pre-cultured[1,2,10,20,21] the microbial communities from each site by growing either *C. maculosa* from the same region used in experiment 1, or a *Festuca* bunchgrass native to the region of soil origin, in pure soil. The two *Festuca* species (*idahoensis* and *ovina*) were chosen because they are similar in size and appearance and co-occur naturally with *C. maculosa* in their respective native lands. The use of a single *Festuca* species might have biased the relationship between the plant and microbes of a different continent in ways that might differ from a plant and microbes from the same region. Five plants were grown for 110 days in each of 40 4-litre pots: $n = 10$ for Montana soil with *C. maculosa*; Montana soil with *F. idahoensis*; French soil with *C. maculosa*; and French soil with *F. ovina*. After the 110-day pre-culturing period, half of the soil in each pot was triple-autoclaved on three successive days to kill the microbial community, and then the non-sterilized and sterilized soil from each 4-litre pot was used to inoculate two sterile 15:85 soil:sand mixtures and two non-sterile 15:85 soil:sand mixtures in 525 cm^3 pots. We used the soil in the 525 cm^3 pots for two experiments: one in which *C. maculosa* was grown alone in sterile and non-sterile soil, and one in which *C. maculosa* was grown with *F. idahoensis* as a competitor. In the no-competition experiment, *Centaurea* seeds were planted in all pots and grown for 91 days during which they were fertilized and watered as in experiment 1. In the competition experiment, *F. idahoensis*, which grows more slowly than *C. maculosa*, was planted first, and after 14 days *C. maculosa* seeds were planted in half of the pots and grown for 91 days. At the end of the experiments all plants were harvested, dried at 60 °C and weighed.

Experiment 4

We compared the post-removal effects of *C. maculosa* on the growth of conspecifics to those of the native grass *Pseudoroegneria spicata*. Ten *C. maculosa* and ten *P. spicata* were planted in random locations outdoors in the Deittart Experimental Gardens in April 2001. The site historically supported native grasslands. These plants were grown until 10 August 2003, when they were harvested aboveground. One *C. maculosa* was grown at each of the 20 sites from 10 August to 14 September 2003 when they were harvested aboveground, dried at 60 °C and weighed.

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Organization of genetic variation in individuals of arbuscular mycorrhizal fungi

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Arbuscular mycorrhizal (AM) fungi (Glomeromycota) are thought to be the oldest group of asexual multicellular organisms. They colonize the roots of most land plants, where they facilitate mineral uptake from the soil in exchange for plant-assimilated carbon[1]. Cells of AM fungi contain hundreds of nuclei. Unusual polymorphism of ribosomal DNA observed in individual spores of AM fungi inspired a hypothesis that heterokaryosis—that is, the coexistence of many dissimilar nuclei in cells—occurs throughout the AM fungal life history[2,3]. Here we report a genetic approach to test the hypothesis of heterokaryosis in AM fungi. Our study of the transmission of polymorphic genetic markers in natural isolates of *Glomus etunicatum*, coupled with direct amplification of rDNA from microdissected nuclei by polymerase chain reaction, supports the alternative hypothesis of homokaryosis, in which nuclei populating AM fungal individuals are genetically uniform. Intrasporal rDNA polymorphism contained in each nucleus signals a relaxation of concerted evolution[4], a recombination-driven process that is responsible for homogenizing rDNA repeats[5]. Polyploid organization of glomeromycotan genomes could accommodate intranuclear rDNA polymorphism and buffer these apparently asexual organisms against the effects of accumulating mutations.

Molecular phylogeny[6] and the fossil record[7] date Glomeromycota to the Ordovician period and indicate that AM-like fungi participated in the transition of early plants to the terrestrial habitat[8]. With no evidence of sexual reproduction, these fungi may represent an ancient asexual lineage that is much older than the asexual bdelloid rotifers[9]. The glomeromycotan reproductive mode, and their reputed position of ancient asexuals, could be verified by methods of phylogenetics and population genetics; however, this work cannot be accomplished until the genetic variation observed in individuals of AM fungi is explained.

