**Supplemental Material**

# "Evolution of social conflict in the bacterium *Myxococcus xanthus*: centimeter vs global scale populations"

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## Experimental Procedures

### Strains

Three sets of strains are distinguished in this study. The first set consists of 78 *M. xanthus* clones isolated from a 16 x 16 cm soil plot in Tübingen, Germany that were described previously [7]. A total of 21 genotypes were distinguished among these clones on the basis of sequence fragments from the *csgA*, *fibA* and *pilA* genes. A second set of strains ("local isolates" above) is a subset of the 78 cm-scale isolates. Rather than being selected randomly from the 78 cm-scale isolates, these nine clones were selected to represent some of the smallest and largest pair-wise genetic distances present among the larger group of 78 isolates. These nine isolates (A9, A23, A30, A41, A47, A75, A85, A94 and A96) were used in the co-development mixing assays described here. Fragments of four additional loci were sequenced in these nine clones in order to calculate pair-wise genetic distances (see 'Sequencing'). The third set of strains ("global isolates" above) consists of nine strains isolated from around the globe that were previously assayed for antagonisms during development [6]. All loci sequenced in the local population clones were also sequenced in the global set.

Antibiotic resistance markers

All nine local isolates used in the co-development assays were marked with antibiotic resistance in order to obtain spore counts for each competitor after mixed development. Kanamycin-resistant variants of strains were obtained for five strains (A23, A30, A47, A75 and A96) by electroporation with the plasmid pREG1727, which integrates into the chromosome at the Mx8 attachment site [33]. (No transformants with pREG1727 could be obtained for A9, A41, A85 and A94.) Rifampin-resistant clones were obtained for all nine strains by selecting for spontaneous mutants. Kanamycin and rifampin concentrations used were 40 and 5 μg/ml, respectively). All antibiotic resistant clones used here exhibited proper fruiting body formation (unpublished data) and similarly high levels of spore production (Fig. S1). No significant marker effects on pure culture spore production were detected (Fig. S1 and unpublished data).

Developmental competition assays

The overall design of the sporulation assays described in Fiegna & Velicer [6] was also employed here with the local isolates. Cells in log phase growth were centrifuged and pellets resuspended in buffer to a density of ~5 x $10^9$ cells per ml. To initiate starvation in the pure culture controls, 100 μl of the cell suspension was dispensed on the center of a buffer agar plate. For co-development treatments, equal volumes of each competitor were mixed and 100 μl of the resulting suspension was dispensed on a TPM plate, making the total cell number and density the same for both pure- and mixed-culture treatments. The development plates were incubated for five days (32 ºC, 90% rH), after which the plate populations were harvested with a sterile scalpel. Harvested cells were incubated in 1 ml of sterile $ddH_2O$ at 50 ºC for two hours to

select for viable spores. Samples were sonicated by microtip to disperse spores, diluted in $ddH_2O$ and plated in soft nutrient agar containing the appropriate antibiotic.

Developmental competition calculations

Spore count data were analyzed as in Fiegna & Velicer [6]. The proportion of cells surviving development as spores in pure culture is the sporulation efficiency (*D*) of a given strain *i*:

$$(1) \qquad D_i = N_i(t_5) \,/\, N_i(t_0)$$

where $N_i(t_0)$ is the number of cells before development is initiated ($5 \times 10^8$ for pure culture assays) and $N_i(t_5)$ is the number of viable spores harvested after five days of development. The sporulation efficiency of strain *i* in mixture with strain *j* is then given as:

$$(2) \qquad D_i(j) = N_i(j, t_5)/N_i(j, t_0)$$

The effect of mixing strains *i* and *j* on the sporulation efficiency of strain *i*, termed the one-way mixing effect, is given as:

$$(3) \qquad C_i(j) = \log(D_i[j]) - \log(D_i)$$

Thus, when strain *i* sporulates less efficiently in the presence of strain *j* than it does in pure culture, $C_i(j)$ will be negative.

