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A hydrogen-based subsurface microbial community dominated by methanogens

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The search for extraterrestrial life may be facilitated if ecosystems can be found on Earth that exist under conditions analogous to those present on other planets or moons. It has been proposed, on the basis of geochemical and thermodynamic considerations, that geologically derived hydrogen might support subsurface microbial communities on Mars and Europa in which methanogens form the base of the ecosystem[1–5]. Here we describe a unique subsurface microbial community in which hydrogen-consuming, methane-producing Archaea far outnumber the Bacteria. More than 90% of the 16S ribosomal DNA sequences recovered from hydrothermal waters circulating through deeply buried igneous rocks in Idaho are related to hydrogen-using methanogenic microorganisms. Geochemical characterization indicates that geothermal hydrogen, not organic carbon, is the primary energy source for this methanogen-dominated microbial community. These results demonstrate that hydrogen-based methanogenic communities do occur in Earth's subsurface, providing an analogue for possible subsurface microbial ecosystems on other planets.

Table 1 Chemical composition of groundwater from Lidy Hot Springs

Constituent or property	Value
Temperature (°C)	58.5
Microorganisms (cells per ml)	$(2.8 \pm 0.7) \times 10^5$
pH	6.77
Dissolved oxygen (mg l^{-1})	<0.05
Iron (mg l^{-1})	<0.01
Sulphide (mg l^{-1})	0.070
Methane (mg l^{-1})	2.0
Hydrogen (nM)	13.0 ± 2.0
Carbon monoxide (nM)	1.3
Dissolved inorganic carbon (mg l^{-1})	666
Dissolved organic carbon (mg l^{-1})	<0.27
^{14}C of dissolved inorganic carbon (per cent of modern value)	<1.20
$\delta^{13}C$ of dissolved inorganic carbon (per ml)	−2.1 ± 0.2
Formate (μM)	<1.0
Acetate (μM)	<1.0
Propionate (μM)	<1.0
Butyrate (μM)	<1.0
Sulphate (mg l^{-1})	131
Nitrate (mg l^{-1})	<0.1
Chloride (mg l^{-1})	6.4
Calcium (mg l^{-1})	91.0
Magnesium (mg l^{-1})	21.5
Potassium (mg l^{-1})	17.1
Sodium (mg l^{-1})	38.3
Ammonium (mg l^{-1})	0.56
Alpha (as ^{230}Th; g l^{-1})	14.4 ± 1.17
Beta (as ^{90}Sr; pCi l^{-1})	12.9 ± 1.03
^{222}Rn (pCi l^{-1})	470 ± 14

Values with error are mean ± s.d.

The lack of water on the surface of extraterrestrial bodies within our Solar System suggests that, if extraterrestrial life exists in the Solar System, it will be found in the subsurface, where liquid water may be present[1–7]. These extraterrestrial subsurface habitats are not expected to contain significant quantities of organic carbon to support life. However, the interaction of hydrothermal fluids with igneous rocks can produce hydrogen or other reduced compounds that may provide an energy source for microorganisms[2,4].

On Earth, microorganisms can gain energy by coupling the oxidation of hydrogen to the reduction of compounds such as oxygen, nitrate, Fe(III), sulphate or carbon dioxide. Molecular oxygen, which is required for the production of significant quantities of nitrate, Fe(III) and sulphate, is available on Earth because of photosynthesis. Although photolysis also produces oxygen on Earth and on extraterrestrial bodies, it is uncertain whether this process delivers oxygen to subsurface environments. This suggests that carbon dioxide is likely to be the most abundant electron acceptor for hydrogen oxidation in the subsurface of extraterrestrial bodies.

It has been suggested[1,2] that life in the subsurface of Mars and Europa might consist primarily of microorganisms that couple the oxidation of hydrogen with the reduction of carbon dioxide to produce methane according to the reaction

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (1)$$

In an ecosystem where they form the base of the food chain, hydrogen-consuming methanogenic microorganisms would be expected to be numerically predominant, although heterotrophic microorganisms may also be present in lesser numbers.

