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Report

Polymorphic Members of the *lag* Gene Family Mediate Kin Discrimination in *Dictyostelium*

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Summary

Self and kin discrimination are observed in most kingdoms of life and are mediated by highly polymorphic plasma membrane proteins [1–7]. Sequence polymorphism, which is essential for effective recognition, is maintained by balancing selection [8–10]. *Dictyostelium discoideum* are social amoebas that propagate as unicellular organisms but aggregate upon starvation and form fruiting bodies with viable spores and dead stalk cells. Aggregative development exposes *Dictyostelium* to the perils of chimerism, including cheating, which raises questions about how the victims survive in nature and how social cooperation persists [11–13]. Dictyostelids can minimize the cost of chimerism by preferential cooperation with kin [14–16], but the mechanisms of kin discrimination are largely unknown. *Dictyostelium lag* genes encode transmembrane proteins with multiple immunoglobulin (Ig) repeats that participate in cell adhesion and signaling [17–22]. Here, we describe their role in kin discrimination. We show that *lagB1* and *lagC1* are highly polymorphic in natural populations and that their sequence dissimilarity correlates well with wild-strain segregation. Deleting *lagB1* and *lagC1* results in strain segregation in chimeras with wild-type cells, whereas elimination of the nearly invariant homolog *lagD1* has no such consequences. These findings reveal an early evolutionary origin of kin discrimination and provide insight into the mechanism of social recognition and immunity.

Results and Discussion

lagC1 and *lagD1* encode similar proteins (59% identity) and have similar developmental functions [19]. *lagC1* resides next to *lagB1*, which encodes another predicted transmembrane protein with several Ig repeats, and *lagD1* resides next to *lagE1*—a close homolog of *lagB1* (68% identity) [23]. In both gene pairs, the ORFs face away from each other, separated by a short intergenic sequence. To test whether these gene pairs could be involved in kin discrimination, we measured their polymorphism levels in wild isolates. We sequenced these genes in wild isolates and found that *lagB1* and *lagC1* are polymorphic (Figure 1), whereas *lagD1* and *lagE1* are nearly invariant (data not shown). The ratio between nonsynonymous (dN) and synonymous (dS) substitutions is an indicator of evolutionary processes, whereby dN/dS > 1 suggests positive selection [24]. We found that *lagB1* and *lagC1* contain regions with dN/dS ratios as high as 2 to 4 (Figure 1), which is comparable with values found in mammalian MHC genes [9]. In *lagD1* and *lagE1*, the ratios were smaller than 1 (data not shown). Therefore, *lagB1* and *lagC1* are probably evolving under positive selection, possibly balancing selection considering the high number of polymorphic alleles [8], and the homologs *lagD1* and *lagE1* must be under purifying selection. Polymorphism is an essential feature of recognition proteins because it provides the molecular basis for self identity. We therefore conclude that *lagB1* and *lagC1* are more likely to be involved in kin recognition than *lagD1* and *lagE1*.

lagC1 and *lagD1* mRNAs are developmentally regulated [19], so we tested the regulation of the respective tandem genes, *lagB1* and *lagE1*. Figure 2A shows that the *lagB1* and *lagC1* expression patterns are nearly indistinguishable, with mRNA levels peaking at 8–12 hr, corresponding to the transition from loose aggregate to tight aggregate. Figure 2B shows that *lagD1* and *lagE1* mRNA levels are also nearly indistinguishable with a peak at 16 hr, corresponding to the finger stage of development. The tandem genes are therefore coordinately regulated, probably as a result of common regulatory elements that reside between their ORFs.

lagC1 is essential for aggregation and for subsequent development [17–22], so we tested whether *lagB1* was also involved in development. We disrupted *lagB1* and compared the mutant to the parental AX4 strain and to *lagC1*$^-$ cells. *lagB1*$^-$ cells failed to progress beyond the loose aggregate stage after 12 and 17 hr of development, similar to *lagC1*$^-$ cells, whereas the wild-type formed tipped aggregates and slugs at the respective times (Figure 2C). A few *lagB1*$^-$ mounds formed small, gnarled fruiting bodies after 40 hr (Figure 2C). Neither mutant produced spores in the first 24 hr of development (data not shown), but 2% of the *lagB1*$^-$ cells formed spores after 30 hr (Figure 2D). Therefore, *lagC1* and *lagB1* are essential for aggregation and for subsequent development, suggesting common functions.