Two models can explain the organization of genetic variation in AM fungi: first, the diverse rDNA variants may be distributed among different nuclei (heterokaryosis, Fig. 1a); or second, all

rDNA variants may be contained in each nucleus (homokaryosis, Fig. 1b). The first model implies that the fungus maintains a stable assemblage of several different genomes during its life cycle and transmits them from generation to generation[2,3]. Such a multigenomic configuration of individual genetic variation would set Glomeromycota apart from other organisms; however, some of the data supporting heterokaryosis have been disputed[10,11]. The alternative hypothesis of homokaryotic organization of genetic variation entails a relaxation of concerted evolution of rDNA in the lineage. Concerted evolution is a universal phenomenon responsible for the homogenization of rDNA repeats within a repeat array, among the arrays dispersed in an individual genome, and throughout a recombining population[5].

To distinguish between these two models, we devised three approaches. First, we investigated maintenance of genetic variation in an AM fungus from one generation to the next during clonal reproduction in laboratory single-spore cultures. Second, we analysed the structure of genetic variation in a natural AM fungal population. Third, we directly examined the rDNA variant distribution among nuclei in spores by amplifying rDNA by polymerase chain reaction (PCR) from individually microdissected nuclei.

To examine genetic variation as it occurs in a natural population of an AM fungus, we sampled a ubiquitous morphospecies, *G. etunicatum* (Glomerales), from an agricultural field in California that had been under cereal cultivation for 80 years. We used four randomly selected field isolates to start single-spore cultures on carrot roots transformed with *Agrobacterium rhizogenes*. A search for genetic markers that varied at the intrasporal (within an individual) level retrieved a marker with 56% amino acid sequence identity to a *Saccharomyces cerevisiae* gene encoding the catalytic subunit of DNA polymerase-α, *POL1*, which is a single-locus gene in yeast and other organisms.

The four *G. etunicatum* field isolates contained 16 variants of the *POL1*-like sequence (PLS), which separated into two distinct phylogenetic clusters, PLS1 and PLS2 (Fig. 2a). In the absence of information about the ploidy level and about the physical organization of the PLS variants in AM fungi in general and in *G. etunicatum* in particular, and to make our approach more specific, we focused on the PLS1 cluster, which contained 14 variants. To distinguish whether the PLS1 variation is a consequence of the neutral accumulation of mutations or selection, we examined differences between the numbers of nonsynonymous and synonymous nucleotide substitutions expected under neutrality, and those observed in PLS1 putative amino acid sequences. By Fisher's exact test[12], we were unable to reject the null hypothesis of the neutral evolution of PLS1.

Each of the four single-spore cultures representing the four field isolates produced many clonal progeny spores. Five progeny spores were collected from each culture, and PLS1 fragments were independently amplified by PCR from each spore, cloned and sequenced. In each culture, all five spores analysed contained the same 13 PLS1 variants (Fig. 2b). This result is consistent with a homokaryotic model of organization of genetic variation (Fig. 1b) and would not be consistent with a heterokaryosis model (Fig. 1a) unless different nuclei, each carrying 1 of the 13 variants, were apportioned to all progeny spores.

To evaluate the probability of recovering all 13 variants in five spores of each of four cultures under the assumption of hetero-

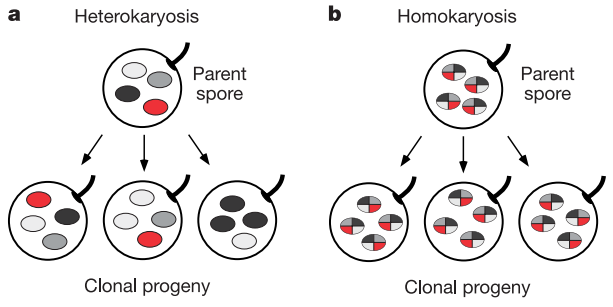

Figure 1 Sorting of variants of a polymorphic marker in single-spore cultures of *Glomus*. **a**, Sorting under the heterokaryotic model; **b**, sorting under the homokaryotic model. Variants of the polymorphic genetic marker contained in nuclei are represented by different colours.

