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Identification of Functions Affecting Predator-Prey Interactions between *Myxococcus xanthus* and *Bacillus subtilis*

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ABSTRACT

Soil bacteria engage each other in competitive and cooperative ways to determine their microenvironments. In this study, we report the identification of a large number of genes required for *Myxococcus xanthus* to engage *Bacillus subtilis* in a predator-prey relationship. We generated and tested over 6,000 individual transposon insertion mutants of *M. xanthus* and found many new factors required to promote efficient predation, including the specialized metabolite myxoprincomide, an ATP-binding cassette (ABC) transporter permease, and a clustered regularly interspaced short palindromic repeat (CRISPR) locus encoding bacterial immunity. We also identified genes known to be involved in predation, including those required for the production of exopolysaccharides and type IV pilus (T4P)-dependent motility, as well as chemosensory and two-component systems. Furthermore, deletion of these genes confirmed their role during predation. Overall, *M. xanthus* predation appears to be a multifactorial process, with multiple determinants enhancing predation capacity.

IMPORTANCE

Soil bacteria engage each other in complex environments and utilize multiple traits to ensure survival. Here, we report the identification of multiple traits that enable a common soil organism, *Myxococcus xanthus*, to prey upon and utilize nutrients from another common soil organism, *Bacillus subtilis*. We mutagenized the predator and carried out a screen to identify genes that were required to either enhance or diminish capacity to consume prey. We identified dozens of genes encoding factors that contribute to the overall repertoire for the predator to successfully engage its prey in the natural environment.

The majority of biomass found in soil consists of highly diverse microbial communities which display complex networks of interspecies interactions ranging from commensalism to competition or cooperation, each of which contributes to the turnover of materials within the environment (1, 2). One important community-shaping determinant is predation. Microbial predators can range from nematodes to highly specialized endobiotic bacteria, such as *Bdellovibrio bacteriovorus* (3). Another ubiquitous microbial predator is *Myxococcus xanthus*, which secrets lytic enzymes and specialized metabolites and hunts in groups and consumes prey (4–6). *M. xanthus* is able to consume a diverse repertoire of microbes ranging from phage to bacterial plant pathogens and clinical isolates, and it uses the resulting nutrients to sustain growth (7–9).

M. xanthus also displays a complex life cycle that requires regulation of motility and intraspecies communication following nutrient starvation, culminating in multicellular aggregation and fruiting body formation (10). *M. xanthus* motility requires type IV pilus (T4P) motility and another machine involved in gliding (e.g., focal adhesion complexes and exopolysaccharide [EPS]). It has been shown that efficient predation requires the regulation of both motility systems, which are controlled, in part, by the Frz chemosensory system (7, 11, 12). Upon encountering prey, *M. xanthus* cells display coordinated rippling in which groups of cells move back and forth trapping prey cells to enhance predation, a phenomenon called predataxis (12).

Most bacteria tested under laboratory conditions are unable to resist *M. xanthus* as a predator. However, we found that *Bacillus subtilis* NCIB3610, an ancestral wild-type Marburg isolate (13), was able to transiently resist predation by *M. xanthus*; the specialized metabolite bacillaene is required for this short-term protection (14). During prolonged exposure to predation, *B. subtilis* produces megastructures composed of viable spores embedded within what appears to be a dense matrix of unknown composition. Our model suggests that megastructure formation provides an opportunity for *B. subtilis* NCIB3610 to produce spores, allowing for long-term survival of cells (7).

In general, microbial communities, such as biofilms, are known to provide protection from predation. For example, biofilm formation of *Escherichia coli* protects cells from predation by *Caenorhabditis elegans* and *M. xanthus* (15). Planktonic *Vibrio cholerae* and *Pseudomonas aeruginosa* cells displayed a reduction in survival against protozoan predators relative to their biofilm-

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associated counterparts (16, 17). Furthermore, sporulation has been shown to provide excellent protection from predation, as *M. xanthus* spores survive the *C. elegans* gut, and spores from both ancestral and laboratory strains of *B. subtilis* escape predation by *M. xanthus* (14, 18).

In other interspecies interactions, specialized metabolites have been shown to act as killing or signaling molecules (2, 19). Shank and coworkers (20) demonstrated that biofilm formation in *B. subtilis* is triggered in response to thiopeptide antibiotics (thiocillins), produced by a member of the same genus, *Bacillus cereus* (20, 21). Interestingly, they show that nonfunctional analogs of thiocillins do not alter biofilm-inducing capabilities. Thus, small molecules with antimicrobial capacity elicit complex behavioral responses in soil communities. Moreover, it is highly likely that antibiotics, such as thiocillins, are present in sublethal concentrations in soil environments, depending on their solubility or light sensitivity, and may have very localized temporal or spatial impact (2).

For *M. xanthus*, the specialized metabolite myxovirescin (also known as TA) is necessary for predation of proteobacteria by inhibiting lipoprotein production (9, 22, 23). Other specialized metabolites found in membrane vesicles or secreted by *M. xanthus* comprise part of the predation machinery. Predation by *M. xanthus* is, therefore, multifactorial and complex in its overall regulation (24–26). Consistent with this perspective, genome analysis indicates that *M. xanthus* employs 18 polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) clusters (8.6% of the genome) to produce a large suite of specialized metabolites, some of which likely affect predation (27–29). Additionally, the *M. xanthus* genome encodes a large number of proteins as part of the type II and type IV secretion systems, as well as ABC transporters (30). These systems are predicted to play a role in the delivery of specialized metabolites or other elements used during predation.

