Natural variation in developmental life-history traits of the bacterium *Myxococcus xanthus*

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**Abstract**

The soil bacterium *Myxococcus xanthus* is a model for the study of cooperative microbial behaviours such as social motility and fruiting body formation. Several *M. xanthus* developmental traits that are frequently quantified for laboratory strains are likely to be significant components of fitness in natural populations, yet little is known about the degree to which such traits vary in the wild and may therefore be subject to natural selection. Here, we have tested whether several key *M. xanthus* developmental life-history traits have diverged significantly among strains both from globally distant origins and from within a sympatric, centimetre-scale population. The isolates examined here were found to vary considerably, in a heritable manner, in their rate of developmental aggregation and in both their rate and efficiency of spore production. Isolates also varied in the nutrient-concentration threshold triggering spore formation and in the heat resistance of spores. The large diversity of developmental phenotypes documented here leads to questions regarding the relative roles of selection and genetic drift in shaping the diversity of local soil populations with respect to these developmental traits. It also raises the question of whether fitness in the wild is largely determined by traits that are expressed independent of social context or by behaviours that are expressed only in genetically heterogeneous social groups.

**Introduction**

Heritable phenotypic variation allows adaptive evolution to occur and the comparative analysis of such variation in natural populations has been the mainstay of evolutionary biology since Darwin and Wallace. Variable phenotypes include both morphological and behavioural traits and often affect fitness-related interactions with other organisms, within or across species. Intra-specific variation in social interactions has been studied extensively in animals (Krebs & Davies, 1997), but microorganisms are now also known to engage in many and diverse social behaviours (Crespi, 2001; Velicer, 2003; West et al., 2007) that are mediated primarily by the synthesis and export of extracellular compounds that harm (Hibbing et al., 2010) or help (Fuqua & Greenberg, 2002; Lewenza et al., 2002) neighbouring cells. Although knowledge regarding the genetics and biochemistry of microbial social traits has expanded considerably, relatively little is known about the degree to which such traits vary in natural populations (Fortunato et al., 2003; Oda et al., 2003; Vogel, 2003; Davelos et al., 2004; Collier et al., 2005; Fiegn & Velicer, 2005; Kadam & Velicer, 2006; Vosahlkova et al., 2007; Vos & Velicer, 2008b, 2009; Stefanic & Mandic-Mulec, 2009). Characterization of intra-specific variation in fitness-related social phenotypes within local natural populations of microorganisms is necessary for understanding microbial social evolution in the wild.

Quantifiable social traits can be examined within genetically homogeneous or heterogeneous social environments and might be constant or variable across such environments. While microbial social phenotypes expressed in heterogeneous groups have received much emphasis in microbial behavioural ecology (Velicer et al., 2000; Griffin et al., 2004; Kohler et al., 2009), relatively little attention has been paid to the social variation among genotypes as expressed in homogenous social groups. Such variation in the social phenotypes of clonal groups is likely to be important for competition outcomes both when physical and behavioural barriers hinder the migration of genotypes across social
groups (Gibbs et al., 2008; Vos & Velicer, 2009) and in heterogeneous social groups as well (Buttery et al., 2009). *Myxococcus xanthus* (order *Myxococcales*, whose species are collectively known as the myxobacteria) is a prominent model organism for the study of microbial social behaviour (Shimkets, 1990; Kaplan, 2003; Fiegna et al., 2006; Wu et al., 2007; Zusman et al., 2007; Berleman et al., 2008; Vos & Velicer, 2009). *Myxococcus xanthus* cells actively migrate through the soil in search of prey or other food sources using two genetically and mechanistically distinct motility systems, one of which involves cell–cell contact mediated by Type IV pili to function (Kaiser, 2008). Cell swarms of *M. xanthus* secrete a variety of compounds that kill and lyse other microorganisms, thus converting them into growth substrates (Rosenberg & Varon, 1984). Perhaps the most extensively studied *M. xanthus* social trait is fruiting body formation. Upon depletion of growth substrate, intracellular signalling cascades cause cells to aggregate into high-density groups that gradually build up into erect fruiting bodies (Singer & Kaiser, 1995; Kaiser, 2001). While a small minority of cells inside this structure form metabolically quiescent spores that are resistant to heat and desiccation, the rest of the cells either remain as undifferentiated rods or lyse (Wireman & Dworkin, 1977). It has been hypothesized that cell lysis within fruiting bodies provides compounds and/or energy that benefit spore differentiation and maintenance (Wireman & Dworkin, 1977; O’Connor & Zusman, 1988). Although various hypotheses have been formulated regarding the possible benefits of forming spores within social fruiting bodies rather than individualistically (Velicer & Vos, 2009), no study has yet tested these hypotheses rigorously.