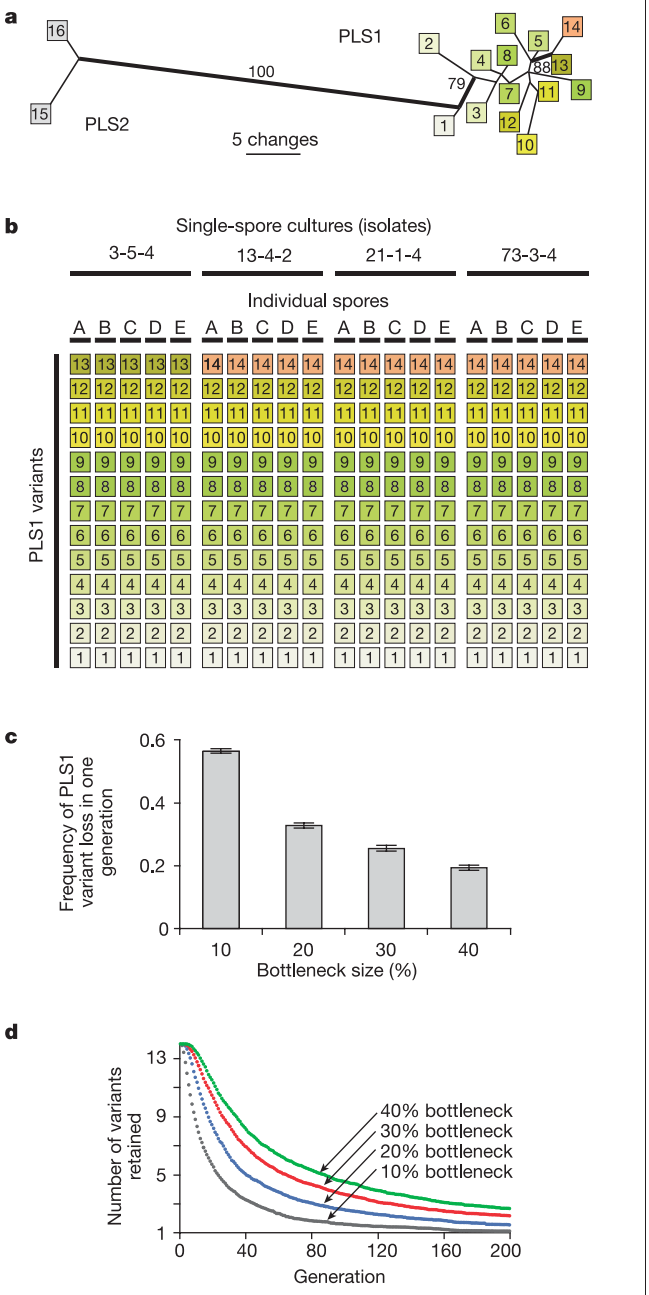

Figure 2 PLS1 variation in *G. etunicatum* field isolates. **a**, Maximum parsimony phylogram of the PLS variants in *G. etunicatum*[25]. Numbers beside thickened branches indicate bootstrap support values. **b**, PLS1 variants detected in natural *G. etunicatum* isolates (single-spore cultures). **c**, Expected frequency (probability) of genetically differentiated spores arising in one generation in single-spore cultures of a heterokaryotic fungus owing to loss of PLS1 variants (from the initial 13 variants) under different bottleneck schemes. Bars represent the mean ± s.e.m. expected frequencies. **d**, Summary of simulated loss of PLS1 variants over many clonal generations in a heterokaryotic fungus experiencing different bottleneck pressures.

karyosis, we modelled the transmission of a neutral polymorphic marker over one generation. To construct the model, we assumed that a spore contains 750 nuclei and that each nucleus carries 1 of the 13 PLS1 variants. We established the number of nuclei per spore by counting nuclei in several *G. etunicatum* spores. Previous work has estimated that 40% of nuclei in a spore migrate into the expanding mycelium[13]. As there are no data on the percentage of nuclei migrating from hyphae into developing spores, we used a range of possibilities (100%, 75%, 50% and 25%) in our model.

Combining the bottlenecks imposed on nuclei at spore germination and spore formation, we modelled the transmission of nuclei from parent spore to progeny spore with bottlenecks of 40%, 30%, 20% and 10%. Under heterokaryosis, our model predicted that the probability of genetic differentiation from spore to spore in one generation by loss of PLS1 variants ranged from 0.1934 (for the 40% bottleneck) to 0.5643 (for the 10% bottleneck; Fig. 2c). For four founding spores, each with five progeny spores, the probability that none of the progeny spores would lose a PLS1 variant ranged from 0.0136 to 6.1×10^{-8}, depending on the assumed bottleneck. Clearly, a model of heterokaryotic individuals is incompatible with our empirical results.

