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Operon Required for Fruiting Body Development in *Myxococcus xanthus*

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We have used mutational analysis to identify four genes, *MXAN3553, MXAN3554, MXAN3555,* and *MXAN3556,* constituting an operon that is essential for normal fruiting body development in *Myxococcus xanthus*. Deletion of *MXAN3553*, which encoded a hypothetical protein, resulted in delayed fruiting body development. *MXAN3554* was predicted to encode a metallopeptidase, and its deletion caused fruiting body formation to fail. Inactivation of *MXAN3555*, which encoded a putative NtrC-type response regulator, resulted in delayed aggregation and a severe reduction in sporulation. Fruiting bodies also failed to develop with the deletion of *MXAN3556*, another gene encoding a hypothetical protein.

Keywords: Myxobacteria, fruiting body development, *Myxococcus xanthus*

Under starvation conditions, the Gram-negative soil bacterium *Myxococcus xanthus* develops multicellular fruiting bodies replete with environmentally resistant spores [28]. Fruiting bodies are developed in *M. xanthus* through the cooperative activities of about 10^5 cells with an orderly expression of genes that is regulated by intercellular signaling. Genetic studies suggest that at least five extracellular signals (A–E) are involved in the development of *M. xanthus* fruiting bodies [6, 9].

Several histidine protein kinases and NtrC-type response regulators, which are components of the bacterial two-component signal transduction system, are essential for normal fruiting body development [31]. For example, *espA*, *espC*, *todK*, *rodK*, *redC*, and *redE* encode putative histidine protein kinases that appear to be involved in the timings of aggregation and sporulation [4, 11, 15, 21, 22]. Null mutations of these genes cause early sporulation, which is similar to a phenotype characteristic of a strain that overexpresses the CsgA protein. The genes *actB* [8], *mrpB* [29], *spdR* [10], *crdA* [13], *nla4*, *nla6*, *nla18*, and *nla28* [2] encode NtrC-type response regulators essential for normal fruiting body development. In particular, *actB* controls the timing and production levels of C-signals [8], a null mutation in *mrpB* blocks aggregation and sporulation [29], and the disruption of *crdA* delays development [13]. Furthermore, *nla18* mutant cells are defective in the production of both the intracellular starvation signal ppGpp and the A-signal [5]. Mutation of the *spdR* gene suppresses the developmental defects of a *bsgA* mutant [10].

In the present study, we report an operon encoding another NtrC-type response regulator that is essential for fruiting body development in *M. xanthus*. This operon also encodes a metallopeptidase and two hypothetical proteins.

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Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was cultured in Luria–Bertani (LB) media, and *M. xanthus* cultured vegetatively in CYE [9]. Fruiting body development was initiated by placing a 20-μl spot of a solution containing 5×10^9 cells/ml on a CF plate with 1.5% agar [3] and incubating this at 32°C.

DNA Manipulation and Sequence Analysis

DNA manipulation was performed using standard protocols [25]. Oligonucleotides were synthesized at Bioneer Co., Korea, and DNA was sequenced at Solgent Co., Korea. The Basic Local Alignment Search Tool (BLAST) was used for homology searches [1]. The Simple Modular Architecture Research Tool (SMART) [26] and CD-Search [18] were used to analyze conserved protein domains.

Plasmid Construction

The pCR2.1 derivatives pDH103, pDH105, pDH106, and pDH107 carried internal PCR fragments of the genes *MXAN3555*, *MXAN3557*, *MXAN3556*, and *MXAN3554*, respectively. The oligonucleotide primers used to amplify the PCR fragments are listed in Table 2. pDH110 was used to create an in-frame deletion of *MXAN3555*, and