The proximity of *lagC1* and *lagB1* raised the possibility that disrupting one gene might have inadvertently disrupted the other. To test that possibility, we measured *lagB1* mRNA in *lagC1*$^-$ cells and vice versa. *lagB1* mRNA was present in the *lagC1*$^-$ cells, although the levels were lower and the mRNA persisted relative to the wild-type (Figure 2E). *lagC1* mRNA was present in the *lagB1*$^-$ cells at levels similar those in the wild-type (Figure 2F). These observations indicate that deleting one gene did not directly inactivate the other. The

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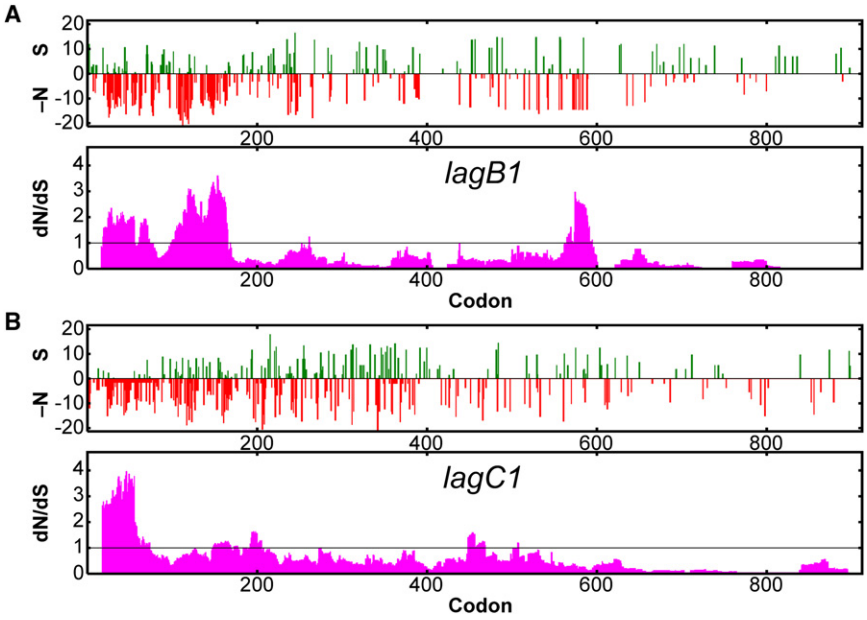

Figure 1. Sequence Polymorphism

We amplified and sequenced genes from several *D. discoideum* strains and aligned the sequences of each gene separately. We determined the position of variable nucleotides and determined whether each variation corresponds to a synonymous (S) or a nonsynonymous (N) variation in the protein sequence. The upper box of each panel represents the synonymous variations as green bars above the zero line and the nonsynonymous variations as red bars below the line. The x axis indicates the codon number in the ORF and the y axis indicates the frequency of the variation in each strain compared to all the other strains. We used the nucleotide variation data to compute the dN/dS ratio at each codon in a sliding window of 31 codons along the entire coding sequence. The data are plotted in the lower box of each panel, the x axis indicates the codon number in the ORF, and the y axis indicates the dN/dS ratio. Data above the line (dN/dS = 1) suggest that the region is under balancing or positive selection.

(A) *lagB1* exhibited a total of 266 polymorphic codons with as many as eight variations per codon (30 strains tested).

(B) *lagC1* exhibited 319 polymorphic codons with as many as seven variants per codon (29 strains tested). We also sequenced *lagD1* and *lagE1*. *lagD1* exhibited only nine polymorphic codons with no more than two variants per codon (15 strains tested), and *lagE1* exhibited ten polymorphic codons with only two variants per codon (six strains tested). The dN/dS ratio was much lower than 1 in both cases (data not shown). Information about the strains and the genes we sequenced is provided in Table S1.

reduced levels and the persistence of *lagB1* mRNA in *lagC1*$^-$ cells probably reflect the delayed development of the mutants, but it is formally possible that they contributed directly to the observed phenotypes.

