Chimaeric load among sympatric social bacteria increases with genotype richness

Helena Mendes-Soares1,†, I-Chen Kimberly Chen1,2, Kara Fitzpatrick1 and Gregory J. Velicer1,2

1Department of Biology, Indiana University, Bloomington, IN 47405, USA
2Department of Environmental Systems Science, ETH Zürich, Zürich, Switzerland

The total productivity of social groups can be determined by interactions among their constituents. Chimaeric load—the reduction of group productivity caused by antagonistic within-group heterogeneity—may be common in heterogeneous microbial groups due to dysfunctional behavioural interactions between distinct individuals. However, some instances of chimaerism in social microbes can increase group productivity, thus making a general relationship between chimaerism and group-level performance non-obvious. Using genetically similar strains of the soil bacterium Myxococcus xanthus that were isolated from a single centimetre-scale patch of soil, we tested for a relationship between degree of chimaerism (genotype richness) and total group performance at social behaviours displayed by this species. Within-group genotype richness was found to correlate negatively with total group performance at most traits examined, including swarming in both predatory and prey-free environments and spore production during development. These results suggest that interactions between such neighbouring strains in the wild will tend to be mutually antagonistic. Negative correlations between group performance and average genetic distance among group constituents at three known social genes were not found, suggesting that divergence at other loci that govern social interaction phenotypes is responsible for the observed chimaeric load. The potential for chimaeric load to result from co-aggregation among even closely related neighbours may promote the maintenance and strengthening of kin discrimination mechanisms, such as colony-merger incompatibilities observed in M. xanthus. The findings reported here may thus have implications for understanding the evolution and maintenance of diversity in structured populations of soil microbes.

1. Introduction

Genetic diversity can affect biological productivity positively or negatively at all levels of organization: community [1–4], population [5], social [1,2,4–7] and organismal [8]. For example, under some conditions yeast strains that differ in their ability to produce the enzyme invertase, which converts sucrose into glucose and fructose, yield a larger total population when they are grown together in chimaeric groups than does any one strain grown in clonal culture under the same conditions [9]. Chimaeric groups of the social bacterium Myxococcus xanthus can produce more or fewer spores than do homogeneous groups [10–12], at least across some frequency ranges. Negative interactions among different phage genotypes co-infecting a host can cause total phage populations to crash due to intense resource competition [13].

Bacterial populations are highly diverse and individual species can be represented by dozens of distinct genotypes at local scales [8,14–17]. Genetically distinct conspecific cells will co-occur in chimaeric local groups due to mutation and possibly migration, and are likely to interact in ways that affect fitness [9,18,19]. Such intra-specific interactions may promote or diminish genetic diversity [20] and may even affect the persistence of whole populations [21]. If interactions among neighbours that are likely to encounter one another are predominantly negative, then the resulting chimaeric load is expected to
increase selection for mechanisms to prevent co-aggregation of distinct genotypes into common social groups.

Myxococcus xanthus is a widely distributed soil proteobacterium [22] that exhibits two major life-history stages: vegetative growth and multicellular fruiting body development. During growth in the presence of nutrients, vegetative cells swarm in groups by gliding motility [23] and cooperatively prey upon other microbial species [24]. Under starvation conditions, the developmental cycle is initiated and fruiting bodies filled with stress-resistant spores are formed [24,25]. Group movement, fruiting body development and predation are all considered to be social behaviours, as they involve coordinated action among cells [26,27].

Total group performance by M. xanthus can be greatly affected by group composition. Using nine M. xanthus clones isolated from distant locations hundreds to thousands of kilometres apart, Fiegna & Velicer [12] showed that mixed M. xanthus groups composed of just two strains tended to suffer substantial chimaeric load [16] (i.e. such mixed groups produced fewer spores than would be expected based on the performance of their constituent clones in single-genotype groups). Such mutual harm between partners forced to socially interact by experimenters after presumably evolving independently for long periods without interacting [28] is perhaps not surprising. Gradual divergence of the spatio-temporal dynamics and molecular details of developmental motility and signalling among geographically (and thus socially) isolated M. xanthus lineages is likely to generate social differences to which such strains respond dysfunctionally when they are experimentally forced to interact.