Table 1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant feature	Source or references
Strains		
M. xanthus		
DZ2	Wild type	[3]
KYC505	DZ2::pDH103, *MXAN3555-103*	This work
KYC507	DZ2::pDH105, *MXAN3557-105*	This work
KYC508	DZ2::pDH106, *MXAN3556-106*	This work
KYC509	DZ2::pDH107, *MXAN3554-107*	This work
KYC545	DZ2 Δ*MXAN3555-110*	This work
KYC734	DZ2 Δ*MXAN3557-114*	This work
KYC735	DZ2 Δ*MXAN3556-115*	This work
KYC749	DZ2 Δ*MXAN3554-116*	This work
KYC750	DZ2 Δ*MXAN3553-118*	This work
E. coli		
DH5α	Host for cloning	Invitrogen
Plasmid		
pCR2.1	Cloning vector, Kan^R	Promega
pDH103	pCR2.1 carrying an internal PCR fragment of *MXAN3555*	This work
pDH105	pCR2.1 carrying an internal PCR fragment of *MXAN3557*	This work
pDH106	pCR2.1 carrying an internal PCR fragment of *MXAN3556*	This work
pDH107	pCR2.1 carrying an internal PCR fragment of *MXAN3554*	This work
pDH110	Derivative of pKY480 used to create the Δ*MXAN3555* mutant	This work
pDH114	Derivative of pKY480 used to create the Δ*MXAN3557* mutant	This work
pDH115	Derivative of pKY480 used to create the Δ*MXAN3556* mutant	This work
pDH116	Derivative of pKY480 used to create the Δ*MXAN3554* mutant	This work
pDH118	Derivative of pKY480 used to create the Δ*MXAN3553* mutant	This work
pKY480	Cloning vector, $SacB^+$, Km^R	[4]

was constructed as reported previously [16]. Primers BN1 and BN2 were used to amplify an N-terminal PCR fragment of *MXAN3555*, and primers BC1 and BC2 were used to amplify a C-terminal PCR fragment of *MXAN3555*. The two resultant PCR products, and primers BN1 and BC2, were then used to amplify the final PCR product carrying an *MXAN3555* in-frame deletion. The product was digested with BamHI and XhoI, and the final PCR fragment ligated into pKY480 to generate pDH110. Four additional plasmids, pDH114, pDH115, pDH116, and pDH118, were generated for in-frame deletions of *MXAN3557*, *MXAN3556*, *MXAN3554*, and *MXAN3553*, respectively. The oligonucleotide primers used to generate these plasmids are listed in Table 2.

RT–PCR

Total RNA was isolated from cells grown under vegetative and developmental conditions with the SV total RNA Isolation System (Promega, U.S.A.). Reverse transcription (RT)–PCR was performed using the Reverse Transcription System (Promega, U.S.A.). A set of DNA oligonucleotide primers, 5'-GCATGTTGGACAGTGGTCAG-3' and 5'-GGCATGTCCAAGGAAGTCAG-3', was used to amplify a region from the middle of *MXAN3557* to the middle of *MXAN3556*. Another set of DNA oligonucleotide primers, 5'-CACCGCATCTAC GAGAAGAC-3' and 5'-GGTGACGTCGAAGCCGTCCC-3', was used to amplify a region from the middle of *MXAN3556* to the middle of *MXAN3555*.

Creation and Characterization of Mutants

Plasmid insertions and in-frame deletions of genes were performed by the aforementioned methods [4]. Fruiting bodies were observed with a Nikon SMZ1000 stereomicroscope, and individual cells and spores observed with a Nikon Eclipse E600 phase-contrast microscope. Heat-resistant spores were counted on CYE plates after inactivating vegetative cells by exposing harvested cells to 50°C for 30 min and sonication.

RESULTS AND DISCUSSION

Identification of *MXAN3555*, an NtrC-Type Response Regulator Encoder

Several research groups have shown that response regulators, such as *actB* [8], *crdA* [13], *mrpB* [29], *spdR* [10], *nla4*, *nla6*, *nla18*, and *nla28* [2], are essential for the normal development of fruiting bodies in *M. xanthus*. However, some response regulators have not yet been characterized. We addressed this in the present study and found 12 relevant genes in the genomic sequence of *M. xanthus* DK1622: open reading frames (ORFs) with locus tag numbers *MAXN1189*, *1279*, *1349*, *3555*, *3711*, *4261*, *4787*, *4977*, *5083*, *5313*, *5995*, and *7143*. Five of these genes, *MAXN1189*, *3555*, *4261*, *4977*, and *7143*, were predicted to encode NtrC-type response regulators carrying a receiver domain, an ATPase domain, and a DNA-binding domain. The other seven genes, *MAXN1279*, *1349*, *3711*, *4787*, *5083*, *5313*, and *5995* appeared to encode response regulators consisting of a receiver domain and a DNA-binding domain only.