To find a subsurface environment that might serve as a suitable analogue for proposed hydrogen-based microbial ecosystems on Mars and Europa, we searched for a subsurface environment that lacked organic carbon, but that had a source of geologically produced hydrogen. These conditions occur at Lidy Hot Springs, Idaho, which is located at the southern extent of the Beaverhead Mountains. The underlying rocks are of volcanic origin and are devoid of allochthonous organic carbon[8], and there are potential sources of geologically produced hydrogen[9–11]. Moreover, there is a well developed system of geothermal springs that tap deep fault zones[12,13]. This deep (200 m below land surface) hydrothermal

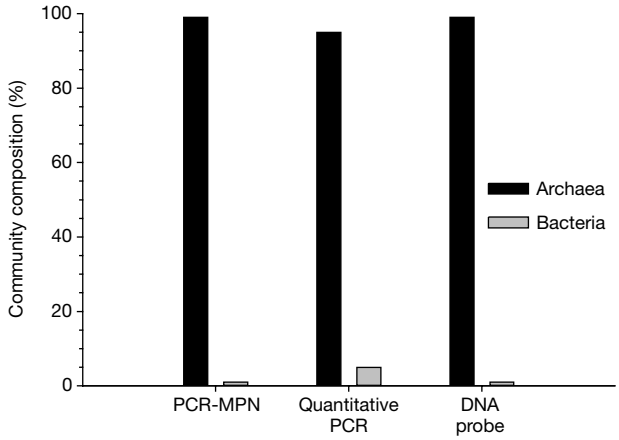

Figure 1 Proportions of Archaea and Bacteria in groundwater from Lidy Hot Springs, as determined by three different molecular techniques.

system was specially instrumented to recover water samples free from surface contamination.

The subsurface water was hot (58.5 °C) and anoxic, and contained negligible concentrations of dissolved organic carbon and short-chain fatty acids (Table 1). Even acetate, the predominant organic acid found in anaerobic environments in which organic matter serves as the primary energy source, was undetectable (Table 1). The low ^{14}C and slightly negative $\delta^{13}C$ content of dissolved inorganic carbon (Table 1) indicated an ancient (>15,000 years), deep source for the water, which is consistent with the mantle-derived 'hot spot' origin of volcanism in this part of Idaho[12]. This further indicated that the water did not contain recent surface-derived organic carbon capable of serving as an energy source for microorganisms.

In contrast to the lack of organic electron donors for microbial metabolism, the water did contain molecular hydrogen (Table 1) at concentrations previously shown to support the activity of hydrogen-oxidizing methanogenic microorganisms in a wide diversity of sedimentary environments[14]. Hydrogen is commonly associated with hydrothermal waters in volcanic terrains[9,10] and is produced by active tectonism[10,11].

Microscopic cell counts indicated that microorganisms were living at depth in this hydrothermal system (Table 1). To initially characterize this microbial community, microorganisms from the ground water were collected on filters[15], and the number of Bacteria and Archaea were estimated by polymerase chain reaction accompanied by a most probable number method (PCR-MPN)[16]. Estimating the relative number of Bacteria and Archaea in this manner might be expected to overestimate the importance of Bacteria, because Bacteria are more likely than Archaea to have multiple copies of 16S rRNA genes[17]. However, the numbers of Bacteria and Archaea in the water were estimated to be 1,230 ± 26 and 227,000 ± 428 per ml (mean ± s.d.) of water respectively. This indicated that Archaea comprised as much as 99% of the microbial community (Fig. 1). Additional analysis of the relative proportions of Archaea and Bacteria with the GeneAmp sequence detection system for quantitative PCR confirmed that the microbial population consisted of more than 95% Archaea (Fig. 1). To evaluate the composition of the microbial community with a technique that did not involve potential biases associated with PCR, samples were also analysed with four radiolabelled probes designed to hybridize with 16S rDNA of either all microorganisms, Archaea, Bacteria or the bacterial order Planctomycetales. When compared with the response to a universal probe designed to hybridize to all microorganisms, about 99% of the 16S rDNA could be attributed to Archaea and 1% hybridized to the probe for Bacteria (Fig. 1). A probe specific for Planctomycetales gave no response.