Although natural M. xanthus populations harbour substantial diversity even in very small areas (e.g. centimetre-scale soil patches [11] and even within individual fruiting bodies [16]), such small-scale diversity is much lower than diversity present across larger sampling scales [11,15,16,28]. Chimaeric load might correlate with the overall genetic distance between interacting strains. Consistent with this hypothesis, Vos & Velicer [11] found no significant effect of chimaerism on the mean total group spore production among pairs of genetically similar centimetre-scale isolates of M. xanthus, a result that contrasts with the substantial chimaeric load previously observed among more divergent strains [12]. Other than genetic distance, the total number of genotypes in a group (i.e. genotype richness) might also affect the degree of chimaeric load on a social trait that occurs during developmental slug migration by the eukaryotic amoeba Dictyostelium discoideum [18].

The centimetre-scale genotypes examined here were isolated from a population in which they may have been likely to encounter one another over time due to both active and passive migration within the sampled soil patch [15]. On agar medium, swarming colonies of these isolates often fail to freely merge [11], suggesting that mechanisms of kin discrimination are pervasive between different genotype groups in the soil patch. The potential for chimaeric load to result from the merger of genetically distinct groups may generate selection favouring such kin discrimination phenotypes. Although no overall effect of chimaerism on the total spore production was previously found in groups composed of just two strains each from this centimetre-scale sample set [11], greater within-group heterogeneity may cause chimaeric load. Using five isolates from our focal centimetre-scale population, we tested whether increasing within-group genotype richness generates chimaeric load by creating both homogeneous groups and chimaeric groups composed of two to five strains and measuring their group-swarming rate on both nutrient agar medium and lawns of prey, as well as spore production upon starvation. We also tested for any correlation between group-level performance and the average within-group genetic distance among these local isolates.

2. Material and methods

(a) Bacterial strains

In this study, the strains referred to as A, B, C, D and E correspond to strains A9, A41, A47, A66 and A85 of Vos & Velicer [15], respectively. The strains from Vos & Velicer [15] represent a collection of 78 isolates that were sampled from a 16 × 16 cm grid of soil in Tübingen, Germany [15]. These five strains were chosen because they existed in close proximity in nature, are phylogenetically diverse with respect to the 78-strain collection from which they were selected [15] (figure 1) and all show similarly high levels of spore production during pure-culture development [11] (figure 2). However, these strains were all found to be genetically distinct based on analysis of three loci involved in M. xanthus social traits (csgA, fibA and pilA [15]) and were also found to each represent distinct colony-merger social compatibility types during swarming on agar medium [11]. We describe these strains as sympatric because distinct csgA-fibA-pilA concanavalin A genotype names used in the paper originally describing these strains [15] are indicated in parentheses. The bar scales for the average number of nucleotide substitutions per site.

![Figure 1. Genealogical tree of the strains used in this study. The identification names used in the paper originally describing these strains (15) are indicated in parentheses. The bar scales for the average number of nucleotide substitutions per site.](http://rspb.royalsocietypublishing.org/ Downloaded from http://rspb.royalsocietypublishing.org/ on April 8, 2017)

(b) Developmental assays

Liquid cultures of M. xanthus were maintained in CTT (1% casitone, 8 mM MgSO4, 10 mM Tris.HCl, 1 mM potassium phosphate, pH adjusted to 7.6) liquid [34] at 32°C with constant shaking (300 rpm). To initiate all experiments, a liquid culture of M. xanthus in mid-growth phase (approx. 2–3 × 10^6 cells ml^-1) were centrifuged for 15 min at 4500 g and re-suspended in TPM starvation buffer (10 mM Tris, pH 8.0, 8 mM MgSO4, 1 mM KPO4) [35] to a density of 5 × 10^6 cells ml^-1. Several mixing treatments corresponding to different degrees of chimaerism were then created (table 1), with all strains at the same frequency in any given mixture. For each assay, 10 μl of resuspended culture was dispensed in the centre of TPM starvation plates (1.5% agar) for starvation, thus holding the total initial cell number and density constant across all pure and chimaeric treatments. Development plates were...
incubated for 5 days, after which the cell populations were harvested with a scalpel blade, transferred into 1 ml ddH2O and heated at 50°C for 120 m to select for viable spores. Samples were then sonicated by microtip and diluted into CTT soft agar (0.5% agar). In cases where no colonies grew at our lowest dilution factor, a value of five total spores per assay was entered for data analysis, corresponding with the lower limit of detection [12].