Although fruiting body development is likely to be very important for fitness in the wild (Velicer et al., 1998), almost nothing is known about how this striking social trait varies and evolves within myxobacterial species in their natural habitats. In a recent study, 78 clones of *M. xanthus* were isolated from a centimetre-scale soil population in Tübingen, Germany (Vos & Velicer, 2006), among which 21 unique multi-locus sequence typing genotypes were identified. Variation among these centimetre-scale isolates has been documented previously for several traits, including swarm expansion rates and morphologies on multiple surface types, predatory efficiency on diverse prey (Morgan, Maclean & Velicer, unpublished data), secondary metabolite production profiles (Krug et al., 2008) and spore production across diverse social environments (Vos & Velicer, 2009). Here, we have tested whether several key developmental traits have diverged among these centimetre-scale *M. xanthus* isolates as well as among more divergent strains isolated from distant locations. The variation uncovered in this study leads to new questions regarding the evolution of *M. xanthus* development in the wild.

### Materials and methods

#### Strains

Strain GJV1 is a laboratory descendant of the commonly studied reference strain DK1622 (Kaiser, 1979) and is distinguished from it by five accumulated mutations of unknown effect (Velicer et al., 2006). The ‘A’ strains examined here (A9, A12, A17, A23, A30, A41, A45, A47, A66, A75, A82, A85, A94, A96 and A98) were isolated by M. Vos from a 16 × 16 cm grid of soil in Tübingen, Germany (Vos & Velicer, 2006). DK836 was obtained from D. Kaiser and isolated from Albany, New York. The Mxx strains were obtained from H. Reichenbach and were isolated from Olympia, Greece (Mxx12 and Mxx15), and Mt Ar-Li, Taiwan (Mxx144).

#### Bacterial cultures

Bacteria were inoculated from frozen stock cultures into 8 mL of ‘TTT’ liquid medium (a slightly modified version of CTT medium (Hodgkin & Kaiser, 1977) in which casitone was replaced by tryptone) and grown at 32 °C while shaking at 300 r.p.m. for 3–4 days. Cultures that grew after 3 days were diluted to prevent entry into the stationary phase. All cultures were diluted into fresh medium after 4 days of growth and incubated overnight to synchronize growth before the initiation of development. Cultures in the exponential growth phase were centrifuged 15 min at 4500 g and resuspended to a density of ~7.5 × 10⁹ cells mL⁻¹ in TPM buffer (Kroos et al., 1986) before being aliquoted onto TPM agar plates. All plate cultures were kept at 32 °C and 90% RH. All experiments were repeated in at least three temporally independent replicate blocks. Statistical analysis of spore production data was performed with log₁₀-transformed data.

#### Timing of developmental morphogenesis and spore production

Ten microliters of each resuspended culture were placed on a TPM 1.5% agar plate (containing no carbon source) to induce development and then allowed to dry. Plated cultures were photographed after 0, 2, 4, 6, 8, 12, 24, 28, 48, 128 and 168 h using a Nikon Eclipse 90i microscope at 20-fold magnification. Under the microscope lighting conditions used, developmental aggregates were initially translucent and subsequently became distinctly opaque. For the sporulation assays, plates for each strain were harvested after 24, 36, 48, 72, 128 and 168 h using a sterile scalpel blade and washed into ddH₂O. Samples were heated at 50 °C for 2 h and sonicated to kill all nonspore cells. Sonicated samples were diluted into TTT soft agar (0.5% agar) and incubated for 8 days before the colonies were counted.
Morphogenesis and sporulation at variable nutrient levels

