Bacillaene and sporulation protect *Bacillus subtilis* from predation by *Myxococcus xanthus*

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**Running Title:** *B. subtilis* Inhibits *M. xanthus* Predation

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Abstract

*Myxococcus xanthus* and *Bacillus subtilis* are common soil-dwelling bacteria that produce a wide range of secondary metabolites and sporulate under nutrient-limiting conditions. Both organisms affect the composition and dynamics of microbial communities in the soil. However, *M. xanthus* is known to be a predator while *B. subtilis* is not. A screen of various prey led to the finding that *M. xanthus* is capable of consuming laboratory strains of *B. subtilis* while the ancestral strain, NCIB3610, was resistant to predation. Based in part on recent characterization of several strains of *B. subtilis*, we were able to determine that the *pks* gene cluster, required for production of bacillaene, is the major factor allowing *B. subtilis* NCIB3610 cells to resist predation by *M. xanthus*. Furthermore, purified bacillaene was added exogenously to domesticated strains resulting in resistance to predation. Lastly, we found that *M. xanthus* is incapable of consuming *B. subtilis* spores even from laboratory strains, indicating the evolutionary fitness of sporulation as a survival strategy. Together, the results suggest that bacillaene inhibits *M. xanthus* predation allowing sufficient time for development of *B. subtilis* spores.
Introduction

Naturally occurring antibiotics are produced by bacteria as secondary metabolites and are typically found at relatively low sub-lethal concentrations suggesting a role in intercellular and interspecies communication (1-3). Bacteria such as *Myxococcus xanthus* and *Bacillus subtilis* are known to produce large numbers of secondary metabolites composed of polyketides and non-ribosomal peptides that may act both as antibiotics and as signaling molecules. Each of these organisms has been described for biofilm formation and for their capacity to differentiate into quiescent spores. Roles for secondary metabolites during development have also been reported for these organisms (4-6).

*M. xanthus* is a δ-proteobacterium and serves as a model organism for the study of gliding motility, intercellular communication and multicellular development. One additional prominent aspect of the *M. xanthus* lifecycle is its capacity to act as a predator (7-10). Upon encountering a suitable source, *M. xanthus* cells penetrate micro-colonies (11) and consume their prey, a process that requires secretion of lytic enzymes and metabolites that target susceptible cells (12, 13). Lytic enzymes such as proteases, lysozyme, amidases and endopeptidases produced by *M. xanthus* are involved in extracellular degradation of cells. Formation of predatory biofilms is frequently described as “wolfpack” behavior, which is thought to facilitate predation by increasing the concentration of secreted lytic factors (14-16).

*M. xanthus* and *B. subtilis* secondary metabolites include both anti-fungal and anti-bacterial properties (17, 18). For *M. xanthus*, myxovirescin was recently demonstrated to inhibit lipoprotein production in proteobacteria thereby defining a role in predation specifically for Gram-negative prey sources (19-21). Another *M. xanthus* metabolite, DKxanthene, has antioxidant properties, is required for developmental sporulation, gives *M. xanthus* its distinctive
yellow color and may also function as an interspecies signal (5). For *B. subtilis*, the lipopeptides plipastatin and surfactin have antimicrobial properties (22) while surfactin also affects surface tension and signaling during biofilm development (23). The peptide/polyketide bacillaene was first discovered to inhibit bacterial protein synthesis and has recently been implicated in interspecies interactions (24-26).

In this study, we sought to refine our understanding of *M. xanthus* predator-prey interactions with various strains of bacteria, specifically by investigating prey preference. We observed that while *M. xanthus* consumes laboratory strains of *B. subtilis*, the ancestral strain NCIB3610 is highly resistant to predation by *M. xanthus*. Further analyses indicated that production of bacillaene by *B. subtilis* inhibits predation by *M. xanthus* and that purified bacillaene could be added exogenously to susceptible strains to provide protection against predation. Additionally, *B. subtilis* laboratory strains were capable of generating spores that are resistant to predation by *M. xanthus*. We conclude that bacillaene is an effective secondary metabolite that transiently 'protects' *B. subtilis* cells from predation during the process of sporulation in its natural environment.
Materials and Methods