Next, we analysed the genetic variation among the four individuals representing the four isolates from the field population of *G. etunicatum*. The set of PLS1 variants was identical in three of the four individuals. These three individuals differed from the fourth individual (isolate 3-5-4) by only the replacement of variant 13 with variant 14 (Fig. 2b). To estimate the probability of finding three individuals with identical PLS1 variants among four randomly collected individuals under the model of heterokaryosis, we simulated maintenance of genetic variation over several generations in a natural population. Our simulation began with a founder spore containing 14 types of nucleus corresponding to the 14 PLS1 variants detected in the field-isolated individuals. In the absence of any evidence for sexual reproduction, and on the basis of evidence that vegetative incompatibility mechanisms present in AM fungi prevent casual flux of nuclei among genetically distinct individuals[14], we assumed strictly clonal nuclear transmission from generation to generation.

In our simulation, the number of variants of a polymorphic marker declined over time in each spore lineage with a rate dependent on the size of the bottleneck (Fig. 2d), and each lineage evolved to a different complement of variants. Under the most conservative scheme, all individuals in the field would have had identical genotypes before the maize plants, whose root systems we sampled to recover the fungus, were planted. As a result, at the time of collection the sampled isolates would be differentiated by only one generation of independent propagation. During this generation and the additional three generations mandated by our procedures between the collection of isolates and their analysis, loss of PLS1 variation could occur at the frequency estimated by our simulations (Fig. 2c).

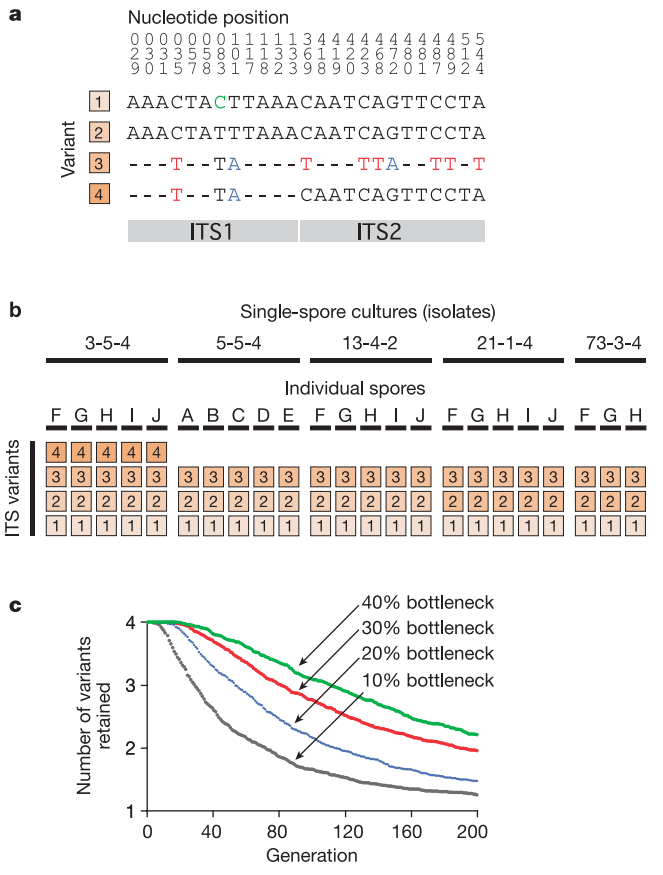

Figure 3 rDNA variation in *G. etunicatum* field isolates. **a**, Alignment of variable nucleotide positions of the ITS1–5.8S–ITS2 rDNA variants in *G. etunicatum*. **b**, ITS1–5.8S–ITS2 rDNA variants detected in natural *G. etunicatum* isolates and single-spore cultures. **c**, Summary of simulated loss of rDNA variants over many clonal generations in a heterokaryotic fungus under different bottleneck pressures.