(c) Swarming assays
Swarming assays were performed to measure rates of colony territory expansion in the presence and absence of prey. On standard nutrient-rich agar medium without prey cells, hydrolyzed casein serves as the growth substrate and does not require extracellular digestion by the predator. In predatory swarming assays, a standardized biovolume of prey was plated on TPM starvation plates as the sole added growth substrate.

To prepare prey suspensions, the prey was inoculated from a frozen stock into 8 ml of fresh R2 medium [36] and grown for 16 h at 32°C with constant shaking (300 rpm). Cells were then re-suspended to a standard biovolume of approximately $10^8$ µl$^{-1}$ in TPM liquid. One hundred microlitres of these cultures were then plated on previously prepared LB 1.5% agar plates (15 ml LB, plates prepared 24 h prior to inoculation of prey) and incubated at 32°C, 90% rH. After 48 h of incubation, cells were harvested with a scalpel blade into 10 ml of TPM buffer, centrifuged and resuspended into fresh TPM buffer to a biovolume of approximately $10^{10}$ µl$^{-1}$.

For the motility assays with CTT agar, plates with either 0.5 or 1.5% agar were prepared. For the predation assays, prey lawns were prepared by spreading 100 µl of prey suspension (approx. $10^{10}$ µm$^3$ml$^{-1}$) onto TPM 1.5% agar plates (15 ml TPM, prepared 24 h prior to inoculation). To initiate all experiments, a liquid culture of M. xanthus in growth phase (approx. $2-3 \times 10^8$ cells ml$^{-1}$) was centrifuged and resuspended in TPM liquid to a density of $5 \times 10^7$ cells ml$^{-1}$. Several treatments corresponding to different degrees of chimaerism were then created (table 1), with all strains at the same initial frequency in any

![Figure 2](http://rspb.royalsocietypublishing.org/Downloaded from http://rspb.royalsocietypublishing.org/)
given treatment. After 30 min at room temperature, 10 μl of total culture suspension (approx. 5 × 10^9 cells ml⁻¹) were spotted on the plate centre and allowed to dry for 60 min. Covered plates were then incubated at 32°C, 90% rH. Swarm edges were outlined 1 and 5 days after inoculation, and distances migrated along two perpendicular diameters at random orientation over this period were measured.

(d) Phylogenetic analysis
The DNA sequences of the genes csgA, fibA and pilA for each of the strains used here [15] were aligned based on their corresponding protein sequences with MUSCLE implemented in MEGA v. 5.0 [37]. Sequences of these genes in each strain were concatenated in the same order as their relative positions in the genome of M. xanthus strain DK1622 (csgA-pilA-fibA). Pairwise evolutionary distances among five strains were estimated using the maximum composite likelihood model in MEGA v. 5.0.

The genealogical tree was reconstructed by Bayesian analysis run in MrBayes v. 3.1.2 for 10 000 generations with two parallel independent searches, using a GTR + invgamma model [38]. The concatenated dataset used was partitioned into gene regions. Within each gene region, sites were partitioned according to their codon positions to allow evolutionary rates to be potentially different across positions.

(e) Mean comparisons and correlation analyses
All assays were performed in at least four, and in most cases five, temporally independent replicates. Statistical analyses were performed with the software package R.

To test for generic effects of chimaerism per se, the mean value of each trait for clonal treatments and the mean for all chimaeric treatments were first calculated for each of the five replicates. Differences between the clonal versus chimaeric cross-replicate means of the within-replicate means were then tested for with paired t-tests. Tests of differences between actual spore production by specific chimaeric groups and expected spore production based on the pure-culture spore production of group constituents were performed with one-tailed Wilcoxon rank-sum tests. Expected spore production for each chimaeric group was calculated as the average of pure-culture spore production values (prior to log-transformation) of group constituents. Tests of differences between spore production by chimaeric groups inclusive versus non-inclusive of specific strains were also performed with Wilcoxon rank-sum tests. All mean comparisons were performed with log_{10}-transformed data.