The relative sporulation efficiency ($W_{ij}$) of two strains is defined as the log difference of the strains' sporulation efficiencies in mixed competition with each other:

$$(4) \qquad W_{ij} = \log(D_i[j]) - \log(D_j[i])$$

The fitness ranks of paired competitors are determined by the direction of $W_{ij}$. Strain $i$ is dominant if $W_{ij}$ is positive, whereas strain $j$ is dominant if $W_{ij}$ is negative.)

The effect of mixing two strains on total group spore production is termed the 'bidirectional mixing effect' ($B_{ij}$). It is the total (log-transformed) number of spores produced by two strains in mixture relative to the total number of spores produced by the two strains in clonal isolation.

$$(5) \qquad B_{ij} = \log\left([N_i(j, t_5) + N_j(i, t_5)]/[N_i(t_5)/2 + N_j(t_5/2])\right)$$

Pure-culture spore counts are divided by two to account for the fact that ~2.5 x $10^8$ cells of each competitor were present in mixed cultures, whereas ~5 x $10^8$ cells of each strain were present in pure-culture controls. If mixing has no effect on spore production, the argument of the log function is one and $B_{ij}$ is zero. A negative $B_{ij}$ value represents a negative effect of chimaeric mixing on total spore production. The average $C_i(j)$, $W_{ij}$ and $B_{ij}$ value for each strain was tested for significant deviation from zero using a one-sample $t$-test ($n = 8$). The three co-development parameters ($C_i(j)$, $W_{ij}$ and $B_{ij}$) were arcsin-transformed when performing regressions with genetic distance to meet model assumptions.

Dominance hierarchy

The overall structure of pair-wise fitness ranks among the nine local isolates could vary from complete linearity to complete circularity. Only with a significantly linear structure of dominance ranks is the competitive relationship between two strains predictive of competitive outcomes between either of those strains and other partners. Kendall's technique [13] was used to quantify the degree of linearity (also termed 'transitivity') among competitive outcomes during co-development. This technique calculates the parameter $K$, which can range from zero (representing complete non-transitivity) to one (complete transitivity). The number of circular triads (or three-strain rank comparisons with non-transitive fitness ranks) in the group, termed $d$, is used to test for the significance of $K$ [13].

Sequencing

The initial genetic characterization of the 78 cm-scale Tübingen isolates was based on sequence fragments of the *csgA*, *fibA* and *pilA* genes [7]. To obtain genetic distance estimates with greater resolution for the nine isolates used in the co-development experiment, fragments of four additional loci were sequenced for this study: *clpX* (ATP-dependent Clp protease ATP-binding subunit), *icd* (isocitrate dehydrogenase), *rpoD* (sigma factor 70) and *sglK* (*dnaK* homologue HSP70 chaperone). A concatemer of all loci (minus the highly variable *pilA* locus that was only used to differentiate between clones) was constructed to calculate genetic distances for all pair-wise strain combinations using the Kimura 2-parameter. The same was done for the set of nine global isolates for which no sequence data had previously been obtained. The use of multiple loci dispersed across the genome buffers for possible past recombination events and allows for a reliable estimate of

genetic distance between strains. NCBI GenBank accession numbers are: EU836739 - EU836831. This excludes several Tübingen isolate sequences that have been deposited previously; their accession numbers can be found in the original publication [7]. Primer sequences are available upon request.

Developmental competition assays

Strains were inoculated from frozen stocks onto CTT hard agar plates [34], incubated for 2-4 days (32 ºC, 90% rH), and then transferred into 6 ml of CTT liquid medium (1% casitone, 8 mM $MgSO_4$, 10 mM Tris-HCL, pH 7.6, 1 mM potassium phosphate, pH 7.6). After two days of growth (32 ºC, 300 rpm, with dilution as necessary to maintain log phase growth) culture cell densities were estimated based on optical density measurements. Culture samples (2 ml) were centrifuged (4 min., 12K rpm, room temperature) and the resulting cell pellets resuspended in TPM buffer [35] to a density of ~5 x $10^9$ cells per ml (TPM, recipe as for CTT but without casitone). TPM agar (1.5% agar) was used for co-development assays.