In this study, we report the identification of a large number of genes required for *M. xanthus* predation when challenged with the *B. subtilis* NCIB3610 ancestral strain as a prey source. We generated and tested a transposon library of *M. xanthus* mutants and found many new factors required for efficient predation, including the specialized metabolite myxoprincomide, an ABC transporter permease, and the clustered regularly interspaced short palindromic repeat (CRISPR) II locus. We also identified genes known to be involved in predation, including those required for production of EPS and T4P-dependent motility, as well as chemosensory and two-component systems thought to affect predation. Deletion of many of these genes confirmed their role during predation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. xanthus* strains used in this study are listed in Table 1. Additionally, *E. coli* DH5α and *B. subtilis* NCIB3610 strains were used as prey strains and were grown in LB at 37°C. *M. xanthus* strains were cultivated in Casitone-yeast extract (CYE) medium at 32°C (31). If required, kanamycin was used at a final concentration of 50 or 100 μg/ml for *M. xanthus* strains.

Construction of *M. xanthus* in-frame deletion mutants. In-frame deletion mutants were constructed as described elsewhere (32). Briefly, 1 kb of the up- and downstream regions of the gene of interest were amplified by PCR using Phusion polymerase (New England BioLabs, MA) and cloned into plasmid pBJ114. Sequencing was performed at the Nevada Genomics Center (Reno, NV). Verified plasmids were transformed into *M. xanthus* DZ2, and clones were selected on CYE agar plates containing kanamycin. Chromosomal insertions were verified by PCR. To obtain clean in-frame deletions, individual clones were plated onto CTT (Casitone, Tris-HCl) agar plates containing 2% galactose for counterselection. In-frame deletion mutants were identified by their ability to grow on CTT agar plates containing 2% galactose and their inability to grow on plates containing kanamycin. Additionally, the in-frame deletions were verified by PCR and sequencing of the genomic region.

TABLE 1 Bacterial strains used in this study

Bacterial strain	Genotype or characteristic	Reference or source
B. subtilis NCIB3610	Ancestral strain	13
E. coli DH5α		Laboratory strain
M. xanthus strains		
DZ2	Wild type	62
JK4633	Δ*difA*	This study
JK4634	Δ*difE*	This study
JK4635	Δ*hsfB*	This study
JK4306	Δ(MXAN_7258-MXAN_7267) (CRISPR II)	This study
JK4296	Δ(MXAN_5713-MXAN_5714) (ABC transporter permease)	This study
JK4294	Δ(MXAN_3778-MXAN_3779) (myxoprincomide)	This study

Transposon mutagenesis of *M. xanthus* and library screen. The EZ-Tn*5* Transposome kit system (Epicentre) was used as a mobile genetic element to create an *M. xanthus* transposon library. *M. xanthus* DZ2 was grown to 25 Klett units (KU) in Casitone-yeast (CYE) liquid medium and spun down, and the pellet was washed two times with water. The pellet was resuspended to 1/1,000 of the initial culture volume. Fifty-microliter aliquots of cells were mixed with EZ-Tn*5*, according to the manufacturer, followed by electroporation at 25 mF, 400 Ω, 0.65 kV. Cells were recovered in 1 ml of CYE medium and incubated at 32°C for 4 h before plating aliquots into CYE Top agar onto CYE agar plates. Kanamycin was used for selection at a final concentration of 50 μg/ml. Incubation for 5 to 6 days at 32°C resulted in single colonies, which were transferred to new CYE-kanamycin agar plates using sterile toothpicks. Growth of kanamycin-resistant clones occurred within 2 days. Individual clones were picked onto new CYE agar plates containing kanamycin and finally onto CYE agar plates to maintain the clones for further use. To screen the transposon mutant library, individual *M. xanthus* clones were transferred using toothpicks into the middle of *B. subtilis* NCIB3610 prey spots, prepared as described below. To conduct the predation screen, we grew *B. subtilis* NCIB3610 to an optical density at 600 nm (OD_{600}) of about 2.0 and washed and spotted cells onto CFL agar plates (predation/starvation medium) in 7-μl spots. A small colony of each individual *M. xanthus* transposon mutant was then taken from a master plate and transferred into the middle of an individual prey spot, thereby generating an inside-out predation assay. Each plate contained both a negative (loss-of-function [LOF]) control, as well as the *M. xanthus* parent for comparison. The LOF control used here is the *mglAB* mutant that was previously characterized for its loss of motility and inability to carry out predation (7). The predation plates were incubated at 32°C, and each individual prey spot was screened by microscopy each day for 5 days. Prey lysis was monitored over time up to 5 days (Fig. 1), allowing us to identify either gain-of-function (GOF) or LOF mutants.

Rescue cloning of predation mutants. To identify the genomic sequence into which the EZ-Tn*5* transposon inserted, we isolated chromosomal DNA of the mutant strains using the DNeasy kit from Qiagen. Subsequently, we digested the chromosomal DNA using the restriction

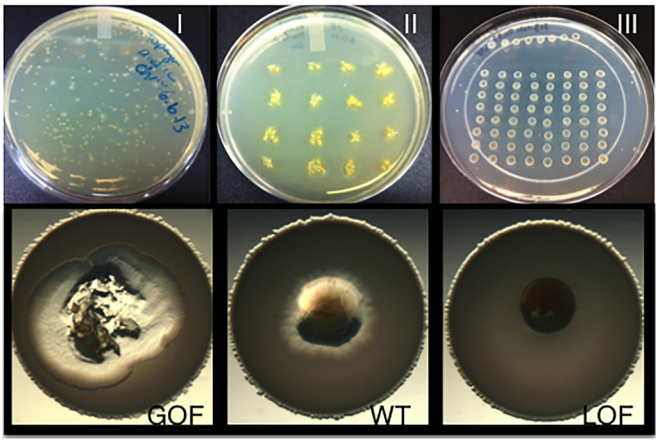

FIG 1 *M. xanthus* transposon screen outline. A library of 6,000 *M. xanthus* transposon mutants was generated using the EZ-Tn5 transposable element. (I) To select for single *M. xanthus* colonies, cells were plated into Top agar on solid CYE agar plates containing kanamycin for selection and incubated for 5 to 6 days. (II) Individual colonies were transferred onto CYE kanamycin agar plates. (III) *B. subtilis* NCIB3610 prey cells were spotted onto CFL agar plates (predation/starvation medium), and the *M. xanthus* transposon mutants were transferred into the middle, showing the inside-out assay. Screening for predation phenotypes occurred for up to 5 days. GOF and LOF strains were selected by comparing them to *M. xanthus* wild type (WT) and an LOF control strain (*mglAB* mutant), as seen in the lower part of the figure.

