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

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Summary

Social interactions among microbes that engage in cooperative behaviours are well studied in laboratory contexts [1,2], but little is known about the scales at which initially cooperative microbes diversify into socially conflicting genotypes in nature. The predatory soil bacterium *Myxococcus xanthus* responds to starvation by cooperatively forming multi-cellular fruiting bodies in which a portion of the population differentiates into stress-resistant spores [3,4]. Natural *M. xanthus* populations are spatially structured [5] and genetically divergent isolates from distant origins exhibit striking developmental antagonisms that decrease spore production in chimaeric fruiting bodies [6]. Here we show that genetically similar isolates of *M. xanthus* from a centimeter-scale population [7] also exhibit strong and pervasive antagonisms when mixed in development. Negative responses to chimerism were less intense, on average, among local strains than among global isolates, although no significant correlation was found between genetic distance at multi-locus sequence typing (MLST) loci and the degree of social asymmetry between competitors. A test for self/non-self discrimination during vegetative swarming revealed a great diversity of distinct self-recognition types even among identical MLST genotypes. Such non-self exclusion may serve to direct the benefits of cooperation to close kin within diverse populations in which the probability of social conflict among neighbours is high.

Keywords

Myxococcus xanthus; social development; cooperation; kin discrimination

Results and Discussion

Biological incompatibilities take diverse forms: reproductive isolation across species [8], non-cooperation between social groups such as insect colonies both across and within species [9] and self/non-self recognition systems that discriminate between closely related individuals [10–12]. Some microorganisms engage in complex forms of social cooperation, such as fruiting body formation in the prokaryotic myxobacteria and eukaryotic slime molds,

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but do so preferentially with identical or highly similar genotypes and exhibit reduced cooperation with (or outright antagonism toward) divergent conspecifics [6,13]. However, little is known about the spatial and genetic scales at which social incompatibilities evolve in natural populations of social microbes.

Myxococcus xanthus is a predatory bacterium that kills and eats other microbes (and also consumes organic polymers), by secreting toxic and lytic compounds [14]. Upon starvation, cells aggregate and exchange signals in the process of forming multi-cellular fruiting bodies [3]. Some cells within a fruiting body become stress-resistant spores, whereas others undergo autolysis or remain undifferentiated [4,15]. This process of social development is likely maintained by kin selection because fruiting body construction can increase group-level fitness among close relatives [16–18].

Fiegna and Velicer (2005) [6] investigated whether cooperation extends across divergent *M. xanthus* genotypes. Nine isolates of *M. xanthus* that originated from distant global locations were mixed in all 36 possible pair-wise combinations and the effect of mixing on their spore production was quantified. Mixing was found to negatively affect the sporulation of one or both strains in most pairings, thus demonstrating that *M. xanthus* has diverged into many socially incompatible genotypes.

Natural populations of *M. xanthus* are spatially structured, with genetic variation within local (e.g. below meter-scale) populations greatly reduced relative to variation between populations separated by large distances ($>10^2 - 10^3$ km) [5]. Such spatial structuring of diversity in large sexual organisms is central to models of how reproductive isolation evolves (i.e. sympatric vs. allopatric modes of speciation [8]). Analogously, spatial structuring of social microbe populations raises the question of whether barriers to cooperation between distinct genotypes originate from divergence in sympatry, allopatry or both. We addressed this question by repeating the experiments of Fiegna & Velicer (2005) [6] with nine isolates collected from a 16 × 16 cm soil plot in Tübingen, Germany and comparing the results from these local cm-scale isolates to those from the global isolates (all strains listed in Fig. 1).

A previous study of 78 *M. xanthus* clones isolated from this cm-scale plot revealed 21 distinct genotypes based on sequences from three loci (*csgA*, *fibA*, and *pilA*) [7]. Seven of those 21 genotypes are represented among the nine isolates examined here, while three strains (A23, A47 and A96) share the same concatemer genotype. The genetic distances for all strain pairs within both the local and global isolate sets were calculated based on a concatemer of six MLST loci (Supplemental Experimental Procedures), which was also used to generate a tree of phylogenetic relationships among strains in which all of the Tübingen cm-scale isolates form a clade that does not include any of the global isolates (Fig. 1). The average pair-wise genetic distance among the local isolates (0.007) was significantly lower than that for global isolate set (0.012, $p = 0.013$ for global vs. local difference, two-tailed t-test, $n = 35$).