The patterns of sequence polymorphism and gene expression and the phenotypes of the null mutants suggested that *lagB1* and *lagC1* are likely to function together in kin discrimination. To test that possibility, we followed cells in chimeras between differentially labeled strains. In control chimeras, AX4-GFP and AX4-RFP cells exhibited equal mixing of green and red fluorescent cells at the aggregation stage (10–12 hr) and at the finger stage (16–19 hr), indicating that the fluorescent markers do not cause segregation (Figures 3A$_i$ and 3A$_{ii}$). Mixing AX4-RFP with *lagB1*$^-$-GFP yielded a different pattern: the red fluorescent wild-type and the green fluorescent mutant co-aggregated at first, but then segregated into regions enriched in either red or green fluorescent cells during mound formation (Figure 3A$_{iii}$). The mounds progressed into slugs that contained cells from both strains, but the *lagB1*$^-$ cells were enriched in the middle of the slugs (Figure 3A$_{iv}$). This enrichment in the prespore region suggested that the mutant may cheat on the wild-type [12], but a direct test did not support this hypothesis (data not shown).

The results observed with *lagC1*$^-$ were even more dramatic. Cells from the two strains coaggregated at first, but then segregated within the loose aggregates (Figure 3A$_v$). Several hours later, the AX4-RFP cells formed migrating slugs that contained a few *lagC1*$^-$-GFP cells and migrated away, leaving behind mounds of mainly *lagC1*$^-$-GFP cells (Figure 3A$_{vi}$). These results suggest that *lagC1* and *lagB1* play a role in kin discrimination, although *lagB1* plays a lesser role than *lagC1*.

To test the kin-discrimination role of *lagD1*, we mixed *lagD1*$^-$-GFP with AX4-RFP cells. The strains mixed well and remained mixed throughout development (Figures 3A$_{vii}$ and 3A$_{viii}$). These results do not support a role for *lagD1* in kin discrimination, even though *lagD1* shares many other properties with *lagC1* [19], highlighting the difference between the polymorphic *lagC1* gene and the nearly invariant *lagD1* homolog.

LagC1 is a cell-adhesion protein [20, 22], so we tested whether other adhesion genes participate in kin discrimination. *cadA* [20, 25] and *csaA* [26] encode two thoroughly studied cell-cell adhesion proteins [20]. We tested the respective null mutants in chimeras with AX4 cells and found no evidence of segregation (Figures 3A$_{ix}$–3A$_{xii}$), arguing against a general role for cell-adhesion genes in kin discrimination. These results are not in conflict with work that described *csaA* as a greenbeard gene [27, 28]. In those experiments, *csaA*$^-$ segregated from the wild-type during development on soil but not on agar. Moreover, the sequence conservation of *csaA* in wild strains (unpublished data) is inconsistent with a direct role in kin discrimination.

The segregation of *lagC1*$^-$ from AX4 could have resulted from differential adhesion. To test the adhesion properties of the strains, we developed them in pure populations for 5 and 12 hr. We disaggregated the cells, mixed them, and allowed them to reaggregate in liquid suspension [22]. We found mixed aggregates in both cases (Figures 3B$_i$ and 3B$_{ii}$), indicating mutual adherence and suggesting that differential adhesion cannot account for the segregation observed in Figure 3A. Moreover, the 12 hr cells became segregated within the mixed aggregates (Figure 3B$_{ii}$), consistent with the proposed role of *lagC1* in kin discrimination.

lagB1, *lagC1*, and *lagD1* have similar developmental roles because mutations in either one cause a developmental arrest at the loose aggregate stage ([17, 19]; Figure 2C). We further tested their roles in kin discrimination by testing chimeras between *lagC1*$^-$ and other mutants (Figure 3C). The control experiment showed that the differential labels did not cause segregation (Figures 3C$_i$ and 3C$_{ii}$). To test whether *lagB1* and *lagC1* participate in one kin-discrimination pathway, we mixed differentially labeled cells of the respective mutants and observed no segregation at any stage (Figures 3C$_{iii}$ and 3C$_{iv}$). Conversely, *lagC1*$^-$ and *lagD1*$^-$ first coaggregated