enzyme NspI, stopped the reaction by Drop dialysis, and religated the chromosomal DNA. The transposon contains the R6Kγ*ori* for rescue cloning in the *pir E. coli* strain and a kanamycin resistance cassette for selection (33). Kanamycin-resistant transformants were subject to plasmid isolation and sequencing performed at the Nevada Genomics Center at the University of Nevada, Reno. Obtained sequences were checked against the *M. xanthus* genome using the NCIB/BLAST tool. Further information about the insertion sites into the *M. xanthus* genome was obtained using the MiST2 database (34).

Predation assays. *M. xanthus* strains were grown in CYE medium to mid-log phase. Cells were harvested and washed twice with MMC buffer (20 mM morpholinepropanesulfonic acid [MOPS] [pH 7.6], 4.0 mM $MgSO_4$, 2 mM $CaCl_2$). *M. xanthus* cells were resuspended in MMC buffer to a final concentration of 250 KU. Prey cells were grown overnight in LB medium to an OD_{600} of about 2. Cells were washed twice with H_2O and resuspended in water to a final concentration of 1×10^{11}/ml of water. Different qualitative and quantitative predation assays were performed. As seen in Fig. 2D, (wild-type) predator and prey cells were mixed in a ratio of 1:50 and spread plated onto CFL agar plates. After about 48 h of predation, megastructure formation of *B. subtilis* as well as the fruiting body of *M. xanthus* were evaluated. Another qualitative assay (inside-out assay, Fig. 2E) was performed to determine lysis of the prey (*B. subtilis* NCIB3610) by the predator (35). Seven microliters of prey cells was spotted onto the CFL plates. After the prey spot was dried, *M. xanthus* predatory cells (2 μl) were spotted into the middle of the prey spot. Predatory lysis is visible as clearing of the prey spot. Semiquantitative predation assays were performed to identify gain-of-function (GOF) and loss-of-function (LOF) mutants. Predator and prey cells were mixed in a ratio of 1:25 (Fig. 2F), 1:50 (Fig. 2G), and 1:100 (Fig. 2H). Seven microliters of predator-prey mixture was spotted onto CFL agar plates, and lysis of the prey spot was observed over time. Assay mixtures were incubated at 32°C, and pictures were taken after different times and magnifications to monitor the progression of predation. GOF can clearly be seen in Fig. 2 for both *difA* and *difE* in-frame deletion mutants in all different mixing ratios (Fig. 2F to H). LOF can clearly be seen for the *hsfB*, ABC transporter/permease, and myxoprincomide mutants at predator-to-prey mixing ratios of 1:25 (Fig. 2F), as prey cells that are darker remain visible compared to the wild type. LOF of the CRISPR II mutant is more visible at higher predator-to-prey cell ratios (Fig. 2G and H) and is indicated by darker prey spots and less clearing of the prey. Quantifications of prey survival and predator growth were performed as previously described (14). Briefly, prey and predator were mixed at a ratio of 50:1 and spread plated onto CFL agar plates. Additionally, both strains were plated individually on CFL agar plates. After 24 h, cells were harvested, and serial dilutions were plated onto LB agar plates to obtain *B. subtilis* NCIB3610 or plated into CYE Top agar onto CYE agar plates (containing 50 μg/ml kanamycin) to obtain CFU for *M. xanthus* strains. Assays were performed in triplicate, and the average CFU and standard deviation were calculated. Predator strains used in this study had a kanamycin resistance cassette inserted into the *attB8* phage attachment site for selection.

Fruiting body formation and motility assays. Fruiting body formation of *M. xanthus* was performed on CFL agar plates, as described before (36). Briefly, *M. xanthus* strains were grown to about 100 to 150 KU at 32°C in CYE medium, washed in MMC buffer, and 10 μl of a 500-KU cell suspension was spotted onto CFL agar plates and monitored for 3 days. Fruiting body formation is indicated by the formation of aggregates (as seen in Fig. 2C, wild type). To assay T4P-dependent motility as well as gliding motility, *M. xanthus* strains were grown as described above, and 10 μl of a 250-KU cell suspension was spotted onto 0.5% or 1.5% CYE agar plates. T4P-dependent motility is visible on soft agar plates (0.5% CYE) after about 48 h, indicated by cells swarms away from the central spot of cells (as seen in Fig. 2A, wild type is swarming, while the *difA* mutant is not). Focal adhesion-mediated motility is observed on hard agar surfaces as individual cells at the corner of the central spot (1.5% CYE) after 24 h (Fig. 2B, wild type).

Microscopy. Phenotypic assays were monitored by microscopy using a Nikon SMZ10000 dissecting microscope. Images were taken using QImaging camera and QCapture software after various times.

RESULTS

Identification of *M. xanthus* predation mutants following a transposon mutagenesis screen. A transposon (EZ-Tn5) mutant library of *M. xanthus* was constructed to screen for mutants that displayed either a gain-of-function (GOF) or a loss-of-function (LOF) phenotype during predation of *B. subtilis* NCIB3610 as the prey source (Fig. 1). *B. subtilis* NCIB3610 was chosen because it displays significant resistance to predation relative to either *E. coli* or laboratory strains of *B. subtilis* under the conditions of our assays (14).