For tests of correlations between trait values and group richness, independent correlation coefficients were calculated for each of five replicates for all six quantified traits. Cross-replicate means of the resulting coefficient estimates were subsequently tested for significant deviation from the null expectation of zero using one-sample t-tests. Possible correlations between the mean pairwise genetic distance of group constituents and trait performance were tested separately for each of the three intermediate richness treatments (two, three and four group members). For swarming assays, mixed-group swarming rate estimates were divided by the pure-culture swarming rate of the fastest swarming member of each respective group and the resulting values were used to test for a correlation with mean pairwise genetic distance between group members across all groups within each richness treatment. For sporulation data, prior to correlation analysis with genetic distance, actual spore production estimates for chimaeric groups were divided by their expected spore production values, calculated as described above.

3. Results
Neighbouring strains isolated from a 16 × 16 cm patch of soil were used to test the effects of within-group diversity on total group performance for several social behaviours observed in the soil bacterium M. xanthus. The genomes of these strains are distinct but nonetheless relatively similar to one another compared with their average divergence from strains isolated from other sites located distances away from our focal patch much greater than the distances between the within-patch isolation points [15,28]. When tested in isolation, these five strains all exhibited a high degree of proficiency at the quantified traits (figure 2) but showed significant variation in swarming rate on prey-free nutrient-rich CTT 1.5% agar (ANOVA F_{4,25} = 14.68, p < 0.001) and prey-free TPM 1.5% agar (ANOVA F_{4,25} = 6.791, p = 0.016), and marginally significant variation in developmental proficiency as represented by spore production (Kruskal–Wallis $\chi^2 = 8.89$, p = 0.064).

Groups composed of two to five different strains (table 1) were then created, and the performance of these chimaeric groups at swarming through lawns of prey bacteria, swarming across three types of prey-free agar plates (substrate-rich hard and soft agar, and starvation buffer plates) and efficiency at producing spores were quantified. All of the clonal and chimaeric treatments had the same total density of cells, and the strains in each mix were present at equal frequencies. Considering all chimaeric treatments combined, chimaerism per se had negative effects on predatory swarming through lawns of each prey type and on swarming rate on TPM starvation agar, but not on swarming rate in either the nutrient-rich prey-free environment or on sporulation (table 2). The estimate of average swarming rate across all chimaeric groups on soft CTT agar was actually more than 10% higher than that of pure groups, but the difference was not significant (table 2).

We then tested whether the magnitude of chimaeric load correlated with the number of strains in a group—genotype richness—for all traits. Swarming rate on both prey species (figure 3), on CTT 1.5% agar and on TPM-buffered agar all exhibited significantly negative correlations with group genotype richness, as did the total group spore production.
whereas swarming on CTT 0.5% agar did not (table 3). Group performance was not found to correlate negatively with the mean pairwise genetic distance among group members (across the csgA-fibA-pilA concatemer) for any trait within any richness level (electronic supplementary material, table S1).

We also examined whether spore production was lower than expected under the null hypothesis of no chimaeric load for each chimaeric combination individually. Only five of the 26 distinct chimaeric groups showed significantly reduced spore production (figure 4). Strain C, but no other strain, was present in all five of these groups, suggesting that this strain may contribute more to chimaeric load during development than others.

The distribution, size and shape of fruiting bodies varied greatly across chimaera treatments (data not shown). In some treatments, small immature aggregates were present concurrently with large mature fruiting bodies, suggesting that chimaerism may have slowed the rate of development in these cases. For chimaera ABC, fruiting bodies formed in only two of the four assays performed.

4. Discussion

In nature, many conspecific genotypes can coexist at small geographical scales and are bound to interact within chimaeric groups [5,6]. We used several socially proficient strains of the soil bacterium M. xanthus isolated from close proximity to one another in the soil [15] to assess the relationship between genotype richness and group performance at multiple cooperative behaviours exhibited by this species. We found that chimaeric load increased with genotype richness for predatory swarming, for group motility on nutrient-rich hard agar and starvation agar, and for developmental spore production, but not for group motility on nutrient-rich soft agar (table 3). The mean pairwise genetic distance among chimaera constituents across the three loci examined here (csgA, fibA and pilA) was not found to predict the magnitude of chimaeric load for any of the six quantified phenotypes (electronic supplementary material, table S1). This latter result suggests that reduced group function may be caused by divergence at a small number of focal social genes that underlie affected phenotypes in a manner not reflected by overall genomic divergence [39]. This finding is also consistent with previous results showing that many centimetre-scale M. xanthus isolates that are genetically identical at csgA, fibA and pilA nonetheless exhibit colony-merger incompatibilities during group swarming [11], although it is not yet known whether the loci responsible for such incompatibilities