Ten microliters of resuspended bacterial cultures were placed on a TPM agar supplemented with 0, 0.32, 3.2 or 10 g L\(^{-1}\) tryptone and allowed to dry. In the morphogenesis assay, plates were photographed at 0, 24, 48 and 72 h after inoculation. In the sporulation assay, developmental cultures were harvested after 72 h, sonicated and diluted into TTT soft agar, and the resulting colonies were counted after 8 days.

Spore heat resistance

Development of strains GJV1, DK836, Mxx144, A66, A75 and A98 was initiated as above. Plates were incubated for 72 h, after which spores were harvested. Samples were heated for 2 h at 50, 55, 60 or 65 °C and subsequently diluted into TTT soft agar after sonication. Colonies were counted after 8 days of incubation.

Phylogenetic analysis

Sequences of the C-signal gene csgA (Hagen & Shimkets, 1990) and putative zinc metalloprotease gene fibA (Kearns et al., 2002) had been obtained previously (Vos & Velicer, 2006) or were generated here (Mxx12) using the same protocol (Genbank accession numbers csgA: DQ411064–DQ411141, fibA: DQ411142–411219). Sequences for forward and reverse primers for csgA and fibA are available upon request. Sequences were aligned using CLUSTALW and adjusted manually. The final alignment included 1196 bp (csgA: 580 bp and fibA: 616 bp). Phylogenetic analyses of each gene individually and both genes combined were performed using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). For the ML approach, we determined the most appropriate model of nucleotide evolution using the Akaike Information Criterion (Hulsenbeck & Rannala, 1997) using MODELCOMP v. 3.7 (Posada & Crandall, 1998). We performed an MP and ML search using a heuristic search setting with random-addition sequences and TBR branch swapping in PAUP*4.0b10 (Swofford, 2003). Support for MP and ML trees was assessed using 1000 bootstrap pseudoreplicates in PAUP*.

BI analyses were performed in MRBAYES v. 3.0b4 software (Hulsenbeck & Ronquist, 2001). For the BI searches, we determined the most appropriate model of nucleotide evolution using the Akaike Information Criterion (Hulsenbeck & Rannala, 1997) using MRMODELTEST v. 1.1b (Nylander, 2004). Four Markov Chain Monte Carlo runs were performed in two simultaneous runs. Analyses were run for 10 million generations, with trees sampled every 1000th generation. Convergence was assessed through the inspection of likelihood values and parameter sample plots using TRACER v. 1.4. The first 10% of the analysis was discarded as burn-in. Bootstrap values > 70% and Bayesian posterior probabilities > 0.95 were interpreted as strong support for a clade (Wilcox et al., 2002).

Results

Variation in the rate of developmental morphogenesis

The 20 strains examined varied significantly in their timing of aggregation and in the darkening of aggregates that subsequently developed into mature, spore-containing fruiting bodies. Although all four global isolates (DK836, Mxx12, Mxx15 and Mxx144) and most local strains showed initial aggregation by 4 h and initial opacity by 6 h (e.g. Fig. 1a), there were notable exceptions. In particular, three local isolates (A66, A75 and A98) exhibited fast morphological
development, with initial opacity occurring after only 4 h (e.g. Fig. 1b). Interestingly, all three of these fast-developing isolates yield higher densities of fruiting bodies than strains that develop more slowly (e.g. Fig. 1b in contrast to Fig. 1a and c).

The lab reference strain GJV1 developed markedly slower than most other strains under our experimental conditions, showing initial opacity after only 8 h and substantial opacity after 12 h. Only one other isolate (A94) consistently showed a similarly slow pattern throughout all replicate blocks and did not become opaque until 12–28 h after the onset of starvation (Fig 1c). Most isolates were highly consistent in the timing and darkening of aggregates across replicates, but a subset of strains (A9, A17, A45) exhibited a large variation in these traits across replicates and yielded zero or very few distinct opaque aggregates in at least one replicate. The overall distribution of the average times of initial aggregation and initial opacity for all strains across all replicate blocks is shown in Fig. 2.