We generated plasmid insertion mutants of each of the 12 genes, to determine if their inactivation resulted in developmentally defective phenotypes. These mutants were incubated in media for inducing fruiting bodies. Whereas 11 of the mutants formed mature fruiting bodies within 72 h, one of them, KYC505, failed to do so. After 96 h, KYC505 had produced less than 0.2% of the amount of spores produced by wild types. The mutant KYC505 was generated by inserting plasmid pDH103 into the ORF with locus tag number *MXAN3555* (Fig. 1). The insertion of plasmids can have polar effects, blocking the expression of downstream genes within an operon. Thus, it is not certain whether the phenotype of an insertion mutant represents an inactivation of the gene at the point of

Table 2. Oligonucleotide primers used to create mutants.

Mutation	Forward primer (5'-3')	Reverse primer (5'-3')
MXAN3557 insertion	CGCCGAGGAGAAGGAGCA (1N)	GGTGAGCGGGTGCGACTC (1N)
MXAN3556 insertion	CTGACTTCCTTGGACATGC (AN)	TAGATGATGGGGATGGTCTT (AC)
MXAN3555 insertion	GGAGGTGCTCGGAACCAT (BN)	TGATCTTGATGAACGGCTTG (BC)
MXAN3554 insertion	AACCTTCCCACACTCGGT (CN)	CGCTGGTAGAGCACCTCC (CC)
MXAN3557 deletion	GACCTCGAGGTAGACGGGAATGAAC-GAGC (1N1)	ACAGGATCGTACTGCGACCGT-CACGTCGTCCAAGTCACTGT (1N2)
	ACGGTCGCAGTACGATCCTGTGGTG-GCGGTGGTTCCGGCAG (1C1)	GACGGATCCACGTCCAGTGGCATGT-TGTG (1C2)
MXAN3556 deletion	GACCTCGAGGCATGTTGGACAGTGGT-CAG (AN1)	ACAGGATCGTACTGCGACCGTGGCAT-GTCCAAGGAAGTCAG (AN2)
	ACGGTCGCAGTACGATCCTGT-GAACGTCGCTAGTTCCGGT (AC1)	GACGGATCCAGGGCTTGGTGATG-TAGTCG (AC2)
MXAN3555 deletion	GACCTCGAGCACCGCATCTAC-GAGAAGAC (BN1)	ACAGGATCGTACTGCGACCGTGGT-GACGTCGAAGCCGTCCC (BN2)
	ACGGTCGCAGTACGATCCTGTGA-CACCACACCAGACGGGC (BC1)	GACGGATCCGCTCACCGATAT-GCGCTCCA (BC2)
MXAN3554 deletion	GACCTCGAGCCGCTCGGTTGCT-TCAAATC (CN1)	ACAGGATCGTACTGCGACCGTAACT-GCGTTGGGCATCTGCG (CN2)
	ACGGTCGCAGTACGATCCTGTAACA-CAGGCCGCAGCTTCGC (CC1)	GACGGATCCCGCAATCCGGGTGAT-GTCCG (CC2)
MXAN3553 deletion	GACCTCGAGAACACAGGCCGCAGCT-TCGC (DN1)	ACAGGATCGTACTGCGACCGTCG-CAATCCGGGTGATGTCCG (DN2)
	ACGGTCGCAGTACGATCCTGTCT-CAACCCCAACTTCGACTG (DC1)	GACGGATCCGGGCACCTGTCT-GAGCTGG (DC2)

insertion or the inactivation of downstream genes by polar effects. To resolve this, we created an in-frame deletion mutant of the ORF *MXAN3555*, from which amino acids 30–461 were deleted. The resultant mutant, KYC545, had a phenotype similar to that of the insertion mutant; it failed to form mature fruiting bodies within 72 h (Fig. 2) and produced only 1.4×10^4 spores/spot after 96 h (vs. 2.0×10^7 spores/spot for the wild-type strain DZ2) (Table 3). This shows that *MXAN3555*, encoding a putative NtrC-type response regulator, is essential for normal fruiting body development in *M. xanthus*.