**Table 2.** Average swarming rates (mm day$^{-1}$) and spore production of clonal and chimaeric groups.

<table>
<thead>
<tr>
<th>condition</th>
<th>clonal average</th>
<th>chimaeric average</th>
<th>p-values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. citreum</td>
<td>0.28</td>
<td>0.13</td>
<td>0.011</td>
</tr>
<tr>
<td>E. coli</td>
<td>4.51</td>
<td>3.52</td>
<td>0.016</td>
</tr>
<tr>
<td>CTT 1.5% agar</td>
<td>2.07</td>
<td>1.84</td>
<td>0.060</td>
</tr>
<tr>
<td>CTT 0.5% agar</td>
<td>0.67</td>
<td>0.76</td>
<td>0.110</td>
</tr>
<tr>
<td>TPM-buffered agar</td>
<td>0.93</td>
<td>0.67</td>
<td>0.002</td>
</tr>
<tr>
<td>log$_{10}$ (no. spores)</td>
<td>5.51</td>
<td>4.76</td>
<td>0.105</td>
</tr>
</tbody>
</table>

*p-Values significant below the p = 0.05 criterion are shown in italic.

**Figure 3.** Effects of chimaerism on predatory swarming. Average swarming rates on C. citreum and E. coli lawns for groups of variable genotypic richness are shown. Error bars represent the standard error of the mean.

**Table 3.** Correlations between trait values and within-group genotype richness.

<table>
<thead>
<tr>
<th>trait</th>
<th>mean r across replicates</th>
<th>p-value for mean r versus 0*</th>
</tr>
</thead>
<tbody>
<tr>
<td>predatory swarming on C. citreum</td>
<td>-0.627</td>
<td>0.057</td>
</tr>
<tr>
<td>on E. coli</td>
<td>-0.930</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>swarming on CTT</td>
<td>-0.633</td>
<td>0.019</td>
</tr>
<tr>
<td>1.5% agar</td>
<td>0.225</td>
<td>0.900</td>
</tr>
<tr>
<td>0.5% agar</td>
<td>-0.705</td>
<td>0.013</td>
</tr>
<tr>
<td>spore production</td>
<td>-0.611</td>
<td>0.033</td>
</tr>
</tbody>
</table>

*p-Values significant below the p = 0.05 criterion are shown in italic.
contribute directly to the chimaeric load documented here (see further discussion below).

(a) Possible mechanisms causing dysfunctional group-level phenotypes

Chimaeric load might be caused by (i) a reduced density of identical neighbours per se (relative to pure groups with the same total density), (ii) dysfunctional responses to the presence of genetically distinct neighbours or (iii) a combination of these two scenarios. The first scenario could occur if interactions between distinct strains within the same group were simply reduced in degree due to non-recognition rather than being dysfunctionally different in character relative to interactions between genetically identical cells in homogeneous groups. Under this scenario, simply reducing the density of pure groups of a focal genotype by a relevant degree would cause the same reduction in group productivity as would be caused by replacement of identical cells with cells of a distinct genotype.

Previous results are consistent with the hypothesis that chimaeric load among the strains examined here is due largely to dysfunctional (i.e. antagonistic) strain interaction rather than simply reduced densities of identical cells. Myxococcus xanthus sporulation efficiency is indeed density dependent, occurring only above a minimum density threshold and increasing with density to a maximum [40,41]. However, many diverse strains sporulate at maximum or near-maximum efficiency over a wide density range that can span more than an order of magnitude. The constant total group density used in our experiments lies squarely within the range of near-maximum sporulation efficiency for most tested strains [41]. Effects on sporulation efficiency of reducing pure-culture densities by factors equivalent to the reduction of individual group-member densities resulting from mixing strains at constant total density (factors of 2, 3, 4 and 5) are therefore expected to be small or non-existent for most strains. We thus consider that antagonistic interaction between mixed-group members is more likely to be primarily responsible for the chimaeric load observed in our development experiments (and by extension in our other assays as well) than is non-interaction.