The co-development experiments performed here (see Supplemental Experimental Procedures) allowed us to test whether patterns of developmental incompatibility among relatively similar isolates at a local scale differ from those among relatively divergent isolates at the global scale. Our co-development assays allow rigorous quantification of social incompatibilities among strains, but were not designed to mimic how distinct genotypes actually interact in the soil. Because direct encounters between conspecifics are likely to occur during vegetative swarming via gliding motility, we also scored whether isolates distinguish self from non-self during active growth on nutrient agar.

Sporulation mixing effects

The effect of mixing two strains *i* and *j* on the sporulation efficiency of strain *i* was quantified by the one-way mixing effect parameter $C_i(j)$ (Supplemental Experimental Procedures). A negative $C_i(j)$ value indicates that strain *i* sporulates less efficiently in the presence of strain *j* than it does in pure culture, whereas positive values indicate that the presence of strain *j* increases the sporulation efficiency of strain *i*. In 11 of the 36 pair-wise mixes performed among the local Tübingen isolates, the reciprocal $C_i(j)$ values for paired competitors were both negative, whereas in the remaining 25 mixes one clone responded negatively to mixing while the winning competitor responded positively. In contrast, only 13 of the 36 global isolate winners had positive $C_i(j)$ values. Strikingly, some instances of strong antagonism associated with positive responses to mixing occurred between identical concatemer genotypes. For example, strains A23, A47 and A96 share a common MLST genotype, but both A47 and A96 strongly inhibit A23 sporulation while themselves sporulating more efficiently in the presence of A23 than in isolation (Fig. 2). On average, genetic chimaerism strongly reduced individual spore production (relative to clonal performance) among the centimetre scale isolates (average $C_i(j) = -1.00$, $p < 0.001$, one-sample *t*-test for difference from 0, $n = 35$) (Fig. 3).

The effect of mixing strains on total group spore production is represented by the bidirectional mixing effect parameter B_{ij} (Supplemental Experimental Procedures). Among the local isolates, B_{ij} was neither significantly negative for any individual strain (Table S1) nor on average across all nine strains (mean B_{ij} local $= -0.13$), indicating that total group productivity was not severely affected by mixing of distinct clones. (For many local isolate pairings, the $C_i(j)$ value for the losing competitor was strongly negative, whereas the corresponding value for the paired winning competitor was very small and/or positive, with the result that total group productivity was largely unaffected. Thus, the average $C_i(j)$ for local strains can be significantly negative while the average of B_{ij} is not (Fig. 3) because the former includes the individual responses from both the winner and loser in each pair.)

Relative sporulation

The fitness parameter W_{ij} quantifies the sporulation efficiency of strain *i* relative to that of *j* during co-development (Supplemental Experimental Procedures). It thus reflects any asymmetry in spore production by two strains when they undergo development together. The average W_{ij} of developmental competition losers among the local isolates was found to be very large (−2.33, Fig. 3) revealing stark fitness differences (>100-fold on average) among these neighboring strains during codevelopment. W_{ij} correlates strongly with the difference between paired $C_i(j)$ and $C_j(i)$ values (parameter C_{ij} in [6], $r^2 = 0.913$, $p < 0.001$), but not with differences between pure-culture sporulation measures ($\log(D_i/D_j)$, Pearsons $r = 0.041$, $p = 0.813$), indicating that fitness asymmetries in mixture are caused primarily by chimerism *per se* rather than by pure-culture sporulation abilities. As for the global isolates [6], the hierarchy of developmental fitness ranks among local isolates (Table S2) was significantly linear (Kendall's technique [19], $K = 0.57$, $p = 0.045$). This result reflects a prevalence of transitive (A beats B and C and B beats C) rather than non-transitive (A beats B and B beats C, but C beats A) fitness relationships among all possible 3-way strain comparisons.