**Bacterial strains, growth and development.** Bacterial strains used in this study are listed in Table 1. *E. coli* and *B. subtilis* strains were grown in LB at 37°C or 32°C. *E. coli* B2155, a diaminopimelic acid (DAP) auxotroph strain, was grown in the presence of 100 μg/ml DAP. *M. xanthus* strains were cultivated in CYE medium at 32°C (27). If required, kanamycin was used at a final concentration of 50 or 100 μg/ml for *M. xanthus* strains. For the cultivation of *B. subtilis* strains antibiotics were used at the following concentrations: chloramphenicol 5 μg/ml, tetracycline 10 μg/ml, spectinomycin 100 μg/ml and erythromycin at 0.5 μg/ml. *Pseudomonas aeruginosa, Rhodobacter capsulatus, Salmonella enterica and Staphylococcus aureus* strains were grown as previously described (28-31).

*B. subtilis* mutant strain construction. All constructs were first introduced into the domesticated strain PY79 by natural competence and then transferred to the 3610 background using SPP1-mediated generalized phage transduction (32). All strains used in this study are listed in Table 1. All plasmids and primers used in this study are listed in Supplemental Table S1. The *ppsC::tet* insertion deletion allele was generated by long flanking homology PCR (using primers 1270 and 1271, 1272 and 1273), and DNA containing a tetracycline drug resistance gene (pDG1515) was used as a template for marker replacement (33, 34). The *pksL* insertion deletion allele was generated by long flanking homology PCR (using primers 1274 and 1275, 1276 and 1278), and DNA containing a tetracycline drug resistance gene (pAC225) was used as a template for marker replacement (34).

**Predation assays.** *M. xanthus* was grown in CYE medium to mid-log phase. Cells were harvested and washed twice with MMC buffer (20 mM MOPS, pH 7.6, 4.0 mM MgSO₄, 2 mM CaCl₂). *M. xanthus* cells were resuspended in MMC buffer to a final concentration of 2x10⁹
cells/ml. Prey cells were grown overnight in strain specific media to an OD_{600nm} of about 2. Cells were washed twice with water and resuspended in water to a final concentration of 1x10^{11}/ml. Qualitative predation assays were performed on CFL (9) Agar plates. Seven µl of prey cells were 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 (inside-out assay). Assays were incubated at 32°C and pictures were taken after different times to monitor progression of predation.

Quantitative predation assays were performed by mixing prey and predator cells in a ratio of 50:1 and spread plating them on CFL Agar plates. Prey and predator cells were prepared as described above. As controls just prey or predator cells were spread plated on CFL Agar plates. After incubation for 24 h at 32°C cells were harvested and resuspended in water. Serial dilutions were plated on strain selective media and incubated at 32°C for 5 days to quantify predator CFU or at 37°C over night to calculate prey survival. Assays were performed in triplicate. Average CFU and standard deviation were calculated for each experiment. Prey survival and predator growth were calculated as % with prey-only or predator-only controls, respectively, set to 100%.