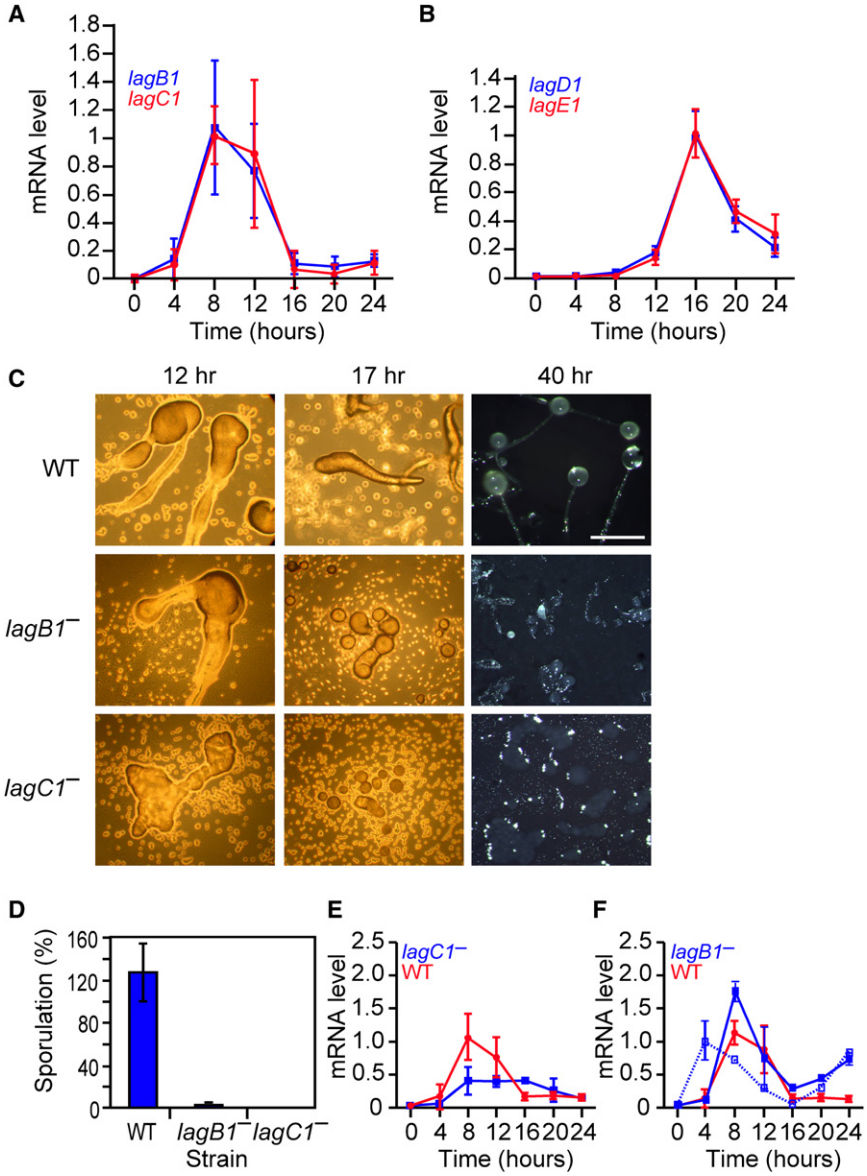

Figure 2. Developmental Regulation and Function of *lag* Genes

In (A) and (B), we used quantitative RT-PCR to measure the mRNA levels of *lag* genes in samples collected at 4 hr intervals from developing wild-type AX4 cells. At 0 hr, the cells are at the vegetative stage, at 4 hr they are starving, at 8 hr they begin to aggregate, at 12 hr they form tight aggregates with differentiated prespore and prestalk cells, at 16 hr they form fingers with prestalk cells in the anterior end, at 20 hr they begin to culminate, and at 24 hr they form mature fruiting bodies with a ball of spores aloft a cellular stalk. The time (hours) is indicated on the x axis and the mRNA level is indicated on the y axis in arbitrary units, relative to the maximal level of expression. The results are presented as averages and standard deviations of three technical replications of each of two biological samples (a total of six measurements).

(A) *lagB1* mRNA (blue) and *lagC1* mRNA (red).

(B) *lagD1* mRNA (blue) and *lagE1* mRNA (red).

In (C) and (D), we mutated the *lagB1* gene, developed wild-type and mutant cells for the indicated time (hours), and analyzed their development.

(C) Morphological analysis: growth and early developmental properties of the three strains were nearly indistinguishable (data not shown). We show morphological differences between cells developed on nonnutrient agar (12 hr, 17 hr) and on dark nitrocellulose filters (40 hr). The genotypes are indicated on the left. Pictures were taken from above the structure. The scale bar represents 0.5 mm.