Local vs. global comparisons

The local and global strain sets differed significantly in two of the three parameters measured here ($C_i(j)$ and B_{ij}). While average $C_i(j)$ was significantly negative within both the local and global [6] data sets, the negative response of the global strains was significantly greater than that for the local isolates (Fig. 3, $p = 0.008$ for difference, 2-tailed *t*-test, $n = 35$). Moreover, the effect of mixing on total group spore production was significantly negative among the global isolates [6] but not among the local strains (Fig. 3, $p < 0.001$). The average W_{ij} of developmental competition losers was slightly lower among global isolates than among local strains, but not significantly so (Fig. 3, $p = 0.12$). The greater negativity of mixing effects on individual and group spore production among the global isolates examined here suggests that social incompatibilities are generally more severe among isolates from allopatric origins than among sympatric strains.

Developmental incompatibility vs. genetic distance

The high degree of antagonism in the local population revealed by the $C_i(j)$ and W_{ij} parameters (Fig. 3) suggests that social incompatibilities often evolve among recently diverged lineages. Indeed, some of the largest competitive asymmetries documented here occur among the least divergent strains examined (A23, A47 and A96, Fig. 1, Fig 2), suggesting that overall genomic divergence (as represented by MLST loci) fails to predict the magnitude of fitness asymmetries among strains. Although average genetic diversity and the magnitude of mixing parameters $C_i(j)$ and B_{ij} were greater among the global isolates (see above and Fig. 3), neither of these parameters nor the fitness asymmetry parameter W_{ij} were found to correlate significantly with pair-wise genetic distance at MLST loci, either for the local and global data sets combined ($C_i(j)$ $r^2 = 0.00$, $p = 0.36$; W_{ij} $r^2 = 0.01$, $p = 0.69$; B_{ij} $r^2 = 0.03$, $p = 0.13$) or within either data set analyzed individually (results not shown).

Kin discrimination during vegetative growth

Different *M. xanthus* genotypes are likely to encounter one another during active swarming through soil. To test whether swarms of different clones merge upon encounter or remain separate through some mechanism of kin discrimination (or ‘allorecognition’), we scored the presence or absence of swarm boundaries between paired clones swarming on nutrient agar (Supplemental Experimental Procedures). This assay is a modified form of the Dienes test, which is a highly discriminatory typing method originally developed to identify swarming *Proteus mirabilis* strains [20, 21]. In this assay, distinct isolates were spotted next to each other on an agar surface and the interface of their respective growing swarms was examined to determine whether they merged into a single continuous swarm or if a line of demarcation formed between them.

Initial experiments indicated that all 21 *csgA/fibA/pilA* concatemer genotypes among the 78 Tübingen cm-scale isolates were distinct allorecognition types (i.e. swarms of distinct genotypes never freely merged). Subsequently, all isolates with identical concatemer genotypes were tested for allorecognition (Table S3). Fourteen genotypes were each represented by multiple (2–15) isolates, whereas seven genotypes were represented by a single clone (A2, A75, A79, A81, A82, A83, A98) and thus could not be staged with a different isolate of the same genotype. A total of 260 such within-genotype comparisons revealed 38 distinct incompatible types among the 71 clones that share a common genotype with at least one other isolate (e.g. between A23 and A47, Fig. 4). Thus, a total of 45 unique allorecognition types appear to be present in our collection of 78 cm-scale isolates (including the seven genotypes unique to single isolates).

Discussion

This study reveals that evolutionary diversification of a highly cooperative bacterium into many antagonistic genotypes has occurred among genetically highly similar individuals living in sympatry. Closely related clones of *M. xanthus* clones occupying the same centimeter-scale soil patch exhibit high levels of conflict when undergoing mixed social development (Fig. 2, Fig 3) and kin discrimination while swarming during vegetative growth (Fig. 4). Such discrimination of self from non-self has also been observed between genetically distinct swarms of the γ-proteobacteria *Proteus mirabilis* [20,21] and *Pseudomonas aeruginosa* [22].

Although interactions among local strains were strongly antagonistic, our data suggest that divergence in allopatry augments incompatibilities that first evolve in sympatry (Fig. 3). Spatially isolated populations may have undergone unique patterns of antagonistic social coevolution and/or some form of local adaptation (e.g. adaptation to distinct prey communities). Distinct evolutionary effects of different local selective conditions may contribute to increased incompatibility across vs. within local populations.

Chimaeric organisms can arise through grafting in vertebrates [23], somatic fusion in invertebrates [24], vegetative fusion in fungi [25,10] or aggregation in slime molds [11,13]. In systems where this process occurs, non-self recognition systems are often in place to limit fusion to genetically identical or closely related individuals. The great diversity of self-recognition types in *M. xanthus* appears to be analogous to these eukaryotic systems.