**Bacillaene extraction and purification.** *Bacillus amyloliquefaciens* CH12 (35) was cultured aerobically at 37°C in LB. Two 4 L flasks containing 1 L each of sterile production medium [100 mM MOPS (pH 7), 5 mM potassium phosphate, 0.5% (wt/vol) sodium glutamate, 0.5% (wt/vol) glycerol, 1 mM MgSO_4, 100 μM CaCl_2, 6 μM MnSO_4, 3 μM FeSO_4] were inoculated with the overnight culture at an initial optical density of OD_{600}=0.008. The flasks were incubated with shaking at 25°C [26 h, 240 rpm, 1 inch throw, light excluded]. All remaining steps were carried out in the dark. Cells were removed by centrifugation (4000 x g, 30 min, 25°C) and the combined supernatants were extracted with 1 volume (2 L) CH_2Cl_2. The organic layer was washed with a saturated NaCl solution to break a partial emulsion and...
evaporated under reduced pressure. The residue was dissolved in 50% MeOH:20 mM NaPi (pH 7) and insoluble particulate was removed by centrifugation (4000 x g, 10 min, 25°C). The clarified culture extract was filtered and chromatographed on an Agilent 1200 HPLC as follows. The crude sample was injected (sequential 50 μl injections) onto a semi-preparative (10 × 250 mm, 5 μm) Phenomenex Luna C18 column equilibrated with 35% ACN:20 mM NaPi (pH 7) and eluted with a gradient program [solvent A: 20 mM NaPi (pH 7), solvent B: acetonitrile; wash post-inject with 35% B for 2 min; ramp to 40% B over 8 min; hold at 40% B for 2 min; return to 35% B over 3 min; re-equilibrate at 35% for 5 min]. Absorbance was monitored at 361 nm, and fraction collection was triggered in the 7-14 minute time window by a minimum threshold absorbance above the baseline. Fractions containing the single major bacillaene peak were pooled and evaporated under reduced pressure. The residue was dissolved in 20% MeOH and passed through a Supelco C18 SPE cartridge for desalting. Bacillaene was eluted from the cartridge with 100% MeOH and the solvent was evaporated under reduced pressure. The residue was dissolved in water and lyophilized. Product was stored desiccated at -80°C until use. Purity was estimated to be ~90% by HPLC, and bacillaene integrity was verified by UV and LC-MS (603 [M+Na]+, 581 [M+H]+, 579 [M-H]-).

**B. subtilis spore preparation.** *B. subtilis* spores were made and purified according to protocols described earlier (36). Briefly, strains were grown for 3 days in DSM sporulation medium (Difco) at 37°C shaking. Sporulated cultures were centrifuged, pellets were washed with ¼ culture volume 1M KCl/0.5 M NaCl and treated with lysozyme (50 μg/ml) at 37°C for 60 min in ¼ culture volume 50 mM Tris-HCl, pH 7.2. Spores were cleaned by alternate washing step with 1 M NaCl, 0.05% SDS, 50 mM Tris-HCL pH 7.2, 10 mM EDTA and 4 washes with H2O. Spores were resuspended to a final concentration of 4x10^8/ml. Predation assays using
mature spores were performed as inside-out assays described earlier on MOPS Agar plates (0.1 M MOPS, pH 7.6).

Microscopy. Predation assays were monitored by microscopy using a Nikon SMZ10000 dissecting microscope. Images were taken using a QImaging Micropublisher CCD camera and processed with QCapture software.
Results

Domesticated Bacterial Strains are Sensitive to Predation by *Myxococcus xanthus*.

To assess whether *M. xanthus* displays a preference for specific prey, we conducted a screen with different bacteria including several isolates of *Staphylococcus aureus*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Rhodobacter capsulatus*, *Escherichia coli* and *Bacillus subtilis* (Figure 1, Table 2). The screen allowed us to verify predation as indicated by *M. xanthus* rippling behavior in the presence of prey on low nutrient agar surfaces (CFL) as described previously (8-10). Prey cells were spotted on agar at high density (7 μl aliquots at 1x10^11 cells/μl). Subsequently, *M. xanthus* predator cells were spotted at a lower density (2 μl aliquots at 2x10^9 cells/μl) into the center of the prey (Figure 1D). The assay conditions provide immediate and direct contact between a minority of predator cells with an excess of prey cells. Successful predation is then indicated by plaque formation or clearing of the prey colony. Predation is accompanied by multicellular rippling (Figure 1E) which is followed by fruiting body formation at the edge of the initial prey spot where consumption of prey generates a step-down in nutrient availability (Figures 1D, 1E) (9). As controls, prey alone (Figure 1A), prey plus buffer (Figure 1B) and prey plus heat-killed *M. xanthus* cells (Figure 1C) were spotted onto CFL agar and did not produce plaques. Thus, plaque formation by *M. xanthus* is an active process that indicates predation has occurred under these conditions (Figure 1D). The results from this screen revealed that *M. xanthus* cells prey on a wide variety of bacteria, including Gram-positive and Gram-negative strains.