We screened a total of approximately 6,000 *M. xanthus* mutants and selected 253 mutants which displayed either enhanced (1.1% GOF) or reduced (2.6% LOF) predation (Fig. 1). From those, we selected 53 mutants that displayed the most obvious predation phenotypes, 17 GOF and 36 LOF mutants, for more thorough analyses. To identify the locus into which the transposon had inserted, chromosomal DNA was isolated, digested with NspI, ligated, and transformed into *E. coli pir* cells capable of propagating the conditional R6Kγ origin of replication. Clones were selected on LB agar containing kanamycin, and plasmid DNA was isolated and sequenced. The results of the 53 insertions are identified in Table 2.

All GOF transposon mutants displayed increased lysis of *B. subtilis* NCIB3610 prey in the inside-out assay (see Fig. S1E in the supplemental material). However, no obvious differences were visible for predation-induced megastructure formation by *B. subtilis* (see Fig. S1D). All LOF transposon mutants displayed reduced lysis of *B. subtilis* NCIB3610 (see Fig. S2E in the supplemental material) while still retaining the ability to induce *B. subtilis* megastructure formation (see Fig. S2D). However, it is worth noting

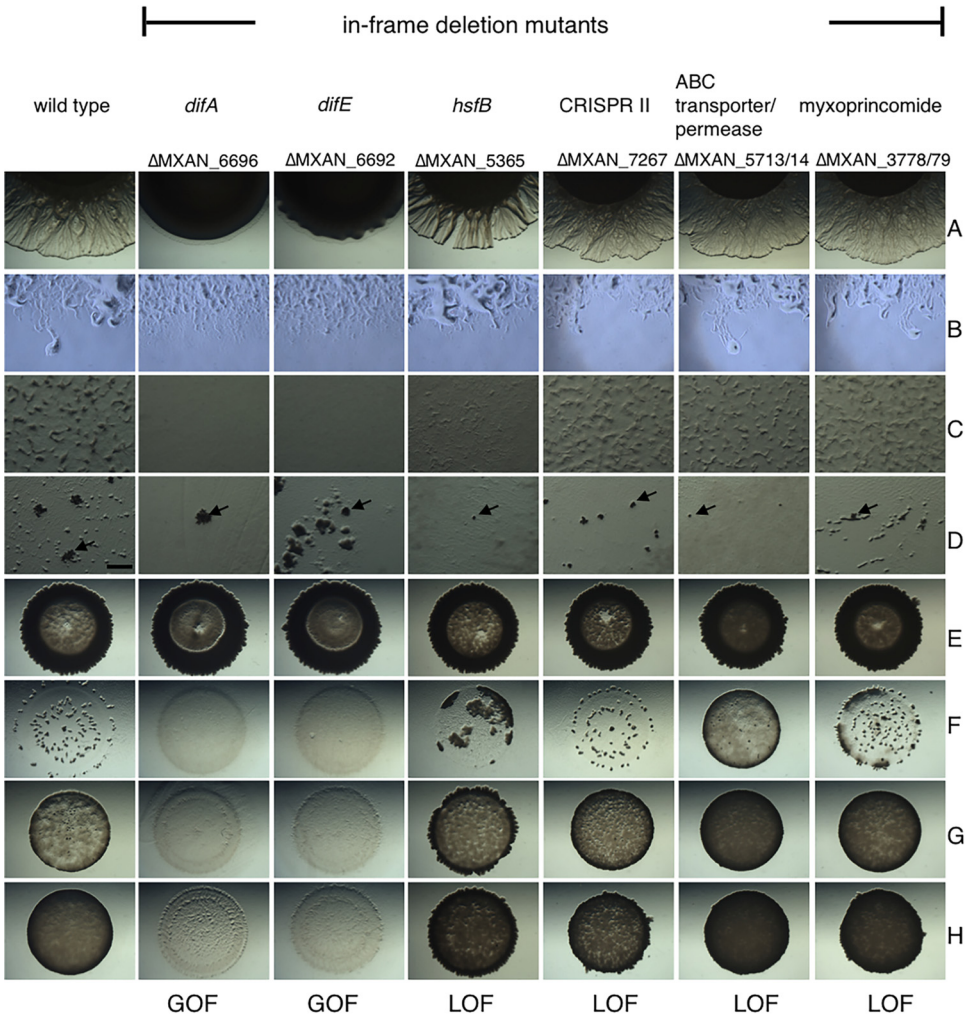

FIG 2 Phenotypic analysis of in-frame deletion mutants. *M. xanthus* transposon mutants were grown to mid-log phase, washed, and resuspended to a cell concentration of 250 KU. (A to C) Phenotypic analysis was performed to assess T4P-dependent motility on 0.5% CYE agar (A), gliding motility on 1.5% CYE agar plates (B), and fruiting body formation on CFL starvation/predation medium (C). (D and E) To investigate predatory ability, 2 μl of *M. xanthus* cells was spotted into the middle of *B. subtilis* NCIB3610 (E), or both predator and prey cells were mixed in a ratio of 1:50 and spread on plates to study megastructure formation on CFL agar plates (D). (F to H) Semiquantitative predation assays. Predator and prey were mixed in a ratio of 1:25 (F), 1:50 (G), or 1:100 (H). Pictures were taken after 24 h (B) and 48 h (A and C to H) at a magnification of ×10 (A, B, and E to H), ×30 (C and D), or ×100 (B). (D) Bar, 0.5 mm; arrows indicate individual megastructures.

that the LOF mutant-induced megastructures appeared to be smaller (see Fig. S2D).

Additionally, we made in-frame deletion mutants in a subset of genes identified during the transposon screen to verify that our findings were not an artifact of the transposon itself (Fig. 2). Below, we describe several transposon and in-frame deletion mutants that were expected targets (e.g., motility systems) and several that represent new loci not previously known to affect predation (e.g., specialized metabolites).