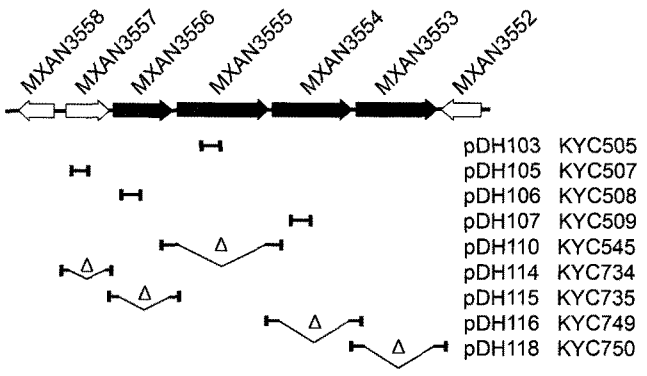


Fig. 1. A physical map of the genes and plasmids used to create mutants.
The slanted labels are ORFs with locus tag numbers corresponding to the genomic DNA sequence of *M. xanthus* strain DK1622 (CP000113).

Characterization of the Operon Carrying *MXAN3555*

Sequence analysis revealed that four ORFs near *MXAN3555* would be transcribed in the same direction as this gene; these were *MXAN3557*, *3556*, *3554*, and *3553* (Fig. 1). *MXAN3558* was predicted to be transcribed divergently from the upstream *MXAN3557* (Fig. 1). On the other hand, *MXAN3552* appeared to be transcribed convergently with the downstream *MXAN3553* (Fig. 1). *MXAN3557* and *MXAN3556* were separated by 46 bp, *MXAN3556* and *MXAN3555* by 30 bp, *MXAN3555* and *MXAN3554* by 76 bp, and *MXAN3554* and *MXAN3553* by 96 bp. There could have been differences between the DNA sequences of the DZ2 strain used in this study and DK1622, the genomic sequence of which was stored in the database. To investigate this, we determined the DNA sequence of these ORFs, *MXAN3553–MXAN3557*, in the DZ2 strain. However, no differences from the DK1622 strain were found.

We investigated the role of the other four ORFs, *MXAN3553*, *3554*, *3556*, and *3557*, near *MXAN3555* in the development process by creating four additional in-frame deletion mutants, KYC734, KYC735, KYC749, and KYC750. KYC734 was an in-frame deletion mutant of *MXAN3557*, from which amino acids 19–155 were deleted. KYC735 was an in-frame deletion mutant of *MXAN3556*, from which amino acids 9–284 were deleted. KYC749 was an in-frame deletion mutant of *MXAN3554*, from which amino acids 57–283 were deleted. KYC750 was an in-frame deletion mutant of *MXAN3553*, from which amino