The assay described above is not quantitative and may not reveal a preference for any given prey. In order to quantify predation efficiency, we used predator and prey strains encoding selectable markers to allow for accurate determination of prey survival and growth of the
predator over time. For these assays, we chose standard laboratory strains of *B. subtilis* and *E. coli* as prey. We mixed prey cells with *M. xanthus* predator cells and then plated on low-nutrient agar. After 24 h, the mixed population was harvested and plated on rich medium with the appropriate antibiotic to determine colony forming units (CFU) for either the predator or prey.

The results (Table 2) indicated that the vast majority of cells for *E. coli* B2155 and two domesticated strains of *B. subtilis*, 168 and OI1085, were consumed efficiently (< 1.0 % prey survival). As expected, *M. xanthus* predation of *E. coli* and *B. subtilis* supported efficient growth as indicated by an increase in CFU for the predator. *M. xanthus* growth varied slightly depending on the prey source, suggesting a slight preference for Gram-negative over Gram-positive strains. This result is consistent with recent observations made by the Wall and Velicer groups which may reflect differences in prey suitability (21, 37).

**Ancestral *Bacillus subtilis* NCIB3610 is Resistant to Predation by *Myxococcus xanthus*.**

Because *M. xanthus* is a soil dwelling organism, it is unlikely to come in direct contact with the domesticated strains or clinical isolates tested above. Thus, we tested the capacity for *M. xanthus* to prey on *B. subtilis* NCIB3610, an ancestor of the lab strain 168 (Figure 1). Strikingly, NCIB3610 was highly resistant to predation as compared to the domesticated strains, 168 and OI1085. Some lysis of NCIB3610 was observed, but only where *M. xanthus* came into immediate contact with *B. subtilis* as a result of directly spotting the predator onto the prey. This assay shows that the ancestral *B. subtilis* strain is naturally resistant to predation by *M. xanthus*.

The quantitative assay also indicated that ancestral *Bacillus* resists predation by *M. xanthus*. The majority of NCIB3610 cells, 67%, survived predation (Figure 2A). However there was some growth (110% relative to the control) and no cell death displayed for *M. xanthus* in this assay (Figure 2B). Such a modest increase suggests that the killing of NCIB3610 cells is due to
conditions of the assay where both predator and prey are mixed immediately prior to plating. The results also indicate that \textit{B. subtilis} does not kill \textit{M. xanthus} cells under the conditions of this assay. Together the results allow us to conclude that ancestral \textit{Bacillus} NCIB3610 actively resists predation, possibly facilitated by secretion of defensive molecules to inhibit predation by \textit{M. xanthus}.

\textit{Bacillus subtilis} Bacillaene Inhibits Predation by \textit{Myxococcus xanthus}.  

The observation that the ancestral \textit{Bacillus subtilis} NCIB3610 is resistant to predation while domesticated strains were susceptible to predation raised the question as to what factors or properties distinguish the two from each other. We hypothesized that resistance by NCIB3610 would most likely be due to production of inhibitory molecules. A key observation came from a recent study where McLoon \textit{et al} (38) identified five loci responsible for observed differences between \textit{B. subtilis} 168 and NCIB3610. Strain 168 is attenuated for biofilm formation on MSgg agar plates and is defective in swarming motility and EPS production (4, 38, 39). Complementation of 168 to restore biofilm formation on MSgg agar confirmed that the observed defects in 168 were attributable to mutations in \textit{sfp} (polyketide production), \textit{swrA} (swarming motility), \textit{epsC} (exopolysaccharide production), the promoter of \textit{degQ} (secretion of degradative enzymes), and \textit{rapP} (plasmid-encoded phosphatase (40)). In addition a detailed study compared \textit{B. subtilis} legacy strains (including NCIB3610 and the most commonly used laboratory strains) and identified 22 SNPs as the major differences between NCIB3610 and 168 (39). Based on these studies, the mutation in \textit{sfp} seemed to be the most likely candidate affecting predation by \textit{M. xanthus}.