Patterns of variation in developmental timing among strains were not found to reflect patterns of phylogenetic relatedness (Fig. 3). For example, the fast-developing isolates A66, A75 and A98 do not cluster together in a common clade, but are distributed across the phylogeny, as are the two slowest-developing strains, GJV1 and A94. Ancestral character states for the rate of development are difficult to infer for several nodes.

**Variation in the temporal patterns of spore production**

A subset of strains representing slow (GJV1, A9, A94), moderate (DK836, Mxx144, A41, A85) and fast (A66, A75, A98) rates of developmental morphogenesis was also examined for variation in the levels and patterns of spore production at six time points after the onset of starvation ranging from 24 to 168 h. Spore production varied significantly across strains (one-way ANOVA, $F = 26.75$, $df = 9$, $P < 0.001$) and across harvest time points ($F = 11.68$, $df = 5$, $P < 0.001$), but not across replicate blocks ($F = 0.79$, $df = 3$, $P = 0.50$). Figure 4 shows the log-transformed average number of spores for all strains at each harvest time. No strains produced viable spores within 24 h. Four strains first produced a significant number of spores at 36 h ($P = 0.025$, 0.01, 0.039 and 0.001 for DK836, Mxx144, A66 and A98, respectively, $n = 4$ in all cases, one-sample, one-sided t-tests) and one each at 48 h (A75, $P < 0.001$, $n = 4$) and 72 h (GJV1, $P = 0.005$, $n = 4$). The spore counts of strains A9, A41, A85 and A94 (dashed lines) never became significantly greater than zero across replicate blocks at any time point. Spore counts for most strains did not begin to level off substantially or decrease until 72 h, but spore production by Mxx144 increased only between 24 and 36 h and remained (statistically) unchanged thereafter.
Intriguingly, a significant positive correlation was found between the rank order of time to initial developmental opacity and the ranked maximum number of spores produced (Spearman’s rank test, $r_S = 0.89$, $n = 10$). In other words, early aggregating strains tended to rank higher in maximum spore production than slow-developing strains, indicating the absence of a trade-off between the rate and the productivity of development.

**Variation in spore heat resistance**

The number of spores produced that remain viable after 2 h of heating at 50, 55, 60 and 65°C was determined for strains GJV1, DK836, Mxx144, A66, A75 and A98 (Fig. 5). Overall, the number of surviving spores was predicted by temperature treatment, strain and temperature by strain interaction (GLM, $F = 78.94$, $df = 2$, $P < 0.001$; $F = 22.52$, $df = 5$, $P < 0.001$ and $F = 11.54$, $df = 10$, $P < 0.001$, respectively). Sporulation of GJV1, DK836, A66 and A98 was reduced only slightly and nonsignificantly at 55 and 60°C relative to the next lower temperature treatment. In contrast, spore production by strains A75 and Mxx144 declined to a greater degree than other strains at 55°C (relative to 50°C) and plummeted more than 100-fold in both cases at 60°C (relative to 55°C) ($P < 0.025$ for both A75 and Mxx144, $df = 2$, one-sided, two-sample $t$-tests for the difference between 60 and 55°C treatments). All strains produced very few or zero spores capable of surviving the 65°C heat treatment. There is no evidence for a trade-off between rapid development and spore quality (as reflected by heat resistance), as two of the three fast-developing strains (A66 and A98) had high spore counts at 60°C.

**Dependence of spore development on nutrient scarcity**

To determine the effects of variable nutrient levels on the initiation and progress of development, we first examined all 20 isolates at five resource concentrations (0–10 g L$^{-1}$ tryptone). Although a clear variation in multicellular morphology was observed among strains at each nutrient concentration (data not shown), it was not always possible to distinguish between multicellular mounds caused by vegetative growth vs. those caused by developmental aggregation. Subsequent analysis therefore focused on the quantification of spore production at different nutrient concentrations for a subset of six strains (GJV1, DK836, A45, A66, A75 and A94).

