rotations did not alter tree structure. The asterisk on the branch leading to Chihaya 20 represents the most likely branch position of DK897 in the MXAN_7267 tree, based on the available sequence information for DK897 (Fig. S1), which covered significantly less of the conserved partial segment of MXAN_7267 than for the other strains and therefore was not used in the tree construction.

**FIG 2** Development of dev mutants under submerged culture conditions. (A) Fruiting body formation by the laboratory strain DK1622 and the indicated dev mutants. Dark fruiting bodies are observed at 96 h into development of DK1622 (an arrow points to one), but not the dev mutants. Bar, 100 μm. Similar results were observed in at least two biological replicates. (B) Cellular shape change. Developing cultures were subjected to gentle dispersion and examined microscopically at the indicated times. Photos show densely packed cell aggregates presumed to be nascent fruiting bodies. Arrows point to round or ovoid cells. Bar, 5 μm. Similar results were observed in at least two biological replicates. (C) Sporulation. Samples were harvested at 72 h into development for measurement of mature spores. Values (log_{10}) are the average of at least three biological replicates, and error bars represent one standard deviation from the mean.

**FIG 3** Development of ΔP_{dev} and ΔdevI mutants. Fruiting body formation by the laboratory strain DK1622 and the indicated mutants under submerged culture conditions. Arrows point to dark fruiting bodies observed at 72 h poststarvation for DK1622 and several of the mutants. Bar, 100 μm. Similar results were observed in at least two biological replicates.
FIG 4  Levels of dev transcripts. (A) Comparison of the laboratory strain DK1622 with the ΔP_dev mutant. At 24 h poststarvation under submerged culture conditions, cultures were harvested, RNA was isolated, and the RNA was subjected to qRT-PCR analysis using primers Pdev-T-F and Pdev-T-R designed to amplify the region from +4811 to +4940 relative to the transcriptional start site of the dev operon. (B) Comparison of DK1622 with the ΔdevS and ΔdevR mutants. Culture conditions were as described for (A). RNA was subjected to qRT-PCR analysis using primers PdevF-2 and PdevR-3 designed to amplify the region from +237 to +440 relative to the dev transcriptional start site. For (A) and (B), values are the average of three technical replicates for at least three biological replicates and are reported relative to DK1622. Error bars indicate one standard deviation from the mean.

FIG 5  Complementation of the ΔdevI ΔdevS double mutant. (A) Map of the dev promoter region and fragments used for complementation. Boxes indicate genes. Numbers are relative to the transcriptional start site (+1) and a bent arrow indicates the direction of transcription. Lines below the map indicate fragments used for complementation. (B) Fruiting body formation by the laboratory strain DK1622 and the indicated mutants under submerged culture conditions. Endpoints of complementing segments relative to the dev transcriptional start site are indicated in parentheses. Arrows point to dark fruiting bodies observed at 72 h poststarvation for DK1622 and several of the mutants. Bar, 100 μm. Similar results were observed in at least two biological replicates. (C) Sporulation. Samples were harvested at 72 h into development for measurement of mature spores. Values (log_{10}) are the average of at least three biological replicates, and error bars represent one standard deviation from the mean. (D) Levels of dev transcripts. At 24 h poststarvation under submerged culture conditions, cultures were harvested, RNA was isolated,
and the RNA was subjected to qRT-PCR analysis using primers 7266-F and 7266-R designed to amplify the region from +36 to +159. Values are the average of three technical replicates for at least three biological replicates and are reported relative to DK1622. Error bars indicate one standard deviation from the mean.

**FIG 6** Effects of a premature stop codon in devI. (A) Fruiting body formation by the laboratory strain DK1622 and the indicated mutants under submerged culture conditions. devI-STOP indicates the single-bp insertion that creates an in-frame stop codon. Arrows point to dark fruiting bodies observed at 72 h poststarvation. Bar, 100 μm. Similar results were observed in at least two biological replicates. (B) Sporulation. Samples were harvested at 96 h into development for measurement of mature spores. Values (log_{10}) are the average of at least three biological replicates, and error bars represent one standard deviation from the mean.