All mixes were performed at least three times in temporally independent blocks. Independent pure-culture controls of paired competitors were performed simultaneously with each mixed-culture assay. In four cases, one (A23-A85 and A47-A85) or two (A30-A85, A30-A96) additional blocks were performed because of relatively high variation in the data from the first three blocks. In cases of mixed cultures where no colonies grew at the lowest dilution factor ($10^1$), a value of ten spores was entered to provide an upper-limit estimate of spore survival. Two mixes were performed simultaneously for competition pairings in which only a rifampin-resistant variant was available for both competitors (A9 vs. A41, A85, and A94, A41 vs. A85 and A94, and A85 vs. A94). In these cases, the response of the rifampin-

resistant variant of each strain to being mixed with the unmarked variant of its paired competitor was quantified [6].

## Swarming allorecognition assays

Swarming allorecognition (or incompatibility) tests were performed on 78 cm-scale isolates clones to investigate whether paired isolates engage in behaviors that prevent merging of their swarms. Two 40 μl spots of log-phase culture of each isolate were placed adjacent to one another on CTT soft (0.5%) agar so that the two spots merged slightly at their interface. In experimental treatments the two spots on a plate contained different isolates, whereas in controls both spots contained the same isolate. Control and experimental treatments were performed simultaneously. Plates were dried in a laminar flow hood, incubated for two days at 32 ºC, 90% rH and then examined for the presence or absence of a clearly discernable line of demarcation between the paired isolate swarms. Seven genotypes were represented by a single clone (A2, A75, A79, A81, A82, A83, A98) and thus could not be staged with a different clone of the same genotype.

Table S1. Average one-way ($C_i[j]$) mixing effect, bidirectional $B_{ij}$ mixing effect and relative sporulation efficiency ($W_{ij}$) by strain

| **Variable** | **A75** | **A47** | **A41** | **A96** | **A94** | **A23** | **A30** | **A85** | **A9** |
|---|---|---|---|---|---|---|---|---|---|
| mean $B_{ij}$ | -0.16 | -0.08 | -0.36 | 0.06 | -0.19 | -0.17 | -0.27 | 0.06 | -0.07 |
| mean $C_i(j)$ | 0.26* | 0.04 | -0.17 | -0.16 | -0.11 | -1.61 | -2.86* | -1.88* | -2.52** |
| mean $W_{ij}$ | 1.58* | 1.71 | 1.52 | 1.96 | 0.72 | -0.96 | -2.00 | -1.84 | -2.68** |

Asterisks denote *p*–values for one-sample *t*-tests for a difference from zero: underlined, $p < 0.10$, * $p < 0.05$, ** $p < 0.01$. Clones are listed in order of their position in the co-development dominance hierarchy (Table S2).

Table S2. Relative sporulation efficiencies ($W_{ij}$)

| Strain | A75 | A47 | A41 | A96 | A94 | A23 | A30 | A85 | A9 |
|---|---|---|---|---|---|---|---|---|---|
| | (7) | (6) | (6) | (5) | (3) | (3) | (3) | (2) | (1) |
| **A47** | 0.87 | | | | | | | | |
| **A41** | 0.40 | -1.06 | | | | | | | |
| **A96** | 0.74 | 1.16 | 1.56 | | | | | | |
| **A94** | 0.50 | 0.68 | -1.23 | 0.59 | | | | | |
| **A23** | 1.41** | 6.16** | 0.82 | 4.83* | -0.23 | | | | |
| **A30** | 4.73* | 3.20P | 4.42*** | 2.58 | 5.43*** | -2.13 | | | |
| **A85** | -0.33 | 2.88 | 3.80 | 5.69** | 1.15 | 2.43 | 1.28 | | |
| **A9** | 4.31* | 1.51 | 2.11 | 5.43*** | -0.05 | 5.05* | 0.93 | 2.15 | |

For each comparison, $i$ represents the column strain and $j$ represents the row strain. Strains are ranked from left to right by the number of wins (i.e. positive $W_{ij}$ values, in brackets) each strain accrued during its pair-wise competitions against the other eight strains. Asterisks denote $p$–values for one-sample $t$-tests for a difference from zero: underlined, $p < 0.10$ (marginally nonsignificant) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Strains A47 and A41 share the same rank number of wins (6), as do A94, A23 and A85 (3) and are listed in rank order of average $W_{ij}$ values (Table S1).