To learn more about the Archaea present in Lidy Hot Springs, 16S rDNA recovered from the waters was cloned and sequenced[18,19]. The ten clones that were initially sequenced all belonged to two similar sequences (IUA5 and IUA6), which are most closely related to known methanogenic microorganisms (Fig. 2). To further evaluate

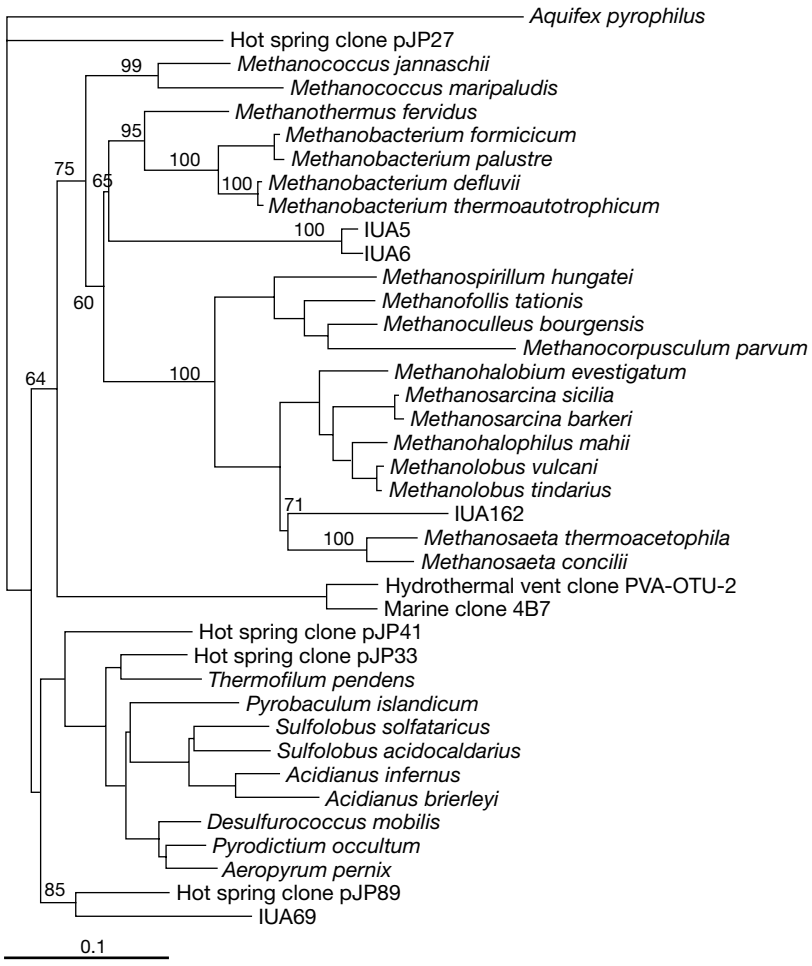

Figure 2 Phylogenetic analysis of archaeal sequences from Lidy Hot Springs. Lidy sequences are designated IUA. The tree was constructed by Jukes–Cantor distance analysis; bootstrap values are shown for key branches.

the predominance of methanogens, 55 additional clones were screened by 16S restriction fragment length polymorphism (RFLP) analysis[20]: 44 of the clones had RFLP patterns identical to IUA5 and IUA6. An additional 8 of the remaining 11 clones had an identical RFLP pattern, designated IUA162, that although different from IUA5 and IUA6 was also related to known methanogens (Fig. 1). The remaining three sequences, designated IUA69, were most closely related to a sequence recovered from a hot spring in Yellowstone National Park[21], California, the physiology of which is unknown. Thus, no fewer than 62 of the 65 archaeal sequences recovered from deep circulating hydrothermal waters underlying Lidy Hot Springs seemed to be from methanogenic microorganisms. To our knowledge, such a predominance of methanogens is unknown in subsurface microbial ecosystems.

It has been suggested that hydrogen produced from water reacting with basalt was the main energy source supporting the microbial community in methanogenic portions of the Columbia River basalt aquifer[22]. However, analysis of 16S rDNA sequences in the groundwater from the site, with the same probes used for analysing the Lidy Hot Springs, demonstrated that less than 3% of the microbial community was composed of methanogenic microorganisms[23], a proportion similar to that found in anaerobic environments in which organic matter is the primary source of energy supporting microbial growth. A likely explanation for this is that little hydrogen is produced from water–basalt interactions under ambient conditions in the Columbia River basalt aquifer, and that organic matter serves as the primary source of energy in this environment[24].