Dysfunctional phenotypes induced by interaction with conspecifics could be due to any number of differences in the identity, quantity and spatio-temporal dynamics of molecules that mediate cell–cell interactions. There are many candidates for such interaction-mediating molecules within the rich M. xanthus secretome, which includes not only all molecules bound to the surface of the outer cell membrane but also a diverse array of unbound secretions, including intercellular signals, toxins, proteases and other secondary metabolites of unknown function [42–44]. Coordinated cell motility is mediated by intercellular communication, and is fundamental for swarming during predation and aggregation of cells during fruiting body formation [45,46]. Thus, interactions between genotypes that have diverged in cell motility dynamics might induce dysfunctional downstream phenotypes during predation or development even if interactants initially share similar biochemical profiles.

The primary goal of this study was to test for general effects of chimaerism on group performance, irrespective of the relative roles of particular strains and biological mechanisms in generating such effects. Nonetheless, further analysis of specific chimaeric load patterns may allow targeted investigations of proximate mechanisms and discernment of particular interactions contributing to chimaeric load. For instance, developmental chimaeric load is greater in the group composed of strains A, B and C than in the group composed of only B and C (figure 4), even though neither the pairwise mix of A and B nor that of A and C suffer chimaeric load (data not shown). This result implies that A interacts with a phenotype that emerges specifically from an interaction between B and C to increase chimaeric load in the three-way mix. More broadly, distinct genotypes are likely to differ in their effects on the social performance of chimaeric groups. Consistent with this

Figure 4. Effects of chimaerism on sporulation. The five cases of significant differences between expected (dark grey bars) and observed (light grey bars) spore production by chimaeric groups are shown. Error bars represent the standard error of the mean.
(b) Evolutionary origins

In principle, traits that induce dysfunctional phenotypes in others might be selected adaptations for competitive success in a diverse social environment. This hypothesis is more likely if the combination of inter-group migration and within-group mutation to new social phenotypes is high, thus reducing mean within-group relatedness [39,47,48]. For example, lineages frequently encountering other genotypes during a cooperative process might, in practice, evolve an adaptation that allows them to actively defect from cooperation when interacting specifically with non-kin. Such defection may induce a dysfunctional response by non-kin individuals and thus lower the total performance of chimaeric groups. No study has yet demonstrated that microbes are capable of such sophisticated adaptive behaviour modification in response to altered social environments, but it is theoretically possible [27,49].

Alternatively, such dysfunction-inducing traits might not be adaptations themselves, even if they happen to be beneficial in some natural or experimental environments that are rarely encountered. First, dysfunction-inducing traits may be pleiotropic side effects of adaptations for something other than social competitiveness [50]. Dysfunction-inducing traits might even be maladaptive, but maintained in a population nonetheless due to hitchhiking with an adaptive mutation of greater positive effect [51]. Finally, dysfunction-inducing traits might be effectively neutral and their fate determined by genetic drift.

This hypothesis is plausible when inter-group migration is low and within-group relatedness is high, as interactions between socially divergent strains that might impose selection would rarely, if ever, occur.

(c) Chimaeric load may promote sympatric social fragmentation

Chimaeric load may be mechanistically and evolutionarily related to the colony-merger incompatibilities that have been documented among *M. xanthus* isolates from the same local centimetre-scale population examined here [11]. Colony-merger incompatibilities evolve readily in both natural populations [16] and experimental lineages (P. Zee & O. Rendueles-Garcia 2012, unpublished results) and might be selectively favoured in soil habitats due to the barriers to inter-migration among non-kin from divergent groups that they create [52]. The potential for chimaeric load to result from the merging of distinct swarms composed of functionally divergent genotypes would contribute to benefits of colony-merger incompatibilities and favour their reinforcement. Such incompatibilities appear to generate a very fine scale of social isolation in natural populations that may lead to long-term divergence among sympatric patches of lineage clusters [16]. In turn, such divergence may increase latent chimaeric load (i.e. unactualized chimaeric load that would occur if relevant chimaeric mixing actually took place). In this scenario, chimaeric load and colony-merger incompatibilities would mutually reinforce one another in a manner analogous to post-speciation increases in genetic incompatibility in sexual organisms [53]. The strains examined here each represent distinct colony-merger compatibility types [11] and may thus have continued diverging at loci responsible for generating chimaeric load after having initially become socially isolated from one another due to prior divergence at loci that govern colony-merger compatibility types. Chimaeric load may be a major determinant of both the extent and spatial structure of genetic diversity observed in natural myxobacterial populations.

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