Specialized metabolite myxoprincomide is required for effective predation. One prominent LOF transposon mutant had an insertion in locus MXAN_3779 encoding a mixed NRPS/PKS biosynthetic module, previously identified for its production of the specialized metabolite myxoprincomide (29). Myxoprincomide was discovered as a linear peptide with some unusual residues but had no known biological function (29). The transposon inserted near the 5′ end of the 42.8-kb gene. It is not yet known whether the resulting mutant produces a modified version of the polyketide.

The myxoprincomide transposon mutant displayed no defects in motility or fruiting body formation compared to the parent (see Fig. S2A to C in the supplemental material). However, *B. subtilis* NCIB3610 cells showed increased resistance to the myxoprincomide mutant, as indicated by less lysis of the prey spot (see Fig. S2E). Additionally, *B. subtilis* megastructure formation was affected such that the structures are smaller than those induced by wild-type *M. xanthus* (see Fig. S2D). Importantly, an in-frame deletion of the myxoprincomide synthesis cluster MXAN_3778-MXAN_3779 verified that the phenotypes observed in Fig. 2A to E were due to the loss of the intact product from the NRPS/PKS module. In addition, semiquantitative predation assays (Fig. 2F to H) clearly show reduced ability for the myxoprincomide mutant to lyse prey relative to the parent. A quantitative assay also showed that about 33% fewer prey cells were consumed by the myxoprin-

TABLE 2 GOF and LOF transposon mutants

MXAN no.	Gene	Gene product description[a]	GOF/LOF
0319		16S rRNA	3× GOF/1× LOF
0322		23S rRNA	2× GOF/1× LOF
5365	*hsfB*	Response regulator sensor histidine kinase HsfB	1× GOF
6673		Hypothetical protein, encoded in operon with GrpE; DnaK chaperone	1× GOF
6696	*difA*	Methyl-accepting protein DifA	1× GOF
0080		Hypothetical protein, DUF1446	1× GOF
1112		Hypothetical protein	1× GOF
7234		Hypothetical protein, DUF946	1× GOF
0517		Acetyltransferase, encoded in operon with TonB-dependent receptor	1× GOF
6679		Hypothetical protein	1× GOF
7509		Inner membrane protein, 60 kDa	1× GOF
6692	*difE*	Histidine kinase DifE	1× LOF
7195		Hypothetical protein	
7267		Hypothetical protein, encoded in operon with CRISPR II	1× LOF
7325		Acetyltransferase	1× LOF
3325	*rpsD*	30S ribosomal protein S4	1× LOF
3779		PKS/NRPS (myxoprincomide)	1× LOF
0091		HAE1 family efflux transport MFP subunit	1× LOF
6127		IS*4* family transposase	1× LOF
0455			
5791		Response regulator/GGDEF-containing protein	1× LOF
0463	*pepP*	Xaa-Pro aminopeptidase	1× LOF
3678		Polyprenyl synthetase	1× LOF
5220		HhH-GPD domain-containing protein	1× LOF
3760	*acdA*	Acyl-CoA dehydrogenase	1× LOF
0180		*fis* family transcriptional regulator	1× LOF
1093		DNA-binding response regulator	1× LOF
5713		ABC transporter/permease	11× LOF
0260		Cation transporter/universal stress protein	1× LOF
7254		Hypothetical protein with NAD-binding domain	1× LOF
7143		*fis* family transcriptional regulator, encoded in operon with histidine kinase	1× LOF
3797		Acyl-CoA dehydrogenase	1× LOF
7466		Hypothetical protein	1× LOF
1449		TonB protein	1× LOF
4659		Putative Flp pilus assembly protein	1× LOF
6785		Hypothetical protein with NaH exchange transporter domain	1× LOF
0925		4-Oxalocrotonate decarboxylase	1× LOF
7492		Hypothetical protein	1× LOF
6908	*pgi*	Glucose-6-phosphate isomerase	1× LOF
6867		Lipoprotein, encoded in operon with two sensor box histidine kinases	1× LOF

[a] MFP, membrane fusion protein; HhH, helix-hairpin-helix; acyl-CoA, acyl coenzyme A.

comide mutant than by the parent (Fig. 3A). Similarly, we observed reduced CFU for the myxoprincomide mutant in the presence of *B. subtilis* prey. Only about 62% survived compared to about 100% of the *M. xanthus* parent (Fig. 3B).

To assess expression of the myxoprincomide NRPS/PKS gene cluster, a *lacZ* fusion was generated with the putative promoter for MXAN_3778. The results indicated that the myxoprincomide NRPS/PKS gene cluster was constitutively expressed under the conditions of our assay, regardless of the presence of prey (see Fig. S3 in the supplemental material). Together, these data indicate that the specialized metabolite myxoprincomide is part of the predatory machinery of *M. xanthus*.

Hsf two-component system affects predation. A prominent GOF phenotype was identified that resulted from a transposon insertion into the gene encoding the hybrid response regulator/histidine kinase HsfB. The transposon inserted downstream of the region encoding the response regulator domain within HsfB (see Fig. S4 in the supplemental material). For this mutant, even though predation was enhanced, we observed a reduction in both T4P-dependent motility and developmental fruiting body formation. In contrast, there was no obvious change in gliding motility, as indicated by the presence of individual cells at the colony edge (see Fig. S1 in the supplemental material). Interestingly, an in-frame deletion of *hsfB* displayed an LOF phenotype in the semiquantitative assays (Fig. 2F to H). However, this mutant also showed reduced motility as well as reduced fruiting body formation. Thus, the transposon insertion may have had an effect on downstream genes or resulted in dysregulation of the HsfBA two-component system.