Spore production after 3 days of development (Fig. 6) varied significantly across nutrient treatments and nearly significantly among strains (one-way ANOVA; $F = 76.06$, $df = 3$, $P < 0.001$ and $F = 2.29$, $df = 5$, $P = 0.053$, respectively), but not among replicate blocks ($F = 0.06$, $df = 3$, $P = 0.979$). No spores were recovered from plates supplemented with 10 g L$^{-1}$ tryptone for any strain. The average spore count for all strains did not differ significantly between the 0 and the 0.32 g L$^{-1}$ tryptone treatments, but was significantly reduced at 3.2 relative to 0.32 g L$^{-1}$. 

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**Fig. 4.** Log-transformed average number of spores of nine natural isolates of *Myxococcus xanthus* and the standard lab strain GJV1 harvested at different points of time after the onset of development. Dashed lines represent strains whose spore count does not deviate from zero; solid lines indicate significant spore numbers. Error bars represent 95% confidence intervals. The lowest limit of spore detection was at 10 spores per sample. □, GJV1; X, DK836; △, Mxx144; ▲, A9; ◆, A41; ◐, A66; ◆, A75; +, A85; ◊, A94; ■, A98.

**Fig. 5.** Heat resistance of spores of five natural isolates and the lab strain GJV1 shown as the log-transformed average number of surviving spores after 2 h incubation at 50, 55, 60 and 65°C. Error bars represent 95% confidence intervals. The lowest limit of spore detection was at 10 spores per sample. □, GJV1; X, DK836; △, Mxx144; ◐, A66; ◆, A75; ■, A98.
natural microbial habitats is reflected by the variation observed in laboratory studies is a difficult, but important open question. Variation documented in the lab may be affected by differential ‘preadaptation’ of strains to laboratory conditions (Velicer & Lenski, 1999) that might mask details of how variation is expressed in natural habitats. Thus, inferences made from phenotypic variation in the lab for understanding variation in fitness strategies in nature ought to be conservative. Nonetheless, it seems unlikely that the major developmental differences observed among the strains examined here are purely laboratory artefacts.

Previous studies have demonstrated large fitness asymmetries between natural M. xanthus isolates mixed at the onset of development (Fiegna & Velicer, 2005; Vos & Velicer, 2009), despite the fact that the strains used in those studies exhibited similar levels of total spore production in clonal culture. While actively antagonistic behaviours between strains are required to explain some of the competition outcomes observed in these studies [e.g. the mutual extinction of paired genotypes (Fiegna & Velicer, 2005)], large fitness asymmetries in mixed cultures might also result from intrinsic behavioural differences (i.e. differences manifest in pure culture) between competitors that are masked by simple assays of spore production in pure culture at a given point in time (Buttery et al., 2009). For example, two strains that produce similar numbers of spores at a given time in pure culture (e.g. strains A75 and A98 at 168 h, Fig. 4) might develop at markedly different rates in a manner unaffected by social environment. In mixed competition, the faster-developing strain might monopolize signalling molecules produced by both strains and thereby largely exclude the slower-developing competitor from producing viable spores.

Discussion

A clear understanding of the evolutionary processes occurring in natural populations of microorganisms requires a detailed characterization of heritable variation in fitness-related traits. We tested whether quantifiable components of M. xanthus development have diverged significantly among several natural isolates since the isolates diverged from their most recent common ancestor. Extensive heritable variation was found in all developmental traits examined, including total spore production, rate of developmental aggregation, temporal patterns of spore formation, effect of multiple nutrient levels on spore production and the stress resistance of spores. This variation was found not only among relatively divergent strains isolated from sites separated by large distances but also among highly similar strains isolated from a centimetre-scale patch of soil. Patterns of phenotypic variation were not found to reflect patterns of phylogenetic relatedness, suggesting that the traits examined here evolve rapidly relative to the sequence concatemer used to measure genetic relatedness. Future work will be required to explain the relative roles of natural selection and genetic drift in determining the range of developmental variation documented here in local soil populations.