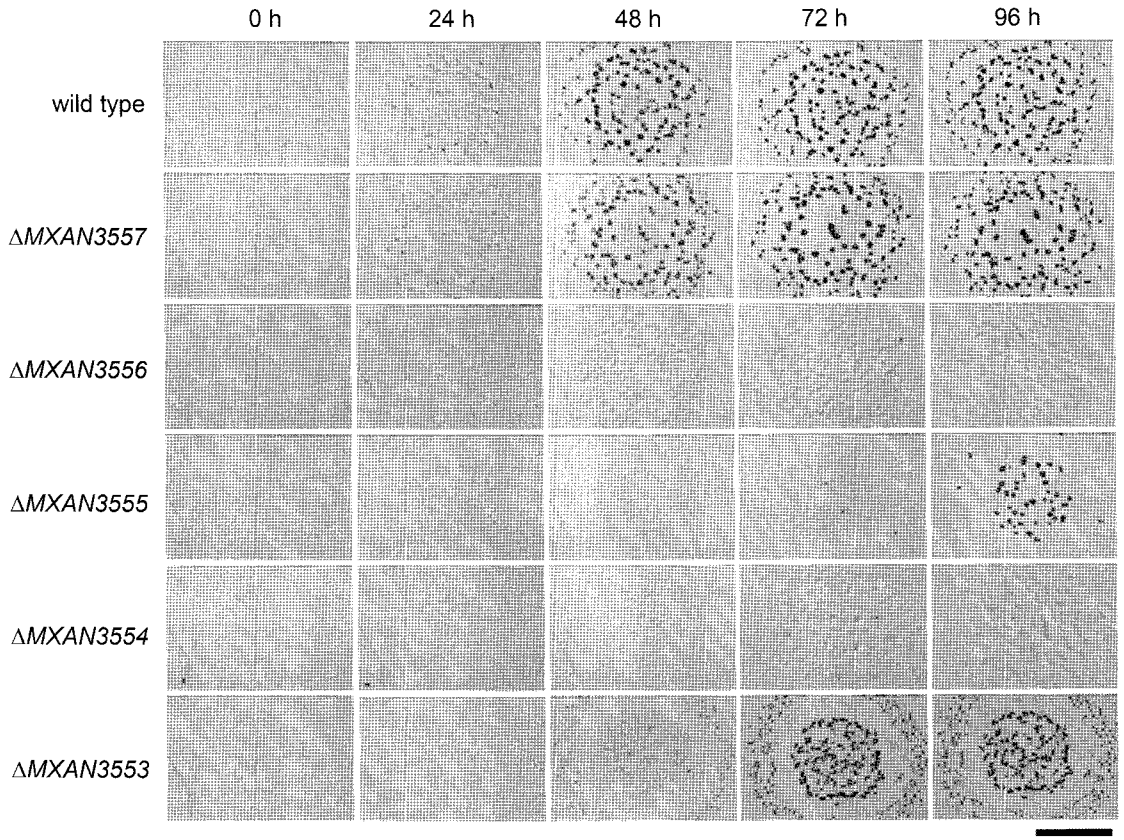


Fig. 2. Developmental phenotypes of the null mutants.
Each strain was cultured in CYE broth to exponential phase, harvested, and resuspended to 5×10^9 cells/ml. Spots (20 μl, 1.0×10^8 cells) of DZ2 (wild type), KYC734 (Δ*MXAN3557*), KYC735 (Δ*MXAN3556*), KYC545 (Δ*MXAN3555*), KYC749 (Δ*MXAN3554*), and KYC750 (Δ*MXAN3553*) were placed on CF plates and incubated at 32°C. Images were obtained after the indicated incubation times. Bar: 5 mm.

acids 29–397 were deleted. The four mutants were plated onto CF plates. Both KYC735 (Δ*MXAN3556*) and KYC749 (Δ*MXAN3554*) failed to form fruiting bodies (Fig. 2), whereas KYC750 (Δ*MXAN3553*) showed a delayed development of fruiting bodies. Conversely, KYC734 (Δ*MXAN3557*) developed fruiting bodies normally.