**FIG 7** Effect of conditioned starvation buffer from the ΔdevS mutant on development of the laboratory strain DK1622 and effect of co-development of the ΔdevS mutant with DK1622. (A) Overlaying conditioned starvation buffer from the ΔdevS mutant at 18, 24, or 30 h under submerged culture conditions was used to replace the overlaying buffer of comparably-treated DK1622 at 18 or 24 h poststarvation (e.g., 24-18 means the conditioned overlay from ΔdevS at 24 h was used to replace the overlay of DK1622 at 18 h). Samples collected at 30 and 36 h poststarvation (left and right bars, respectively, for each replacement regimen) were sonicated and spores were quantified microscopically using a Neubauer counting chamber. Values (log_{10}) are the average of at least three biological replicates. Error bars represent one standard deviation from the mean. (B) Effect of co-development on fruiting body formation. A derivative of strain
DK1622 with a kanamycin resistance marker (DK1622 Kmr), the ΔdevS mutant, and mixtures of the two strains at the indicated ratios, were subjected to starvation under submerged culture conditions. Arrows point to dark fruiting bodies observed at 96 h poststarvation. Bar, 100 μm.

Similar results were observed in at least two biological replicates. (C) Effect of co-development on sporulation. Samples were harvested at 96 h for the measurement of mature spores. Values (log₁₀) are the average of at least three biological replicates, and error bars represent one standard deviation from the mean.
FIG 5

A

MXAN_7267
-934 -93 +1 +43 +165 +367 +833 +219 +833
cas6
devI

B

DK1622
ΔdevS
ΔdevIΔdevS

ΔdevIΔdevS (-934 to +219)
ΔdevIΔdevS (-934 to +833)

C

Log Colony-forming units/ml

0 1 2 3 4 5 6 7 8

DK1622 ΔdevS ΔdevIΔdevS ΔdevIΔdevS (-934 to +219) ΔdevIΔdevS (-934 to +833)

D

Relative dev transcript level

0 1 2 3 4 5 6 7

DK1622 ΔdevS ΔdevIΔdevS ΔdevIΔdevS (-934 to +219) ΔdevIΔdevS (-934 to +833)
FIG 6

A

DK1622  \(\Delta\text{devI}\)  devI-STOP

\(\Delta\text{devS}\)  \(\Delta\text{devI}\Delta\text{devS}\)  devI-STOP \(\Delta\text{devS}\)

B

<table>
<thead>
<tr>
<th></th>
<th>Log Colony-forming units/ml</th>
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<tr>
<td>DK1622</td>
<td>7</td>
</tr>
<tr>
<td>(\Delta\text{devI})</td>
<td>6</td>
</tr>
<tr>
<td>devI-STOP</td>
<td>7</td>
</tr>
<tr>
<td>(\Delta\text{devS})</td>
<td>6</td>
</tr>
<tr>
<td>(\Delta\text{devI}\Delta\text{devS})</td>
<td>7</td>
</tr>
<tr>
<td>devI-STOP (\Delta\text{devS})</td>
<td>7</td>
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</tbody>
</table>
FIG 7

A

Log Sonication-resistant cells/ml

DK1622 ΔdevS 18-18 24-18 30-18 18-24 24-24 30-24

DK1622 Km$^r$:ΔdevS (1:1) DK1622 Km$^r$:ΔdevS (1:10)

B

DK1622 Km$^r$ ΔdevS

DK1622 Km$^r$:ΔdevS (1:1) DK1622 Km$^r$:ΔdevS (1:10)

C

Log Colony-forming units/ml

DK1622 Km$^r$ DK1622 Km$^r$:ΔdevS (1:1) DK1622 Km$^r$:ΔdevS (1:10) ΔdevS