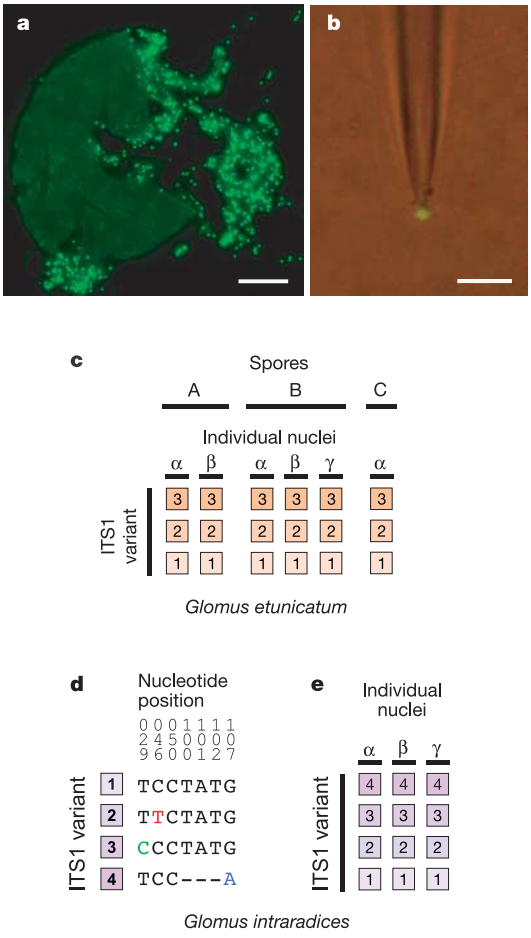

Figure 4 Distribution of rDNA variation among individual nuclei in two *Glomus* species. **a**, *G. etunicatum* spore crushed to release nuclei. Scale bar, 50 μm. **b**, Micropipette with a nucleus attached to its tip. Scale bar, 10 μm. **c**, ITS1 rDNA variants detected in individual nuclei of *G. etunicatum*. **d**, Alignment of variable nucleotide positions of the ITS1 rDNA variants in *G. intraradices*. **e**, ITS1 rDNA variants detected in individual nuclei of *G. intraradices*.

Consequently, the probability of recovering three isolates with identical genotypes under a model of heterokaryosis ranged from 0.0759 (40% bottleneck) to 4.7×10^{-5} (10% bottleneck). A more realistic model assumes one fungal generation per year for the 80 years since the establishment of the field (Fig. 2d) and a limited dispersal ability of AM fungal propagules[15]. Under this model, the probability of recovering three of four heterokaryotic isolates with identical genotypes ranged from 4.0×10^{-23} (40% bottleneck) to 2.0×10^{-87} (10% bottleneck). Thus, our empirical data are inconsistent with a model of heterokaryosis.

In addition to the analysis of PLS1 variation, we applied a similar strategy to examine the patterns of rDNA variation among and within five *G. etunicatum* field isolates using the internal transcribed spacer (ITS) region ITS1–5.8S–ITS2 as a marker. We detected four ITS1–5.8S–ITS2 variants (Fig. 3a). Variants 1 and 2 differed at a single nucleotide position (Fig. 3a). Variants 1 and 2 differed from variant 3 at ten or nine nucleotide positions, respectively, representing 8 million years of divergence (assuming independent variant evolution and a substitution rate for ITS of 1.0×10^{-9} nucleotide substitutions per site per year[16]). Variant 4 was best described as a recombinant between variants 2 and 3. As in the case of PLS1, clonal spores that were formed in each of the five single-spore cultures representing field isolates were genetically identical within a culture (Fig. 3b). Our simulations indicated that the rDNA variant composition should differ among the field sampled individuals if the fungus was heterokaryotic (Fig. 3c). But four of the five field isolates contained the same complement of the three ITS1–5.8S–ITS2 variants (Fig. 3b). The probability of such a sampling outcome in a heterokaryotic fungus ranged from 0.0151 (40% bottleneck) to 1.9×10^{-5} (10% bottleneck) under the assumptions described above for PLS1 with 80 generations of independent lineage propagation. Again, the empirical data do not support heterokaryosis.

To test further the hypothesis that genetic variation in AM fungi is distributed among different nuclei, we developed a technique to microdissect nuclei from individual spores of *G. etunicatum* (Fig. 4a, b). Microdissecting individual nuclei, while preserving the ability to use them as PCR templates, is technically challenging, but we were able to amplify by PCR, clone and sequence directly the ITS1 rDNA region from six individual nuclei, representing three separate spores. Each of the six nuclei contained all three of the ITS1 variants that we previously detected in the *G. etunicatum* field isolates (Fig. 4c), providing direct evidence for the homokaryosis model in which each nucleus contains all of the rDNA variation present in a spore. PCR amplification and analysis of ITS1 variants from individually microdissected nuclei of a laboratory strain of *Glomus intraradices* (Fig. 4d, e) also showed that polymorphic rDNA sequences are present in individual nuclei, confirming that homokaryosis is not the exclusive property of *G. etunicatum* but constitutes normal genetic structure in Glomerales.