(D) Sporulation efficiency: we counted the number of spores collected after 30 hr and present the data as a fraction (%) of the number of amoebas deposited for development. The genotypes are indicated below the bars. Results are the means and standard deviations of three independent replications. For gene expression, we used quantitative RT-PCR to measure mRNA levels in samples collected at 4 hr intervals from developing cells. The graphs are as above and the results are presented as averages and standard deviations of three technical replications of each of two biological samples (a total of six measurements) except as indicated.

(E) *lagB1* mRNA in wild-type cells (red) and in *lagC1*$^{-}$ cells (blue).

(F) *lagC1* mRNA in wild-type cells (red) and in *lagB1*$^{-}$ cells (blue). The *lagB1*$^{-}$ cells exhibited large differences between biological samples, probably because the cells do not develop synchronously, so we show two biological experiments out of four that we have performed (solid and dashed blue lines). The data are averages and standard deviations of three technical replications.

(Figure 3C$_{v}$) but then segregated into clusters consisting mainly of one or the other strain (Figure 3C$_{vi}$). The latter observation suggests that segregation is not a result of differential developmental progression because the strains segregate even though both progress to the same developmental stage.

These results suggest that *lagB1* and *lagC1*, but not *lagD1*, function in one kin-discrimination pathway. This conclusion is also supported by the finding that *lagB1* and *lagC1* are physically mapped near each other, so they are likely to be inherited together in a syntenic block as seen in other kin-discrimination genes [2], and by the observation of developmental coregulation (Figure 2A), which provides the temporal opportunity for common function.

We also tested the role of other cell-adhesion genes by mixing *lagC1*$^{-}$ with *cadA*$^{-}$ or with *csaA*$^{-}$. In both cases, the cells coaggregate initially but then segregated into structures that consisted mainly of one strain or the other (Figures 3C$_{vii}$–3C$_{x}$). These results suggest that the kin-discrimination roles of *lagB1* and *lagC1* are specific to these genes rather than a general property of cell-cell adhesion genes.

Mixing AX4 cells with genetically dissimilar cells results in partial segregation, implying a kin-discrimination mechanism [16]. The properties of *lagB1* and *lagC1* suggest they might participate in that mechanism. To examine the correlation between the *lagB1* and *lagC1* sequence polymorphism and segregation, we computed the dissimilarities between the AX4 LagB1 and LagC1 sequences and the respective sequences in 11 wild isolates. We then computed the correlation between these dissimilarities and the published strain segregation data [16]. We observed a positive correlation between sequence dissimilarity and segregation, although it was weaker than the correlation with genetic distances inferred from microsatellite length (Table S2 available online). We then searched and found within LagB1 and LagC1 protein

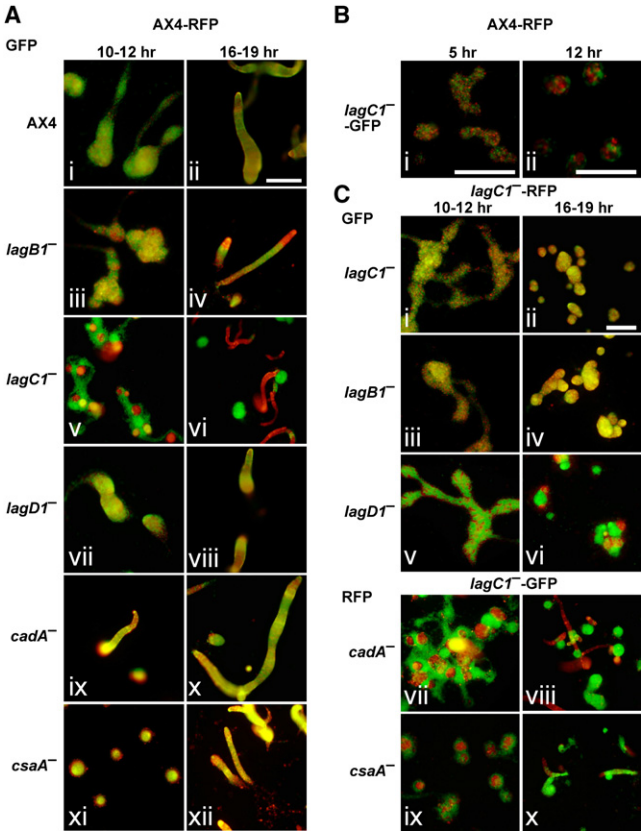

Figure 3. Segregation of Cell-Cell Adhesion Mutants from Wild-Type and from *lagC1*$^-$ Cells

(A) We mixed wild-type (AX4) cells labeled with RFP with different strains labeled with GFP. We developed the cells on a solid substratum, photographed the aggregates after 10–12 hr and after 16–19 hr of development with fluorescence microscopy at the appropriate wavelengths, and merged the red and green images. The scale bar represents 0.5 mm.