The methanogen-dominated microbial community present at Lidy Hot Springs is unlike any previously described on Earth, and the predominance of hydrogen-oxidizing methanogens in this hydrogen-containing subsurface environment, poor in organic carbon, is consistent with geochemical scenarios proposed for microbial communities that may inhabit the subsurface of Mars and Europa[1–6]. Therefore, terrestrial ecosystems such as Lidy Hot Springs provides a useful analogue for the kinds of hydrogen-based, subsurface microbial communities that may exist elsewhere in the Solar System. □

Methods

Groundwater samples

The well instrumentation was designed and built by C. E. Wilson, the owner of Lidy Hot Springs, as a means to capture and regulate spring flow. At the request of the US Geological Survey, C. E. Wilson modified the instrumentation to enable representative water samples to be obtained directly from deep fracture zones. A hole was drilled through the overburden (~25 m thick), and the overburden was sealed off with steel well casing cemented to the top of the underlying igneous rocks. A drill-rod assembly was equipped with a valve system for restraining the pressure of the hydrothermal waters, and an open bore hole was drilled about 200 m into the underlying igneous rocks. About 100 m of polyethylene casing was then inserted into the bore hole, extending about 75 m below the steel casing into the fractured igneous rocks. Because there is a constant upward flow of groundwater through the plastic casing (upward pressure at the top of the casing is ~275 kPa, or ~40 p.s.i.), and because this water flows continuously from the deep fractures, this configuration makes it possible to obtain water samples suitable for detailed geochemical and microbiological characterization of the deep hydrothermal ecosystem.

Geochemical analyses

Anions, cations and volatile fatty acids were analysed by the US Geological Survey using ion chromatography (anions, volatile fatty acids) and inductively coupled plasma spectroscopy (cations). Dissolved inorganic carbon (DIC) and methane were determined by headspace gas chromatographic analysis of water samples injected into sealed serum bottles. DIC was measured after acidifying the sample. Dissolved hydrogen was measured by the bubble-strip method[25]. Water samples were screened for the presence of volatile and semi-volatile organic compounds with gas chromatography–mass spectrometry, but no compounds were detected other than internal standards. Microorganisms in the groundwater were enumerated on black filters with epifluorescent microscopy[26].

Microbiological characterization

For molecular studies, groundwater was filtered as described previously and DNA extracted[15]. For enumeration of Archaea with PCR-MPN[16], 16S rDNA was initially amplified with the primers 25F and 1392R followed by a second round of amplification with 344F and 915R. To enumerate Bacteria, the initial amplification was with 8F and 1392R followed by amplification with 338F and 907R. This enumeration technique was verified with standards of archaeal and bacterial DNA. Quantitative PCR was performed with the GeneAmp 5700 sequence-detection system (ABI). DNA was amplified with the Bacteria-specific primer 338F or the Archaea-specific primer 344F and the universal primer 907R. Incorporation of the fluorescent dye SybrGreen into double-stranded DNA provided a fluorescent signal, which was detected with the GeneAmp 5700. Relative proportions of archaeal or bacterial targets were determined by interpolation from a standard curve generated with standard additions of archaeal or bacterial DNA. Oligonucleotide probing was carried out as previously described[19]. Samples were hybridized with either the universal probe UNIV-1392, archaeal probe Arch-915, bacterial probe EUB-338 or *Planctomycetes* probe PLA886.

Phylogenetic analyses

For phylogenetic analysis, DNA from the hot spring was recovered and amplified as described above, cloned, and sequenced by standard procedures[18]. BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) was used to identify and obtain similar sequences from GenBank (http://www.ncbi.nlm.nih.gov/Genbank)[18]. Distance matrix, maximum likelihood and bootstrap analyses were performed using 508 homologous bases for the archaeal clones. For RFLP analysis, 55 clones were amplified with M13 forward and reverse primers. The resulting PCR product was digested overnight with the restriction enzyme *Msp*I. Digests were run on a 3% Metaphor gel (FMC Products) and restriction patterns were visualized with ethidium bromide staining.