The highly divergent rDNA variants present in individual nuclei of AM fungi signify a molecular pattern of relaxation in the concerted evolution of rDNA. Polymorphism of rDNA sequences may reflect an incomplete homogenization of diverse rDNA arrays that were brought together by an interspecific hybridization event[17], or it may result from impediments in meiotic recombination leading to independent evolution of arrays located on homologous chromosomes[18]. Independent rDNA evolution may also occur in arrays distributed among nonhomologous chromosomes if these arrays do not participate in interchromosomal genetic exchanges[19]. The recombinant rDNA chimaeras in *G. etunicatum* indicate that recombination, which underlies the concerted evolution process, occurs in AM fungi. It remains to be seen whether this is solely mitotic recombination within an individual, or whether it also involves genetic exchanges and cryptic sexual recombination.

The rDNA heterogeneity combined with the large number of PLS variants in *G. etunicatum* may be an expression of a duplicated genome structure[20], which would explain the relatively large genome size of about 10^8 base pairs estimated for Glomerales[21]. Genome polyploidization has been detected in many asexual taxa[22], and it is an effective way in which to buffer the genome against the effects of accumulating mutations[23]. In the absence of sexual reproduction in Glomeromycota, genome polyploidization accompanied by periodic changes in the ploidy level[24] might be the mechanism accounting for their long-term evolutionary persistence. The understanding that genetic variation in individuals of AM fungi is contained within each nucleus provides a foundation to address the glomeromycotan reproductive mode in nature and to examine their ancient asexual status. □

Methods

Experimental procedures

Experimental details are given in the Supplementary Information.

Computer simulations and probability estimates

To simulate clonal transmission of PLS1 variants over several generations under the heterokaryosis scheme, we populated spores with 750 nuclei, each carrying a single PLS1 variant. The founder spore contained 14 PLS1 variants distributed at random among the nuclei, and each nucleus was sampled with replacement from the 14 types of PLS1 variant. To create the t generation spore, nuclei were sampled with replacement from the $t - 1$ generation spore. The number of sampled nuclei corresponded to the size of a bottleneck imposed on the nuclear population. Mutations in nuclei were introduced by assigning a new variant to a random nucleus with a frequency of 2×10^{-4} mutations per generation[16]. The nuclei deposited in the t generation spore were multiplied at random to reach the count of 750. We replicated the simulation 250 times for each bottleneck size. Transmission of nuclei carrying different ITS variants over generations was simulated in a similar manner. Each nucleus was assigned a label representing one of the four different ITS1–5.8S–ITS2 rDNA variants. Mutations occurred at a rate of 3×10^{-4} per generation[16]. Transmission of rDNA under each bottleneck condition was replicated 300 times.

We also estimated a frequency (probability) of genetic differentiation of spores formed in single-spore cultures of a heterokaryotic fungus over one generation owing to loss of PLS1 and ITS1–5.8S–ITS2 rDNA variants. A parent spore was populated with 750 nuclei, each carrying 1 of 13 variants for PLS1, or 1 of 3 variants for rDNA. Distributions of variant frequency in the parent spores were generated in the multigeneration simulations described above (starting with 14 variants for PLS1 and 4 variants for rDNA). The number of nuclei determined by the bottleneck parameter was transferred to each progeny spore by sampling with replacement. We implemented mutations as before. Under each bottleneck scheme, 750 and 1,100 single-spore cultures, yielding 100 progeny spores each, were simulated for PLS1 and rDNA, respectively. Details of probability estimates are given in the Supplementary Information.

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Multistability in the lactose utilization network of *Escherichia coli*

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Multistability, the capacity to achieve multiple internal states in response to a single set of external inputs, is the defining characteristic of a switch. Biological switches are essential for the determination of cell fate in multicellular organisms[1], the regulation of cell-cycle oscillations during mitosis[2,3] and the maintenance of epigenetic traits in microbes[4]. The multistability of several natural[1–6] and synthetic[7–9] systems has been attributed to positive feedback loops in their regulatory networks[10]. However, feedback alone does not guarantee multistability. The phase diagram of a multistable system, a concise description of internal states as key parameters are varied, reveals the conditions required to produce a functional switch[11,12]. Here we present the phase diagram of the bistable lactose utilization network of *Escherichia coli*[13]. We use this phase diagram, coupled with a mathematical model of the network, to quantitatively investigate processes such as sugar uptake and transcriptional regulation *in vivo*. We then show how the hysteretic response of the wild-type system can be converted to an ultrasensitive graded response[14,15]. The phase diagram thus serves as a sensitive probe of molecular interactions and as a powerful tool for rational network design.