(B) To test the adhesion properties of the cells, we developed separately wild-type (AX4) cells labeled with RFP and *lagC1*$^-$ cells labeled with GFP. We disaggregated the cells after 5 hr ($[B_i]$, the scale bar represents 1.0 mm) and after 12 hr ($[B_{ii}]$, the scale bar represents 0.5 mm) of development, as indicated, mixed the cells with unlabeled counterparts, and allowed them to reaggregate in shaking suspension. We photographed the mixed aggregates as above.

(C) To test interactions between pairs of mutant strains, we mixed *lagC1*$^-$ cells labeled with RFP with different strains labeled with GFP (C_i–C_{vi}) and *lagC1*$^-$ cells labeled with GFP with different strains labeled with RFP (C_{vii}–C_x), as indicated, developed them on a solid substrate, and photographed them as above. The scale bar represents 0.5 mm. The genotypes are indicated on the left of each row.

domains that exhibited better correlations between sequence dissimilarity and strain segregation (Table S2). In LagB1, the correlation was best between amino acids 239–259 and in LagC1 it was between amino acids 180–197 (Figure S1). Both regions reside near the first Ig-fold of the extracellular domain, suggesting that specific extracellular domains may have a function in segregation.

Because we tested many correlations, one or more could have been strong just by chance. However, if these correlations reflect a causative relationship, they should be predictive of strain segregation, which would be less likely if they were serendipitous. Ostrowski et al. mixed three wild strains, QS32, QS33, and QS38, all of which segregated from AX4. QS32 and QS33 are genetically close to each other and distant from QS38. In that experiment, QS32 did not segregate from itself or from QS33, but segregated from the genetically dissimilar strain QS38 [16]. Table 1 shows that our sequence data predict low segregation of QS32 from QS33 and high segregation of QS38 from QS32. Therefore, the predictions of the sequence-based segregation model correlate well with the observations, supporting the hypothesis that LagB1 and LagC1 play a direct role in kin discrimination.

Table 1. Prediction of Strain Segregation from *lagB1* and *lagC1* Sequence Dissimilarity

Strains	Reported Segregation[a]	Predicted Segregation	
		lagB1 Sequence	*lagC1* Sequence
QS32/QS32	0.9	0	0
QS33/QS32	2.5	0	0.3
QS38/QS32	201.5	61.3	257.4
Pearson's Correlation		0.9999	0.9999
p Value		0.0044	0.0038

[a] Recalculated from [16].

Our data suggest that *lagB1* and *lagC1* participate in a common self- or kin-recognition mechanism in *D. discoideum*. In other organisms, genetic crosses between organisms with polymorphic alleles have provided support for the role of such genes in self/nonself recognition [3, 29]. Because *D. discoideum* is not amenable to these types of studies, tests of the causative relationships between *lag* genes and kin recognition would have to be accomplished by other means. Nevertheless, our findings suggest that the molecular mechanisms that regulate kin recognition evolved before the evolutionary departure of the amoebazoa from the evolutionary line leading to animals, illustrating the critical role of these mechanisms in multicellularity and in sociality.

Experimental Procedures

Cell Growth and Development

Cells were grown in shaking suspension, harvested at the logarithmic growth phase and developed on filters [30] or on agar [31] as indicated. Wild isolates (Table S1) were grown in association with bacteria. The *cadA*$^-$ strain TL97 [25] and the *csaA*$^-$ strain T10 [32] were obtained from the *Dictyostelium* stock center. The *lagD1*$^-$ strain was described before [19]. Detailed information is provided in the Supplemental Data.

Construction of New Strains

We constructed and used vectors for deletion of *lagB1* and *lagC1* by homologous recombination (see Supplemental Data for detailed information). The knockout plasmids were confirmed by sequencing, linearized, and transformed into AX4 cells by electroporation. Clones were identified by PCR analysis and confirmed by Southern blot analysis [30].

For labeling cells with fluorescent proteins, we constructed several new vectors for expression of RFP and GFP under the actin15 promoter (see Supplemental Data for complete detail). The vectors were transformed into AX4 and into the various mutants.