We propose that non-self exclusion between distinct swarms of *M. xanthus* may be a selected trait that benefits genotypes that would be inferior competitors under conditions of direct cell-cell contact within genetically heterogeneous groups. Our experiments show that strains co-existing in the soil often have dramatically asymmetric fitness values under conditions of direct cell-cell competition during development and that fitness ranks among these strains are strongly hierarchical under our laboratory conditions. Were distinct clonal groups to merge freely in the soil, our data suggest that a small number of competitively superior genotypes would come to dominate within local areas and thus reduce local diversity relative to a scenario in which different clonal groups are able to maintain swarm integrity upon encounter. The ability to prevent territorial invasion by antagonistic genotypes could serve as a general xenophobic strategy [16] to prevent susceptible genotypes from being overtaken by superior competitors that engage in antagonistic and/or exploitative behaviors [6,26].

In a manner analogous to *M. xanthus*, starvation induces individual amoebae of the predatory slime mold *Dictyostelium discoideum* to aggregate and form multicellular fruiting bodies. Aggregating populations first form migrating slugs, which then transform into stalked, spore-bearing fruiting bodies [28,27]. Despite their similar responses to starvation, *M. xanthus* and *D. discoideum* differ in their predatory phase. *M. xanthus* cells usually remain in dense swarms bound together by extracellular polysaccharides [29] and individuals rarely leave established groups to move alone [30]. Such social cohesion promotes high relatedness when cells deplete local resources and initiate development. In contrast, *D. discoideum* predation is individualistic [31], making genotypic interspersion during predation and the probability of chimeric coaggregation at the onset of development more likely than in *Myxococcu*s. Even with solitary predation, only a minority of *Dictyostelium* fruiting bodies in nature appear to be chimeric [17]. When *D. discoideum* genotypes do coaggregate in nature, the degree of cooperation between them has been found to decrease with increasing genomic divergence [32]. This result stands in stark contrast to our findings with *Myxococcus*, in which mixing of swarms is strongly binary, with even very closely related strains being able to discriminate and exclude each other.

The molecular basis of the developmental antagonisms and territorial exclusion among *M. xanthus* clones documented here remains to be characterized, but might involve differential production of diffusible secondary metabolites [33], altered cell-surface chemistry [21] or both. [34]. The strains examined here exhibit a wide variety of secondary metabolite production profiles, and even the genetically most similar strains (A23, A47 and A96) produce different metabolite combinations [34]. Consistent with their higher level of genetic diversity, the global isolates from Fiegna & Velicer (2005) [6] exhibit a greater diversity of metabolite profiles than do the local Tübingen isolates [30], which may contribute to the larger reduction in total spore production observed among mixtures of the global isolates.