The bistability of the lactose utilization network has been under investigation since 1957 (refs 16, 17). The basic components of this network have been well characterized[13], making it an ideal candidate for global analysis. The *lac* operon comprises three genes required for the uptake and metabolism of lactose and related sugars (Fig. 1): *lacZ*, *lacY* and *lacA*. *lacZ* codes for β-galactosidase, an enzyme responsible for the conversion of lactose into allolactose and subsequent metabolic intermediates. *lacY* codes for the lactose permease (LacY), which facilitates the uptake of lactose and similar molecules, including thio-methylgalactoside (TMG), a non-metabolizable lactose analogue. *lacA* codes for an acetyltransferase, which is involved in sugar metabolism. The operon has two transcriptional regulators: a repressor (LacI) and an activator (the cyclic AMP receptor protein, CRP). Inducers, among them allolactose and TMG, bind to and inhibit repression by LacI, whereas cAMP binds to and triggers activation by CRP. The concentration of cAMP drops in response to the uptake of various carbon sources, including glucose and lactose[18]; glucose uptake also interferes with LacY activity, leading to exclusion of the inducer[18]. Together these effects mediate catabolite repression—the ability of glucose to inhibit *lac* expression. Crucially, cAMP levels are not affected by TMG uptake. Therefore, the extracellular concentrations of TMG and glucose can be used to independently regulate the activities of LacI and CRP, the two *cis*-regulatory inputs of the *lac* operon[19]. However, the response of the operon must be considered within the broader context of the network. The uptake of TMG induces the synthesis of LacY, which in turn promotes further TMG uptake; the resulting positive feedback loop creates the potential for bistability[20,21].

To probe the network's bistable response, we incorporated a single copy of the green fluorescent protein gene (*gfp*) under the control of the *lac* promoter into the chromosome of *E. coli* MG1655 (Fig. 1). We placed this reporter in the chromosome rather than on a multicopy plasmid to minimize the titration of LacI molecules by extraneous LacI-binding sites. The cells also contained a plasmid encoding a red fluorescent reporter (HcRed) under the control of the galactitol (*gat*) promoter. This promoter includes a CRP-binding site, as well as a binding site for the galactitol repressor,

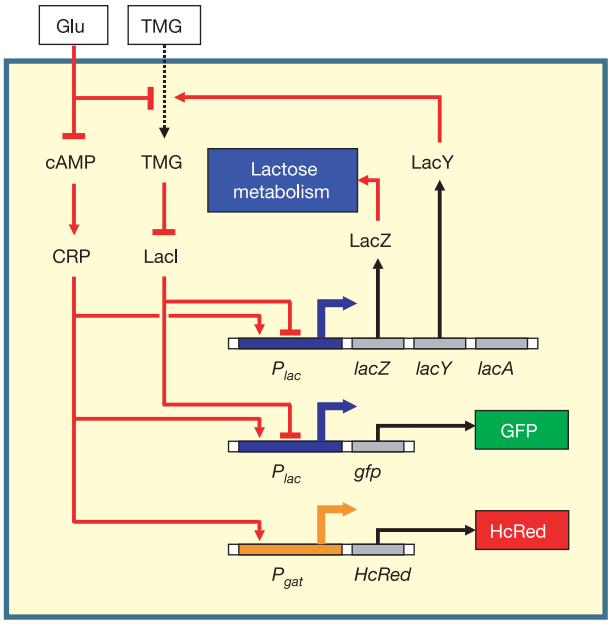

Figure 1 The lactose utilization network. Red lines represent regulatory interactions, with pointed ends for activation and blunt ends for inhibition; black arrows represent protein creation through transcription and translation, and dotted arrows represent uptake across the cell membrane. In our experiments we vary two external inputs, the extracellular concentrations of glucose and TMG, and measure the resulting levels of two fluorescent reporter proteins: GFP, expressed at the *lac* promoter, and HcRed, expressed at the *gat* promoter. LacY catalyses the uptake of TMG, which induces further expression of LacY, resulting in a positive feedback loop.