The frequency with which M. xanthus engages in multi-cellular development in natural habitats is unknown, as is the contribution of development to overall fitness (Velicer & Hillesland, 2008). Relaxed selection on developmental proficiency rapidly leads to the evolution of socially defective genotypes (Velicer et al., 1998; Zhang et al., 2005), so the globally widespread presence of developmentally competent strains (Vos & Velicer, 2008a) indicates that social proficiency is highly beneficial in many habitats. Variations in selective forces across space and time may have generated the diverse developmental phenotypes documented here, either directly by acting on the traits per se or indirectly by acting on other traits linked by pleiotropy to those examined here.

The degree to which the phenotypic variation present in natural microbial habitats is reflected by the variation observed in laboratory studies is a difficult, but important open question. Variation documented in the lab may be affected by differential ‘preadaptation’ of strains to laboratory conditions (Velicer & Lenski, 1999) that might mask details of how variation is expressed in natural habitats. Thus, inferences made from phenotypic variation in the lab for understanding variation in fitness strategies in nature ought to be conservative. Nonetheless, it seems unlikely that the major developmental differences observed among the strains examined here are purely laboratory artefacts.

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Fig. 6. Log-transformed average number of spores harvested after 72 h development on plates containing four different concentrations of the nutrient tryptone (0, 0.32, 3.2 and 10 g L\(^{-1}\)) for six local and global isolates and the lab strain GJV1. Tryptone concentration is plotted on a log scale. Error bars represent 95% confidence intervals. The lowest limit of spore detection was at 10 spores per sample. □, GJV1; X, DK836; ●, A45; ○, A66; V, A75; Ø, A94.

tryptone (two-sample, two-sided \(t\)-test; \(df = 17, P < 0.001\)). DK836 and A94 were the only strains to show significant declines in spore production at 0.32 g L\(^{-1}\) tryptone relative to zero tryptone (\(P < 0.025\) for DK836 and A94, one-sided \(t\)-tests, \(df = 3\)). Declines in spore production at 3.2 g L\(^{-1}\) tryptone (relative to lower tryptone levels) were relatively small for strains DK836, A66 and A75, but were very large for GJV1 ( > 1000-fold decrease relative to 0.32 g L\(^{-1}\) tryptone), A45 and A94 (complete loss of spore production at 3.2 g L\(^{-1}\) tryptone in both cases).
Such a scenario could result in a competitive outcome indistinguishable from that between two strains that exhibit similar temporal patterns of spore production in pure culture, but engage in some form of interference competition in chimeric groups. Our data demonstrate the existence of variation in intrinsic developmental traits (e.g. rate of development) that might cause major fitness asymmetries in chimeric groups without interference competition.

The relationship between the amount of carbon substrate available for vegetative growth and developmental spore production varied dramatically even among the centimetre-scale natural isolates examined here (Fig. 6). Thus, these strains have diverged in the operation of the key regulatory pathway that controls how M. xanthus transitions from vegetative growth to fruiting body development. While some of the major components of this pathway have been identified (Harris et al., 1998; Crawford & Shimkets, 2000; Yu & Velicer, 2010), an understanding of the genetics and biochemistry of the transition from growth to development remains incomplete. Analysis of the natural variation in this transition and future identification of the genetic diversity that underlies this phenotypic variation may help elucidate the fundamental biology of the gateway between growth and development.

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References


Collier FA, Elliot SL & Ellis RJ (2005) Spatial variation in Bacillus thuringiensis cereus populations within the phyllosphere of broad-leaved dock (Rumex obtusifolius) and surrounding habitats. FEMS Microbiol Ecol 54: 417–425.


Kaiser D (1979) Social gliding is correlated with the presence of pili in Myxococcus xanthus. P Natl Acad Sci USA 76: 5952–5956.