In \textit{B. subtilis}, \textit{sfp} encodes phosphopantetheinyl transferase that post-translationally modifies a serine residue within carrier domains of peptide synthetases (41). Thus, Sfp provides a
necessary early step in the production of several small molecules including the lipopeptide antibiotic surfactin, the phospholipase A$_2$ inhibitor plipastatin and the bacterial protein synthesis inhibitor bacillaene (41-44). To investigate if Sfp is required for production of a small molecule inhibitor, we first generated a NCIB3610 $sfp$ mutant and assayed cells for resistance to predation by $M. xanthus$. The inside-out predation assay indicated that both lysis of prey and subsequent fruiting body formation had occurred (Figure 3A), confirming that Sfp is required for resistance to predation by $M. xanthus$. Furthermore, quantification revealed that the $sfp$ mutant cells cannot survive when mixed with $M. xanthus$ cells under the conditions of this assay (Figure 3B). Likewise, growth of the predator (Figure 3C) corresponded with elimination of the $sfp$ prey cells.

Sfp is required for production of surfactin, plipastatin and bacillaene. Therefore, we generated mutations in genes encoding components required for production of each molecule, $srfAC$, $ppsC$ and $pksL$, respectively. Cells from each mutant strain were tested in the predation assay and quantified for survival and capacity to promote growth of the predator (Figure 3). $M. xanthus$ formed large plaques at the center of the prey colony (Figure 3A) when introduced to $B. subtilis$ cells carrying the mutation in $pksL$ but not when introduced to NCIB3610 cells. Furthermore, quantitative assays indicated that the survival of $B. subtilis$ cells mutated for $pksL$ was reduced to only 1.4% survival following $M. xanthus$ predation. By contrast, the survival of $B. subtilis$ cells mutated for $ppsC$ was 24% and survival for the $srfAC$ mutant was similar to the parent (68%) following $M. xanthus$ predation.

Mutations were also generated to assess the possibility of combinatorial effects. The $ppsC$ $pksL$ double mutant and the $srfAC$ $ppsC$ $pksL$ triple mutant strains displayed 1.74% and 1.1% survival, respectively, similar to the levels displayed by the $pksL$ (1.4%) and $sfp$ (1.6%) single mutant strains (Figure 3B). Thus, cells that cannot produce bacillaene are diminished greatly for
their ability to escape predation by *M. xanthus* cells. In addition, the results indicate that combinations of mutations in *pksL* with either *srfAC* or *ppsC* do not display synergy under the conditions of this assay, revealing that bacillaene, synthesized in part by PksL, promotes *B. subtilis* survival when challenged with *M. xanthus*.

Furthermore, *M. xanthus* growth correlated well with its capacity to kill and utilize *B. subtilis* strains for nutrients. This is evident by the increased growth observed for *M. xanthus* where CFUs following predation correspond to 825% for *sfp*, 407% for *pksL* and 584% for *srfAC ppsC pksL* mutant cells (Figure 3C). These results indicated that *M. xanthus* kills and utilizes susceptible strains and that *B. subtilis* mutants lacking the capacity to produce bacillaene result in the greatest growth for the predator.