This study, as well as previous work on *P. mirabilis* [20 and 21 and *P. aeruginosa* [22], suggests that intra-specific allorecognition is commonplace in natural populations of swarming bacteria. Moreover, our results show that such non-self exclusion, as well as antagonism during multicellular development, evolves among very closely related, sympatric genotypes. This finding is consistent with the discovery of single mutations that strongly affect social interactions between mutants and their parents in both *M. xanthus* [26,35] and *P. mirabilis* [21]. Exclusion of genetically distinct strains promotes high relatedness within local groups, which in turn favors the evolution and maintenance of social cooperation and multicellular complexity [1,12].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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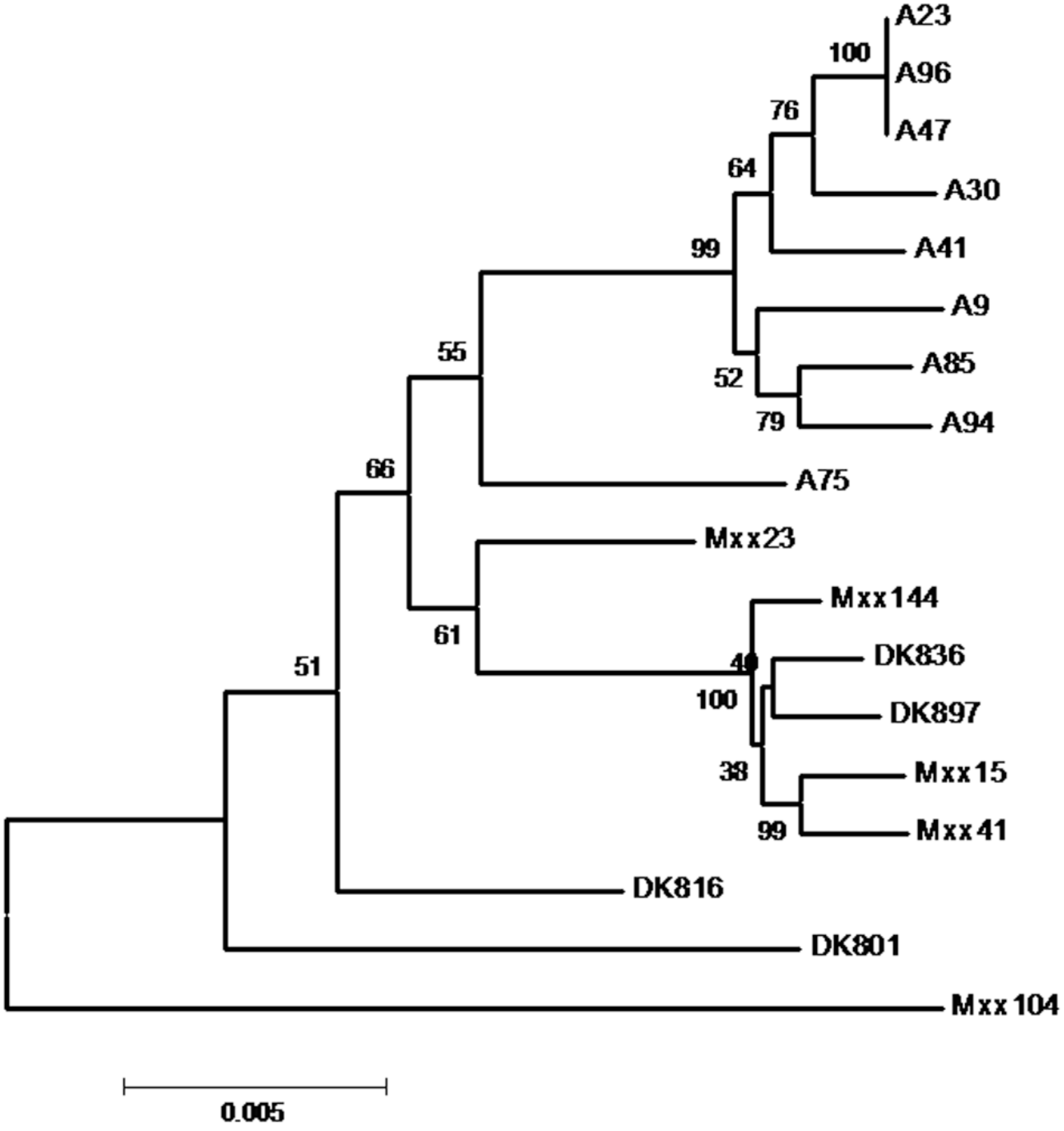

Figure 1.

Neighbor-joining tree of nine local strains from Tübingen (prefix A) and nine global isolates (prefixs Mxx and DK). Bootstrap values (10,000 replicates) are indicated next to nodes. Number of base substitutions per site is indicated below the scale bar.