Table S3. Swarming allorecognition isolate pairings

Each table below represents a set of clones from the 78 cm-scale isolates that share a common *csgA-fibA-pilA* concatemer genotype. Colors represent distinct allorecognition types within each genotype set. All distinct genotypes are also distinct allorecognition types. Isolate pairings that generated a clear line of demarcation between clonal swarm groups are indicated by an 'X' and pairings in which swarms merged into one visually undifferentiated swarm are indicated by the "=" symbol.

MLST genotype 1

| | A0 | A6 | A7 | A23 | A26 | A32 | A39 | A46 | A47 | A49 | A58 | A60 | A64 | A92 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A0 | | | | | | | | | | | | | | |
| A6 | X | | | | | | | | | | | | | |
| A7 | X | X | | | | | | | | | | | | |
| A23 | = | X | X | | | | | | | | | | | |
| A26 | X | X | = | X | | | | | | | | | | |
| A32 | = | X | X | = | X | | | | | | | | | |
| A39 | X | X | X | X | X | X | | | | | | | | |
| A46 | = | X | X | = | X | = | X | | | | | | | |
| A47 | X | X | = | X | = | X | X | X | | | | | | |
| A49 | = | X | X | = | X | = | X | = | X | | | | | |
| A58 | X | X | X | X | X | X | X | X | X | X | | | | |
| A60 | = | X | X | = | X | = | X | = | X | = | X | | | |
| A64 | X | X | X | X | X | X | X | X | X | X | X | X | | |
| A92 | = | X | X | = | X | = | X | = | X | = | X | = | X | |
| A96 | X | = | X | X | X | X | X | X | X | X | X | X | X | X |

MLST genotype 2

| | A15 | A16 | A30 | A31 | A34 | A36 | A44 | A51 | A56 | A59 | A62 | A69 | A72 | A77 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A15 | | | | | | | | | | | | | | |
| A16 | X | | | | | | | | | | | | | |
| A30 | X | X | | | | | | | | | | | | |
| A31 | X | X | X | | | | | | | | | | | |
| A34 | X | X | X | = | | | | | | | | | | |
| A36 | X | X | X | X | X | | | | | | | | | |
| A44 | X | X | = | X | X | X | | | | | | | | |
| A51 | X | X | X | X | X | X | X | | | | | | | |
| A56 | X | X | X | = | = | X | X | X | | | | | | |
| A59 | X | X | X | X | X | X | X | X | X | | | | | |
| A62 | X | X | X | X | X | X | X | X | X | X | | | | |
| A69 | X | X | X | X | X | X | X | X | X | X | X | | | |
| A72 | X | X | = | X | X | X | = | X | X | X | X | X | | |
| A77 | X | X | X | = | = | X | X | X | = | X | X | X | X | |
| A93 | X | X | X | X | X | X | X | = | X | X | X | X | X | X |