We note, however, that there is a difference for predator growth upon consumption of *sfp* mutant cells (825%) versus *pksL* mutant cells (407%), about 2 fold, possibly reflecting a modest level of synergy. Of the single mutants tested, those cells deficient in surfactin production (*srfAC*) resulted in only 106% predator growth, similar to the ancestral parent. In contrast, cells deficient in plipastatin production (*ppsC*) resulted in modest gains, about 210% predator growth, while those cells deficient in bacillaene production (*pksL*) resulted in the greatest amount of predator growth at 407%. Thus, the combination of Sfp dependent secondary metabolites may be additive where plipastatin and bacillaene have the greatest individual affects on predator growth. Nevertheless, the overall affects of individual mutations for these loci result in predator growth that is reciprocally related to their affects on survival. In summary, bacillaene provides a critical contribution to prey survival and corresponding prevention of predator growth, suggesting that production of bacillaene by the ancestral strain, *B. subtilis* NCIB3610, effectively functions as a significant defensive molecule protecting cells from predation by *M. xanthus*.
Support for this conclusion was obtained by mixing purified bacillaene with sensitive prey and challenging those cells with the predator (Figure 4). Cells from predation sensitive, domesticated strains *E. coli* β2155, *B. subtilis* OI1085 and the *B. subtilis* 3610 *pksL* mutant were mixed with bacillaene. The predator was spotted into the middle of the prey spot as described above (Figure 4). As controls, prey cells were mixed with either methanol (used for solubilizing bacillaene) or MMC buffer (Figure 4). The plates were incubated in the dark to avoid light-induced degradation of bacillaene and pictures were taken at 24 h. Addition of bacillaene was observed to provide protection for otherwise sensitive prey relative to controls (Figure 4). Importantly, purified bacillaene did not affect vegetative growth of *M. xanthus* while fruiting body formation was transiently delayed (Figure S1). Together, these results indicate that bacillaene protects susceptible cells from predation without affecting growth of the predator.

**Bacillus subtilis** Spores are Resistant to Predation

One factor affecting interpretation of the quantitative assays is that *B. subtilis* is capable of sporulating under stressful conditions which might render cells resistant to predation by *M. xanthus*. To test this, we assayed the *B. subtilis* spores for their capacity to resist predation by *M. xanthus*. The assays were performed on MOPS agar, completely lacking nutrients, to prevent germination of the *B. subtilis* spores being tested. Spores were purified from *B. subtilis* domesticated OI1085, the ancestral strain NCIB3610 and the NCIB3610 *pksL* mutant using the method described previously (36). Spore suspensions were spotted on MOPS agar and did not germinate under these conditions (Figure 5A). When *M. xanthus* cells were spotted onto the spores, no predation was observed for any of the strains tested (Figure 5B). As a control, vegetatively growing cells for each strain were tested on MOPS agar as prey. As expected, OI1085 and NCIB3610 *pksL* were susceptible to predation on MOPS agar while the NCIB3610...
parent was resistant (Figure 5C). We conclude that sporulation enables *B. subtilis* to escape predation. It is also worth noting that the NCIB3610 *pksL* mutant is competent for sporulation.

Lastly, we assayed for predation of spores on low-nutrient CFL medium. In the control assay, we observed *B. subtilis* outgrowth from spores on CFL agar plates (Figure S2), due to the presence of nutrients in the medium. Because spores from all stains tested were completely resistant to predation, the results indicate that *M. xanthus* preys upon live cells or cellular debris. Furthermore, CFL promotes outgrowth of spores which would render prey susceptible to predation by *M. xanthus*. Thus, any spores formed by *B. subtilis* during predation assays on CFL medium would subsequently germinate, thereby eliminating sporulation as a confounding factor in the quantitation described above.
Discussion

*Myxococcus xanthus* and *Bacillus subtilis* are ubiquitous soil bacteria that produce a wide range of secondary metabolites and sporulate under nutrient-limiting conditions (3, 7, 45). From an ecological perspective, *M. xanthus* is likely to affect the composition and dynamics of microbial communities due to its capacity for predation (21, 37). In this study we observed that bacillaene-producing *B. subtilis* cells are effectively resistant to predation by *M. xanthus* cells. The inhibitory effect of bacillaene provides ample time for those cells to develop into mature spores without becoming prey for *M. xanthus* cells. This conclusion is supported by the fact that addition of purified bacillaene resulted in limited protection of sensitive prey such as *E. coli*. We also conclude that sporulation enables *B. subtilis* to escape predation. It is worth noting that the NCIB3610 *pksL* mutant is competent for sporulation, indicating that *pksL* is not required for sporulation even though Spo0A is known to be required for bacillaene production (46).