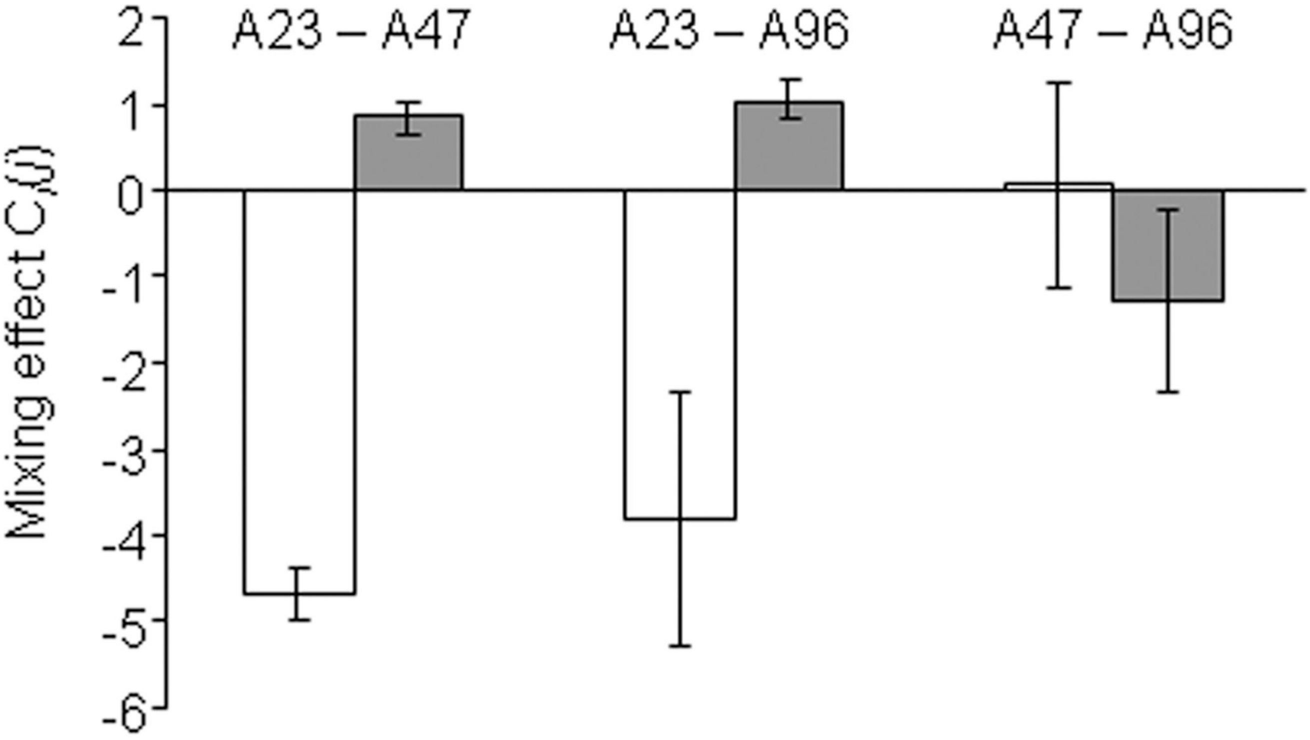

Figure 2.
Co-development interactions among the three most closely related local isolates (A23, A47 and A96). The effect of mixing two clones on the (log-transformed) sporulation efficiency each is given as $C_i(j)$ (with '*i*' referring to whichever strain is being considered). Open bars show the mixing effect on the first clone in each pair and grey bars show the effect on the second clone. Error bars represent 95% confidence intervals.