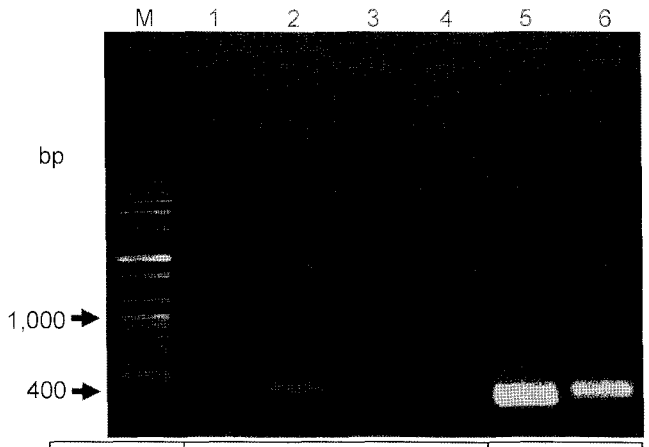
We also created insertion mutants of *MXAN3557*, *3556*, and *3554* (Fig. 1). It was expected that if *MXAN3557* formed an operon with *MXAN3556* or other ORFs, the insertion mutant would show a polar phenotype, with defects in development. However, KYC734, the insertion mutant of *MXAN3557*, showed a wild-type phenotype. This suggests that *MXAN3557* might not have been transcribed with other downstream ORFs.

Table 3. Sporulation levels of mutants.

Strains	Genotype	Number of spores[a]
DZ2	Wild type	2.0×10^7
KYC735	Δ*MXAN3556*	$<1.0\times10^1$
KYC545	Δ*MXAN3555*	1.4×10^4
KYC749	Δ*MXAN3554*	$<1.0\times10^1$
KYC750	Δ*MXAN3553*	1.3×10^7

[a]Each strain was cultured in CYE broth to exponential phase, harvested, and resuspended to 5×10^9 cells/ml. A spot (20 μl) of each strain was placed on a CF plate and incubated at 32°C for 96 h. Cells were then scraped from the plate, incubated at 50°C for 30 min in water, dispersed by sonication, diluted, and plated on CYE plates. The number of spores shown in the table is the number of germinated spores in the spot after heating and sonication. Results represent the average of duplicate experiments.

It seemed likely that the other ORFs, *MXAN3556–MXAN3553*, would form an operon, which we aimed to establish using RT-PCR. Total RNA was isolated from cells of the wild-type strain DZ2 grown under vegetative and developmental conditions. We then tested whether a set of two primers, one localized in each of *MXAN3556* and *MXAN3555*, amplified a DNA product, using the isolated RNA as a template: DNA fragments were indeed produced (lanes 2 and 4 of Fig. 3). Similar results were found using other primer sets, one with primers localized in each of *MXAN3555* and *MXAN3554*, and another with primers localized in each of *MXAN3554* and *MXAN3553* (data not shown). This suggests that the four ORFs from *MXAN3556* to *MXAN3553* are transcribed as one transcript, and that they form an operon. However, DNA amplification did not occur for a primer set with primers localized in each of *MXAN3557* and *MXAN3556*, suggesting that



DNA template	cDNA				Genomic DNA	
	from vegetative cells		from developing cells			
Amplified region	*3557* ~ *3556*	*3556* ~ *3555*	*3557* ~ *3556*	*3556* ~ *3555*	*3557* ~ *3556*	*3556* ~ *3555*

Fig. 3. Detection of transcripts by RT–PCR.
Two sets of primers, one amplifying a region from the middle of *MXAN3557* to the middle of *MXAN3556* (lanes 1, 3, and 5) and the other amplifying a region from the middle of *MXAN3556* to the middle of *MXAN3555* (lanes 2, 4, and 6), were used to amplify the PCR product with genomic DNA (lanes 5 and 6) or cDNAs, from either vegetative (lanes 1 and 2) or developmental (lanes 3 and 4) cells, as DNA templates. The vegetative cells were prepared by growing cells in CYE to 5×10^8 cells/ml. The developmental cells were prepared by incubating cells on CF plates for 24 h. M, 100-bp size marker (Bioneer, Co., Korea).

MXAN3557 may not be a part of the operon (lanes 1 and 3 of Fig. 3). We found this pattern of results for RNAs isolated from vegetative cells as well as for RNAs isolated from developmental cells. This indicates that the operon is expressed under both vegetative and developmental conditions, consistent with the DNA microarray analysis finding of Shi *et al.* [27].