Production of secondary metabolites from different soil bacteria has been shown to regulate interactions with their neighbors. For example, *Streptomyces* strain A3 was shown to upregulate secondary metabolite production in the presence of certain bacteria leading to the consumption or lysis of the inducing strains (47). In another example, competition experiments identified surfactin from *B. subtilis* as a negative regulator of aerial hyphae formation and sporulation in *S. coelicolor* (25). Likewise, *M. xanthus* was found to induce secondary metabolite production and aerial mycelium formation in *S. coelicolor* (48). Additionally, *B. subtilis* bacillaene was found to inhibit the production of prodiginines by *S. coelicolor* and to inhibit growth of *S. avermitilis* (25, 26, 49) and, similarly, a co-culturing experiment demonstrated that a bacillaene mutant strain was more susceptible to lysis by *Streptomyces* (50). Together with our results, it is clear that
bacillaene plays a significant role in protection for \textit{B. subtilis} in the natural environment and displays a broad range regarding host susceptibility.

With a large repertoire of genes dedicated to secondary metabolite production, we suspected that \textit{M. xanthus} would be an efficient predator against many bacteria. Our screen to test different species of prey revealed that \textit{M. xanthus} is able to consume both Gram-negative and Gram-positive bacterial species, indicating that cell wall structure is not sufficient for protection from predation. Interestingly, \textit{M. xanthus} was able to consume a wide variety of pathogenic, clinical isolates such as \textit{Salmonella} and \textit{Staphylococcus} strains. Even though \textit{M. xanthus} does not readily prey upon the ancestral \textit{B. subtilis} strain, it efficiently consumes related domesticated strains. The common denominator regarding predation for the domesticated strains we tested here is a defect in phosphopantetheinyl transferase activity. Thus, it appears that sensitive \textit{B. subtilis} strains have simply lost their capacity to produce secondary metabolites which otherwise confer resistance to predation. It is likely that clinical isolates or human commensals which do not naturally encounter \textit{M. xanthus} have developed alternative antimicrobials suited their competitors.

Overall, our results show that bacillaene is a primary defensive molecule generated by \textit{B. subtilis} conferring substantial resistance to predation by \textit{M. xanthus}. Both the structure and biosynthetic pathway for bacillaene have been determined (43, 49) and its regulation is under the control of multiple factors to allow for dynamic control (46). Bacillaene was first described as an inhibitor of bacterial protein synthesis (24) even though our results indicate that predator growth is not affected by the addition of purified bacillaene. Because both \textit{Bacillus subtilis} and \textit{Bacillus amyloliquefaciens} are known to generate bacillaene, it appears that the \textit{pks} cluster has been conserved in this clade, thereby implying a broader role for bacillaene as a defensive molecule.
for some *Bacillus* species (35). It remains unknown as to whether other *Bacillus* isolates will be found to produce bacillaene or a similar derivative with defensive properties. Based on previous studies and our findings presented here, bacillaene appears to play a central role in the regulation of interspecies interactions between *B. subtilis* and *Streptomyces* or *Myxococcus*. Bacillaene transiently inhibits the predatory capacity of *M. xanthus* cells, which enables *B. subtilis* to form spores in the presence of *M. xanthus* cells.
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Figure Legends

Figure 1. *M. xanthus* predation of various prey strains. Shown are predation assays using different Gram-positive and Gram-negative strains as prey for *M. xanthus* predator cells. Efficient predation results in clearing of the prey spot with *M. xanthus* fruiting body formation occurring at the edge of the original prey spot. The prey strains tested were *E. coli* DH5α and B2155, *B. subtilis* OI1085, 168 and NCIB3610, *Rhodobacter capsulatus*, *Salmonella enterica*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Strains resisting predation show only minimal lysis at the center of the prey spot. (A) prey only, (B) prey with buffer spotted at center, (C) prey with heat-killed predator, (D) prey with predator. Pictures were taken at 48 h after spotting at 10x magnification (A-D) or at 30x magnification (E). (A)-(D) bar = 0.5 cm, (E) bar = 0.1 cm.