The Hsf two-component system is encoded by an operon spanning MXAN_5365 to MXAN_5362 (see Fig. S4 in the supplemental material) and includes genes encoding HsfB, HsfA (NtrC-like response regulator), an NAD kinase, and a protein with a conserved domain of unknown function (DUF218). In previous work, we demonstrated specific phosphotransfer between HsfB and HsfA (37). In other previous work, HsfA was shown to affect

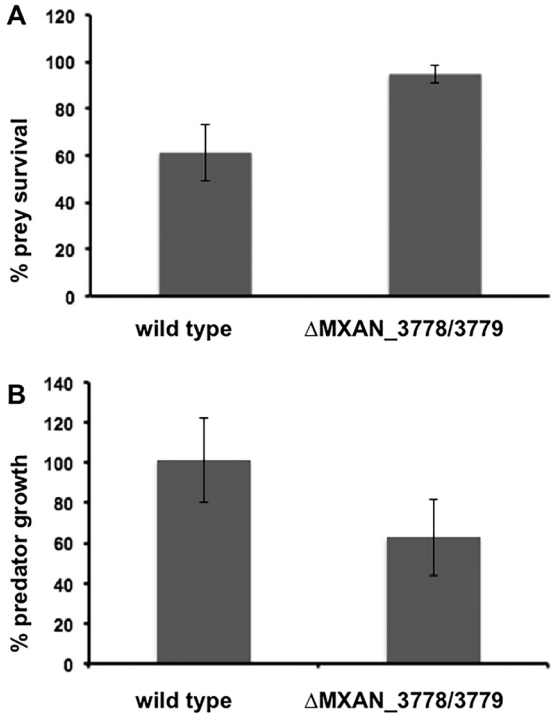

FIG 3 Quantification of prey survival and predator growth. Predator *M. xanthus* wild-type or myxoprincomide mutant [Δ(MXAN_3778-MXAN_3779)] cells were mixed with *B. subtilis* NCIB3610 prey cells in a ratio of 1:50, and the mixture was spread plated onto CFL agar. After incubation for 24 h at 32°C, cells were harvested and serial dilutions were plated onto selective medium. Predator and prey alone were used as controls. CFU were used to calculate the percentage of prey survival and predator growth relative to the controls. (A) The myxoprincomide mutant shows a reduced ability to consume *B. subtilis* NCIB3610. (B) The *M. xanthus* wild type shows no significant growth, whereas the loss of the specialized metabolite myxoprincomide results in reduced CFU. The data represent the average of the results from 3 individual eating experiments, and the error bars show the standard deviation.

the production of specialized metabolites, including DKxanthene and myxovirescin. HsfA was found to bind the putative promoter region of the gene clusters responsible for the production of each of these two molecules (38). Thus, the HsfBA system appears to be a critical regulator for the production of specialized metabolites by *M. xanthus* and for the regulation of predation.

Dif chemosensory system is required for predation. The transposon insertion screen led to the identification of three mutations in the *dif* chemosensory system, two LOF mutants and one GOF mutant. The *dif* cluster encodes a chemosensory system with homologs to a prototypical chemotaxis signaling system and includes a methyl-accepting protein (MCP), DifA; a coupling protein (CheW), DifC; the response regulator DifD; the histidine kinase DifE; and a phosphatase, DifG (see Fig. S4 in the supplemental material) (39, 40). The Dif system has been well characterized and shown to regulate EPS production, T4P-based motility, and fruiting body formation in *M. xanthus*.

The GOF mutant resulted from an insertion into *difA* at bp 970, corresponding to amino acid 321 within the MCP signaling domain of the resulting protein (see Fig. S4 in the supplemental material). As an MCP, the DifA mutation could result in a truncated receptor that retains its signaling domain. This *difA* insertion mutant displays reduced T4P-dependent motility and is defective in fruiting body formation, while gliding motility was not affected (see Fig. S1A to C in the supplemental material). Increased lysis of the prey *B. subtilis* NCIB3610 was observed, while megastructure formation was not dramatically changed compared to the wild type (see Fig. S1D and E). A GOF phenotype was also obtained with an in-frame deletion mutation in *difA* (Fig. 2F to H). The semiquantitative predation assay clearly shows increased predation even at high prey concentrations which might otherwise enhance resistance (Fig. 2F). Additionally, the Δ*difA* mutant displays no T4P-dependent motility and forms no fruiting bodies (Fig. 2A and C), as described previously.

The insertion sites for the transposons for the two LOF mutants occurred near the 5′ end of the gene encoding DifE, a CheA homolog and the central histidine kinase of the Dif chemosensory system. The transposon insertions occurred either upstream or downstream of the Hpt domain-encoding region within *difE*, thereby likely disrupting the production of an intact kinase (see Fig. S4 in the supplemental material). The corresponding mutants displayed reduced T4P-dependent motility and loss of fruiting body formation. However, in these cases, lysis of *B. subtilis* NCIB3610 was also reduced, as was induction of megastructure formation (see Fig. S2 in the supplemental material). A Δ*difE* mutant, however, displayed the opposite phenotype and was a GOF mutant (Fig. 2F and G), suggesting complex regulation of predation by the Dif chemosensory system. All *dif* mutants described here, whether transposon insertions or in-frame deletions, displayed defects in EPS production (see Fig. S5 in the supplemental material).