Phenotypes of the Null Mutants

As described above, the Δ*MXAN3556* and Δ*MXAN3554* mutants failed to form normal fruiting bodies, and had not produced any viable heat-resistant spores after 96 h of incubation. Somewhat differently, each of the Δ*MXAN3555* and Δ*MXAN3553* mutants showed a delayed developmental phenotype. For the Δ*MXAN3555* mutant, fruiting body development was delayed by at least 48 h in comparison with the wild-type strain (Fig. 2). The sporulation level of the Δ*MXAN3555* mutant was 0.1% that of the wild type after 96 h of development, but had increased to 10% after 120 h. The fruiting body development of the Δ*MXAN3553* mutant was delayed by approximately 24 h in comparison with the wild-type strain (Fig. 2), but the sporulation level of these was similar after 96 h of development (Table 3).

Sequence Analysis of Predicted Proteins

Sequence analysis using BLASTP indicated that the putative 287-amino-acid-long protein encoded by *MXAN3556* was homologous to both linocin M18, a bacteriocin produced by *Brevibacterium linens* [30] (19.1% identical), and CFP29, a 29-kDa protein from *Mycobacterium tuberculosis* culture filtrate recognized by mouse memory effector cells [24] (19.5% identical). However, with the identity in both cases being less than 20%, the properties of the protein encoded by *MXAN3556* remain unknown.

The putative 474-amino-acid-long MXAN3555 protein was predicted to consist of a receiver domain (amino acids 5–17), an ATPase domain (amino acids 160–297), and a Fis-type helix-turn-helix binding motif (amino acids 417–458). It thus appeared that *MXAN3555* could be an NtrC-type response regulator. *MXAN3555* was 42.6% and 39.6% identical to *Escherichia coli* AtoC [12] and NtrC [19], respectively.

Developmental genes in *M. xanthus* often have sigma-54 promoters, and several NtrC-type response regulators, presumably regulating the expression of the sigma-54 promoters, have been identified: *actB* [8], *crdA* [13], *mrpB* [29], *spdR* [10], *nla4*, *nla6*, *nla18*, and *nla28* [2]. Inactivation of these NtrC-type response regulators results in a developmentally altered phenotype. In particular, *actB*, *nla18*, and *spdR* are implicated in the extracellular signaling process essential for normal fruiting body development: *actB* controls the timing and level of C-signal production [8], *nla18* mutant cells are defective for the production of both the intracellular starvation signal ppGpp and A-signal [5], and the *spdR* mutation suppresses the developmental defects associated with *bsgA* mutants [10]. The putative MXAN3554 protein was predicted to be 345 amino acids long. The C-terminal region of *MXAN3554*, comprising amino acids 205–307, was 30.1% identical to the C-terminal region of LytM, an autolytic enzyme produced by *Staphylococcus aureus* [20], and 39.6% identical to the C-terminal region of lysostaphin, a metalloendopeptidase of *Staphylococcus simulans* [23]. *MXAN3554* carries a 32-amino-acid-long signal peptide at its N-terminus.

Proteases are heavily involved in the extracellular signaling of *M. xanthus*. A-signal is essential for fruiting body development, and is generated by the action of proteases [14]. The *bsgA* gene, which is involved in developmental signaling, encodes an intracellular protease [7]. The 17-kDa CsgA protein, known as the C-signal and essential for fruiting body development, is synthesized by N-terminal proteolytic processing of the 25-kDa protein encoded by *csgA* [17].

The putative MXAN3553 protein was predicted to be 431 amino acids long. *MXAN3553* appears to be conserved in myxobacteria, as homologous proteins were found in *Stigmatella aurantiaca* (EAU64940), *Anaeromyxobacter* sp. (ABS26153), *Plesiocystis pacifica* (EDM78688), and

Sorangium cellulosum (CAN96652). However, no homologous protein was found in any other organism listed in the database. The MXAN3553 protein carries a 22-amino-acid-long signal peptide at its N-terminus.

In conclusion, the genes characterized in the present study are essential for normal fruiting body development in *M. xanthus*, and further study of these genes will help elucidate the mechanisms underlying this process.

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