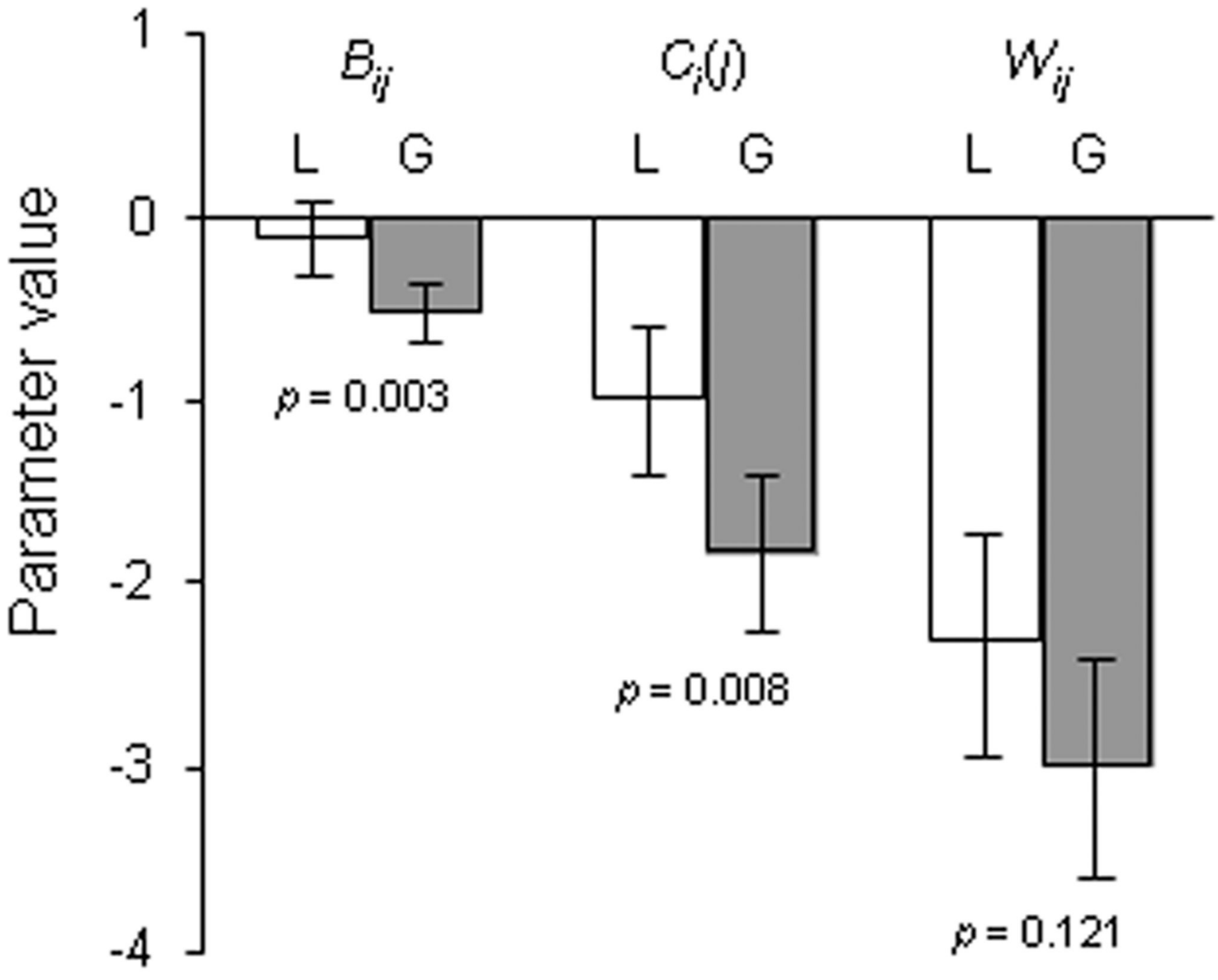

Figure 3.

A comparison of average one-way ($C_i[j]$) mixing effect, bidirectional B_{ij} mixing effectand relative sporulation efficiency (W_{ij}) parameters between the local (L) isolates and global (G) isolates. Error bars represent 95% confidence intervals.

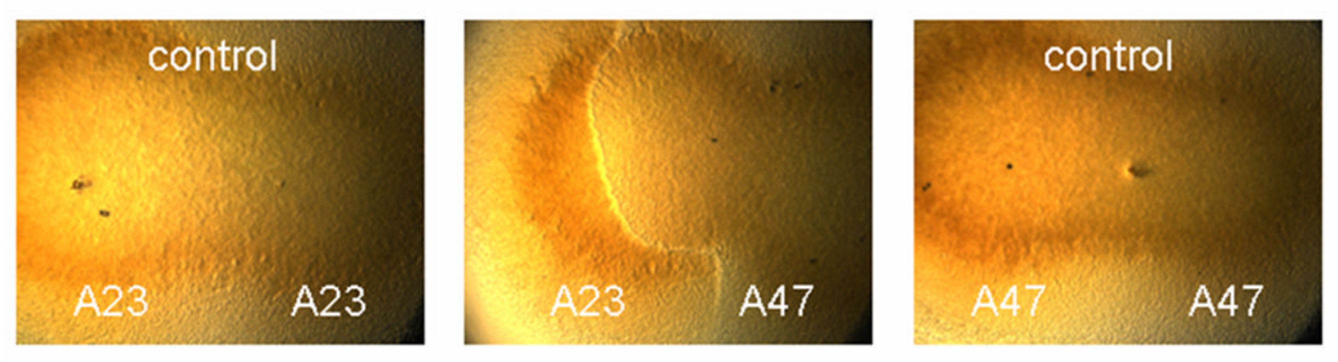

Figure 4.

Kin recognition between vegetatively growing swarms. A clear line of demarcation is evident between swarms of isolates A23 and A47. The control treatments show two swarms of a single isolate that have merged into a continuous swarm with no line of demarcation.