MLST genotype 3

| | A3 | A10 | A13 |
|---|---|---|---|
| A3 | | | |
| A10 | X | | |
| A13 | X | = | |
| A27 | X | = | = |

MLST genotype 4

| | A5 | A24 | A80 |
|---|---|---|---|
| A5 | | | |
| A24 | X | | |
| A80 | = | X | |
| A91 | X | X | X |

MLST genotype 5

| | A9 | A19 | A61 |
|---|---|---|---|
| A9 | | | |
| A19 | = | | |
| A61 | = | = | |
| A71 | = | = | = |

MLST genotype 6

| | A11 | A21 | A57 |
|---|---|---|---|
| A11 | | | |
| A21 | = | | |
| A57 | X | X | |
| A94 | X | X | X |

MLST genotype 7

| | A42 | A53 | A85 |
|---|---|---|---|
| A42 | | | |
| A53 | = | | |
| A85 | = | = | |
| A86 | = | = | = |

MLST genotype 8

| | A1 | A33 |
|---|---|---|
| A1 | | |
| A33 | = | |
| A45 | = | = |

MLST genotype 9

| | A4 | A67 |
|---|---|---|
| A4 | | |
| A67 | = | |
| A95 | X | X |

MLST genotype 10

| | A11 | A21 |
|---|---|---|
| A11 | | |
| A21 | X | |
| A57 | = | X |

MLST genotype 11

| | A17 | A76 |
|---|---|---|
| A17 | | |
| A76 | X | |
| A89 | = | X |

MLST genotype 12

| | A38 | A65 |
|---|---|---|
| A38 | | |
| A65 | = | |
| A73 | = | = |

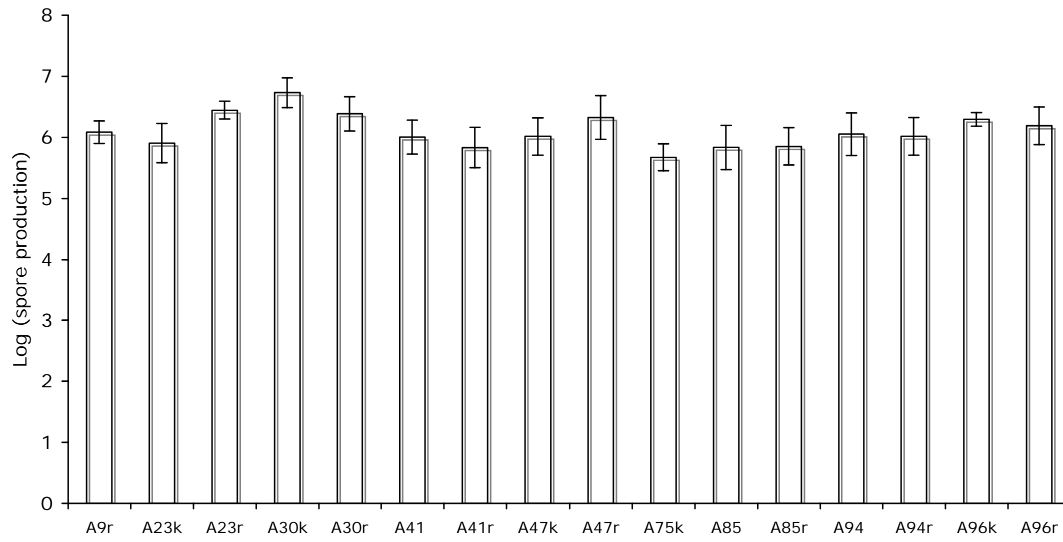
MLST genotype 13

| | A66 | A88 |
|---|---|---|
| A66 | | |
| A88 | X | |
| A99 | X | X |

MLST genotype 14

| | A25 |
|---|---|
| A25 | |
| A41 | X |

Figure S1. Pure culture sporulation of strains used in developmental competition experiments. Kanamycin and rifampin resistance is represented by ‘k’ and ‘r’, respectively. Error bars represent 95% confidence intervals.



**Additional results: swarming allorecognition assay**

The patterns of swarm exclusion between similar local isolates are also evident when swarms undergo starvation on buffered (TPM) agar in the absence of a carbon source for growth (data not shown). In addition to forming fruiting bodies, starving populations swarm outward, allowing us to observe that swarms exclude one another under developmental conditions as well as under conditions favourable for growth.

In a previous study, we estimated that a total of 26 *csgA*-*fibA*-*pilA* concatemer genotypes were present in the 16 x 16 cm sampling plot of soil based on the sampling accumulation curve of the 21 genotypes present among the 78 isolates [7]. Extrapolation from the genotypic diversity suggests that at least 55 distinct *M. xanthus* allorecognition types were present in the 256 $cm^2$ patch of soil at the time of sampling ((26/21) x 45 chemotypes).