An ABC transporter/permease is required for predation. One of the most frequent transposon insertion sites (11 independent isolates) occurred in MXAN_5713, encoding an ABC transporter/permease predicted to be involved in branched-chain amino acid (BCAA) transport. BCAAs are essential to *M. xanthus* and function as precursors for fatty acid synthesis as well as specialized metabolite production (31, 41, 42). All 11 transposon insertion mutations in MXAN_5713 displayed an LOF phenotype (see Fig. S2 in the supplemental material). MXAN_5713 appears to be part of an operon, which includes a second ABC transporter/permease gene (MXAN_5714) and two genes located upstream encoding a putative lipoprotein (MXAN_5711) and an ABC transporter/ATP-binding protein (MXAN_5712). According to the MiST2.2 database, the *M. xanthus* genome contains a total of four ABC transporter/permease genes that are located in two operons, the MXAN_5711 to MXAN_5714 operon described above and the MXAN_6667 to MXAN_6659 operon (34). No transposon insertions were identified in the MXAN_6667 to MXAN_6659 cluster. Overall, these data indicate that BCAA import, possibly impacting fatty acid and specialized metabolite production, is intertwined with *M. xanthus* physiology to affect the efficiency of predation. An in-frame deletion mutant of the ABC transporter/permease genes MXAN_5713 and MXAN_5714 verified that loss of the transporter resulted in the observed phenotypes (Fig. 2F to H). In the semiqualitative predation assays, this in-frame deletion mutant displays the strongest LOF phenotype relative to all mutants presented in this study.

CRISPR locus affects predation. Another LOF mutant phenotype resulted from transposon insertion into the CRISPR II locus (see Fig. S2 in the supplemental material). CRISPR/CRISPR-associated protein (Cas) systems protect prokaryotes from foreign genetic elements, like plasmids and phages, and act as prokaryotic

immune systems (43). The *M. xanthus* CRISPR II locus contains the prototypical CRISPR/Cas genes (MXAN_7257 to MXAN_7266) of prokaryotic immunity systems (see Fig. S6 in the supplemental material). This locus also includes genes previously annotated as development genes in *M. xanthus*, *devR* (a *cas7* homolog) and *devS* (a *cas5* homolog), both of which were shown to affect sporulation levels (44, 45). The transposon mutant that we identified was inserted into MXAN_7267 (encoding a hypothetical protein) at the 5′ end of the CRISPR locus, just upstream of the newly described *devI* (MXAN_7266). DevI is a small protein that inhibits sporulation when overexpressed (see Fig. S6) (45). An in-frame deletion of the CRISPR II locus [Δ(MXAN_7258-MXAN_7267)] resulted in an LOF phenotype and thus verified the involvement of this locus in *B. subtilis* NCIB3610 predation, as seen in the semiquantitative assay (Fig. 2G and H).

DISCUSSION

Interspecies interactions can produce a variety of outcomes ranging from cooperation to competition or commensalism (1). Predation by *M. xanthus* influences the environment, as it provides nutrients for itself and other bacteria but also changes the composition and diversity of the soil community (8, 9, 14). *M. xanthus* utilizes a broad range of prey found in soil environments worldwide and is also known to produce a large suite of unique specialized metabolites thought to facilitate predation (5, 28, 46). In soil, *B. subtilis* would likely be a source of prey for *M. xanthus*. Our previous work demonstrated that the ancestral strain of *B. subtilis*, NCIB3610, is transiently resistant to *M. xanthus* predation due to its production of the specialized metabolite, bacillaene (14). Following long-term interactions, *B. subtilis* goes on to produce megastructures that contain *B. subtilis* spores embedded within a matrix, allowing cells to escape predation altogether (7). In contrast, many factors utilized by *M. xanthus* during predation are less well characterized. Thus, we performed a transposon mutagenesis of the predator and screened 6,000 *M. xanthus* mutants for their ability to consume *B. subtilis* NCIB3610. We identified both GOF and LOF mutants based on their capacity to consume *B. subtilis* cells relative to the rate of consumption by the parent *M. xanthus* cells.

The predicted functions of genes affecting *M. xanthus* predation capacity were predominantly signal transduction, motility, transport, and the production of specialized metabolites. Loss of production of the specialized metabolite myxoprincomide (either by transposon insertion or in-frame deletion) greatly reduced predation by *M. xanthus*. A quantitative experiment revealed that the myxoprincomide mutant survives or grows at a lower rate in the presence of prey, as only about 62% of cells were recovered after a 24-h competition experiment relative to the parent. This result may reflect an inability to lyse the NCIB3610 prey cells or be due to a killing effect from *B. subtilis* itself. The possibility of a killing effect should be considered because myxoprincomide was not required for the predation of sensitive strains, including *E. coli* or the *pksL* mutant of *B. subtilis*, which is incapable of producing bacillaene (see Fig. S7 in the supplemental material). Taken together, it is clear that specialized metabolites have an important function in interspecies interactions between *M. xanthus* and *B. subtilis*. Furthermore, the observation that myxoprincomide NRPS/PKS gene expression is constitutive strongly suggests that the production of this specialized metabolite is critical for the survival of *M. xanthus*.

Specialized metabolites or natural products that can act as antibiotics are known to have a variety of different functions. They can act as defensive molecules shaping microbial communities by killing or by inhibiting growth (2, 19). However, many recent publications show that the role of specialized metabolites may be more complex, as they can induce biofilm formation and act as signaling molecules (47, 48). Therefore, it was not surprising that we identified a transposon insertion mutant in the *M. xanthus hsf* gene cluster known to regulate specialized metabolite production (38). HsfB and HsfA form a classical two-component system (37); however, it is not known what activates this pathway or if HsfB is capable of cross talk to another response regulator. This result is of great interest, as *M. xanthus* is known for its large repertoire of two-component systems regulating its complex lifestyle (36–38). It is also important to point out that the *hsf* cluster encodes a putative GGDEF-containing protein, suggesting that local concentrations of the second messenger, cyclic di-GMP (c-di-GMP), may affect predation. Because c-di-GMP has been implicated in many processes for *M. xanthus* (49–51), we speculate that predation, specialized metabolite production, two-component signaling, and c-di-GMP sensing are interconnected processes. Additional experiments to investigate the metabolite profile of the *hsf* mutant strain during predation are under way.