Segregation Assays

Strains were grown in pure populations, washed and mixed at 1:1 ratios, and deposited on nonnutrient agar (see Supplemental Data for detailed information). Developmental structures were photographed with epi-fluorescence microscopy at the appropriate wavelengths and images were overlaid without further manipulation. The results are shown as color photographs.

Sporulation Efficiency

Cells were grown and then developed for 30 hr. Sporulation efficiency was measured as described [33]. Each strain was tested in two independent

biological replications. Each sample was developed on three filters and samples from each filter were counted thrice. Sporulation efficiency was calculated as the fraction (%) of spores recovered relative to the number of cells that were developed.

RNA Extraction and Quantitative RT-PCR

For RNA production, vegetative cells (5×10^7 total) were resuspended in 500 μl Trizol reagent (Invitrogen). Developing cells were collected and resuspended in 500 μl Trizol, incubated at room temperature for 5 min, and stored at –80°C. RNA was extracted according to the manufacturer's recommended protocol. RNA concentration was calculated by absorbance at 260 nm. cDNA was produced by reverse transcription and quantified by real-time PCR. Each strain was developed in two independent replicates and each RNA sample was analyzed thrice by quantitative PCR. Data and standard deviations were calculated across the six replicates (see Supplemental Data for detailed information).

Sequencing Genes from Wild Strains

Genomic DNA was extracted as described [30]. To sequence *lagB1*, *lagC1*, *lagD1*, and *lagE1*, we used one set of primers to amplify the entire structural gene from genomic DNA and another set of primers to amplify smaller fragments from the initial PCR product. Each nested PCR primer pair contained universal primer tags for sequencing. The PCR products were purified and sequenced with an Applied Biosystems 3730xl automatic sequencer. Detailed information and the sequences of all the oligonucleotides are provided in the Supplemental Data.

Analysis of Polymorphic Sequences

The regions of the genes used for analysis are provided in Table S1. Multiple sequence alignments were performed with the Clustal W algorithm in MacVector 7.2.3. Intronic sequences were deleted manually and the alignments were adjusted accordingly. We used the Nei and Gojobori method to calculate the number of synonymous (dS) and nonsynonymous (dN) nucleotide substitutions per site for a given pair of homologous sequences [34]. The gene coding sequences of a given pair of strains were scanned with a sliding window spanning 101, 81, and 31 amino acids with essentially identical results (31 amino acid windows shown), and the dN/dS ratio was calculated at each position. The dN/dS ratios reported are an average across all pairwise comparisons.

Correlation between Sequence Polymorphism and Segregation

We calculated the Spearman's rank correlation between the calculated protein sequence dissimilarity and the segregation variance values reported by Ostrowski et al. [16] for 12 strains (AX4, NC4, QS32, QS33, QS34, QS36, QS37, QS38, QS40, QS41, QS45, and QS113). Both the sequence similarity and the segregation were measured against a common reference strain (AX4). Sequence dissimilarity was calculated in all subintervals between 5 and 13 amino acids over each protein position. We used three protein similarity matrixes (PAM250, BLOSSUM, and GONNET) to calculate similarity and then negating it to obtain dissimilarity. We then fitted a linear model relating sequence dissimilarity and segregation for each subinterval with high positive Spearman's rank correlation ($r \geq 0.65$ and $p < 0.025$). The final model is a composite (ensemble) of linear models (140 for *lagB1* and 24 for *lagC1*), each represented by the coordinates of the identified subinterval, sequence similarity matrix used, and coefficients for linear regression. When the composite model was applied to a new sequence, each linear model predicted the level of segregation on the basis of the sequence similarity in its specific subinterval. The final prediction of the composite model is an average of the predictions of all the linear models. We built two composite models, one for each gene (*lagB1* and *lagC1*) and present the final prediction as the relative contribution of each model among all models (y axis) that span each position (x axis) (Figure S1; see Supplemental Data for additional details).

We also adapted the models built on the AX4 reference data and applied them to predict the segregation of strains QS32, QS33, and QS38 from strain QS32. In that case, the models were adjusted (shifted by a constant value) to predict zero segregation when the reference strain was mixed with self.

Accession Numbers

GenBank accession numbers of all the sequences we used are provided in Table S1.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, one figure, and two tables and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(09)00747-7.

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