Figure 2. Quantification of prey survival and predator growth. (A) Prey and predator cells were mixed in a ratio of 50:1, plated onto CFL agar plates and incubated at 32°C for 24 h. Prey and Predator alone were used as controls. CFUs were determined and % prey survival and % predator growth were calculated relative to the controls. The majority of *E. coli* B2155 and *B. subtilis* strains OI1085, 168 were consumed whereas about 68% of *B. subtilis* NCIB3610 survived. (B) *M. xanthus* was able to grow significantly on *E. coli* B2155 and *B. subtilis* OI1085 but not the ancestral *B. subtilis* NCIB3610 strain.

Figure 3. Bacillaene inhibits *M. xanthus* predation. (A) Predation assays using the domesticated, ancestral and mutant strains of *B. subtilis* are shown. Mutations in *sfp* and *pksL* reveal the requirement for bacillaene as the major factor inhibiting *M. xanthus* predation. Pictures were taken 24 h after spotting *M. xanthus* predator cells in the center of the prey source. Bar = 0.1 cm. (B) Prey survival was quantified as described above and normalized to the
NCIB3610 control spotted without the predator (C) Growth of the *M. xanthus* predator was quantified after 24 h. and normalized to *M. xanthus* cells spotted without prey.

**Figure 4. Bacillaene protects sensitive prey.** Predation assays were conducted as described above using sensitive prey mixed with purified bacillaene (left), methanol (center) and MMC buffer (right) on CFL agar plates. Photographs were taken after 24 hours. Sensitive prey were protected in the presence of bacillaene (A). Bar = 0.5 cm.

**Figure 5. Bacillus subtilis spores resist predation.** Spores were made and purified as described in Materials and Methods (36). Spores were spotted on MOPS agar plates lacking any nutrients to prevent spore germination. Predation assays were conducted as shown in Figure 1. Shown are (A) spores alone (B) spores with predator cells and (C) vegetative prey cells with the predator. Domesticated strains as well as the NCIB3610 *pksL* mutant cells are capable of producing predation resistant spores. Bar = 0.5 cm.
Table 1

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<tr>
<td><strong>Myxococcus xanthus</strong></td>
<td></td>
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<tr>
<td>Myxococcus xanthus DZ2</td>
<td>wild type</td>
<td>(51)</td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis OI1085</td>
<td>domesticated strain</td>
<td>(52)</td>
</tr>
<tr>
<td>Bacillus subtilis 168</td>
<td>domesticated strain</td>
<td>(53)</td>
</tr>
<tr>
<td>Bacillus subtilis NCIB3610</td>
<td>ancestral strain</td>
<td>(39)</td>
</tr>
<tr>
<td>Bacillus subtilis DS4085</td>
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<td>this work</td>
</tr>
<tr>
<td>Bacillus subtilis DS4114</td>
<td>ppsC::tet</td>
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<tr>
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<td>(55)</td>
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<td><strong>Other</strong></td>
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<tr>
<td>Escherichia coli DH5</td>
<td>laboratory strain</td>
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<tr>
<td>Escherichia coli Ø2155</td>
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<td>(56)</td>
</tr>
<tr>
<td>Salmonella enterica serovar Typhimurium 6704</td>
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<td>(28)</td>
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<tr>
<td>Pseudomonas aeruginosa 388</td>
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<td>(29)</td>
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<tr>
<td>Staphylococcus aureus MN8</td>
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<td>(30)</td>
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<tr>
<td>Rhodobacter capsulatus SB1003</td>
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<td>(31)</td>
</tr>
<tr>
<td>Prey</td>
<td>% prey survival</td>
<td>% predator growth</td>
</tr>
<tr>
<td>---------------</td>
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<tr>
<td>E. coli ß2155</td>
<td>0.0007 +/- 0.00006</td>
<td>2501 +/- 468</td>
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<tr>
<td>B. subtilis OI1085</td>
<td>0.046 +/- 0.01</td>
<td>1912 +/- 307</td>
</tr>
<tr>
<td>B. subtilis 168</td>
<td>0.293 +/- 0.06</td>
<td>524 +/- 47</td>
</tr>
<tr>
<td>B. subtilis NCIB3610</td>
<td>68 +/- 12.8</td>
<td>110 +/- 20</td>
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<thead>
<tr>
<th></th>
<th>bacillaene</th>
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<tr>
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