M. xanthus is well known for its capacity to develop and produce spore-filled fruiting bodies. This complex process requires the production of exopolysaccharides (EPS) and coordination of two motility systems, T4P-dependent motility and gliding motility (10). These complex processes are regulated by various two-component systems (TCS) and chemosensory systems, including Frz and Dif (11, 52, 53). While we did not identify insertions in the Frz system, we identified three transposon insertion mutants in the *dif* cluster. Two LOF mutations mapped to the DifE (CheA) homolog, and one GOF mutant mapped to the C-terminal end of the DifA (MCP) homolog. The site of insertion in DifA is consistent with the current understanding of MCP function, suggesting that a truncated DifA receptor may produce a constitutive output; a similar mutation was isolated in *M. xanthus* decades ago in FrzCD (*frzCD*^{c}), which produced a hyperreversing phenotype in contrast to the corresponding deletion (54, 55). Interestingly, both Δ*difA* and Δ*difE* mutant cells display a GOF phenotype, despite the fact that both mutants lack the ability to produce EPS. We conclude that the transposon insertion in *difA* led to dysregulation of the signal transduction pathway and that EPS is not required *per se* for predation. Additional evidence for motility as a key predation factor was the finding that disruption of a putative Flp pilus assembly protein (MXAN_4659) (Table 2) resulted in an LOF phenotype. Our results also indicate that several other putative signaling proteins are required for predation by *M. xanthus*, including an operon comprising a lipoprotein and two sensor histidine kinases (MXAN_6866 and MXAN_6865) (Table 2).

One critical finding was the role for the ABC transporter/permease-encoding gene cluster MXAN_5711 to MXAN_5714. The ABC transporter is most likely involved in the import of branched-chain amino acids, like leucine, isoleucine, and valine. Leucine is essential for *M. xanthus* growth and becomes available following the lysis of prey (31). We speculate that the transporter mutant is unable to utilize BCAAs, preventing growth, and it therefore displays a deficiency in predation. Moreover, it has been shown that BCAAs, such as leucine, serve as the precursor for fatty acid biosynthesis and also as starter molecules for polyketide synthases (41, 42). Reduced levels of available BCAAs due to disrup-

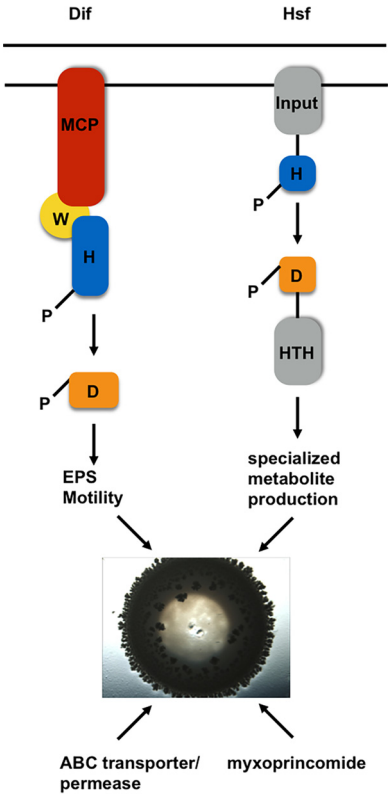

FIG 4 Factors and pathways involved *M. xanthus* predation. Our transposon mutagenesis screen identified 2 two-component systems, the Dif chemosensory system (left) and the Hsf system (right), that are involved in regulating predation. Each system utilizes a histidine kinase (blue) that becomes autophosphorylated upon activation to regulate outputs via response regulators. Upon activation, phosphoryl groups are transferred to response regulators (orange) to generate the physiological responses, such as EPS production, motility, and specialized metabolite production. Additionally, we found an ABC transporter/permease and the specialized metabolite myxoprincomide to be involved in the predation of *B. subtilis* NCIB3610. HTH, helix-turn-helix.

tion of the ABC transporter could result in an altered specialized metabolite or fatty acid profile and generate deficits in growth on otherwise-suitable prey. Metabolite profiling of this mutant is also under way.

Other *M. xanthus* functions affecting predation were identified and include a CRISPR system. The *M. xanthus* genome encodes three CRISPR systems, each composed of a typical arrangement of CRIPSR/Cas genes with repeat/spacer regions (44, 45, 56). The CRIPSR/Cas systems are prokaryotic immune systems that confer resistance to foreign genetic elements, such as plasmids and phages, and provide a form of acquired immunity. Because we identified an LOF mutant that mapped to one particular locus, CRIPSR II (spanning MXAN_7267 to MXAN_7258), it is possible that *M. xanthus* utilizes CRISPR systems during predation, since the predator is exposed to foreign DNA during prey lysis. The CRISPR II locus has been studied previously for its regulation of the development in *M. xanthus* and is known to contain *devTRS* and *devI*, encoding a small protein that inhibits sporulation (45, 57–59). In our hands, the LOF mutant displayed defects in predation but did not display altered motility or sporulation. It has been reported that CRISPR systems can regulate alternative functions, such as biofilm formation and swarming in *Pseudomonas aeruginosa*, EPS production in *M. xanthus*, and lipoprotein production in *Francisella novicida* (56, 60, 61).

In summary, our screen revealed many new genes regulating predation by *M. xanthus*. We also identified several functions that were expected or predicted to regulate predation. Results from the transposon insertions were independently confirmed by constructing in-frame deletion mutants. Overall, *M. xanthus* predation is a multifactorial process, requiring several signaling pathways, regulation of motility, branched-chain amino acid transporters, and production of specialized metabolites (Fig. 4). Myxoprincomide appears to be a key determinant to enhance the capacity of *M. xanthus* to prey upon *B. subtilis*. Further analysis is ongoing to investigate the complexity of gene regulation and specialized metabolite production during predation.

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