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1. Boston, P. M., Ivanov, M. V. & McKay, C. P. On the possibility of chemosynthetic ecosystems in subsurface habitats on Mars. *Icarus* **95,** 300–308 (1992).
2. McCollom, T. M. Methanogenesis as a potential source of chemical energy for primary biomass production by autotrophic organisms in hydrothermal systems on Europa. *J. Geophys. Res.* **104** (E12)**,** 30729–30742 (1999).
3. Carr, M. H. in *Evolution of Hydrothermal Ecosystems on Earth (and Mars?)* (eds Bock, G. R. & Goode, J. A.) 249–265 (Wiley, Chichester, 1996).
4. McKay, C. P. in *Subsurface Microbiology and Biogeochemistry* (eds Fredrickson, J. K. & Fletcher, M.) 315–327 (Wiley, New York, 2001).
5. Fisk, M. R. & Giovannoni, S. J. Sources of nutrients and energy for a deep biosphere on Mars. *J. Geophys. Res.* **104** (E12)**,** 11805–11815 (1999).
6. Shock, E. L. in *Evolution of Hydrothermal Ecosystems on Earth (and Mars?)* (eds Bock, G. R. & Goode, J. A.) 40–60 (Wiley, Chichester, 1996).
7. Farmer, J. D. Hydrothermal systems: Doorways to early biosphere evolution. *GSA Today* **10** (7)**,** 1–9 (2000).
8. Embree, G. F., McBroome, L. A. & Soherty, D. J. Preliminary stratigraphic framework of the Pliocene and Miocene rhyolite, Eastern Snake River Plain. *Idaho Bur. Mines Geol. Bull.* **26,** 333–346 (1982).
9. Sugisaki, R. & Sugiura, T. Gas anomalies at three mineral springs and a fumarole before an inland earthquake, Central Japan. *J. Geophys. Res.* **91,** 12296–12304 (1986).
10. Lilley, M. D. & Olson, E. J. in *Abstr. 11th Annu. V. M. Goldschmidt Conf.* Abstract no. 3682, LPI contribution no. 1088 (Lunar and Planetary Institute, Houston, 2001) (CD-ROM).
11. Wakita, H. *et al.* Hydrogen release: New indicator of fault activity. *Science* **210,** 188–190 (1980).
12. Pierce, K. L. & Morgan, L. A. The track of the Yellowstone hot spot: Volcanism, faulting, and uplift. *Geol. Soc. Am. Mem.* no. 179 (1992).
13. Knobel, L. L. *et al.* Chemical constituents in ground water from 39 selected sites with an evaluation of quality assurance data, Idaho National Engineering and Environmental Laboratory and vicinity, Idaho. *US Geol. Surv. Op. File Rep.* no. 99-246 (1999).
14. Lovley, D. R. & Goodwin, S. Hydrogen concentrations as an indicator of the predominant terminal electron accepting reactions in aquatic sediments. *Geochim. Cosmochim. Acta* **52,** 2993–3003 (1988).
15. Orphan, V. J., Taylor, L. T., Hafenbrandl, D. & Delong, E. F. Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. *Appl. Environ. Microbiol.* **66,** 700–711 (2000).
16. Picard, C. *et al.* Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Appl. Environ. Microbiol.* **58,** 2717–2722 (1992).
17. Klappenbach, J. A. *et al.* rrndb: the ribosomal RNA operon copy number database. *Nucleic Acids Res.* **29,** 181–184 (2001).
18. Amann, R. I., Ludwid, W. & Schleifer, K. H. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59,** 143–169 (1995).
19. Massana, R., Murray, A. E., Preston, C. M. & Delong, E. F. Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara channel. *Appl. Environ. Microbiol.* **63,** 50–56 (1997).
20. Moyer, C. L., Dobbs, F. C. & Karl, D. M. Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active hydrothermal vent system, Loihi Seamount, Hawaii. *Appl. Environ. Microbiol.* **60,** 871–879 (1994).
21. Barns, S. M. *et al.* Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proc. Natl Acad. Sci. USA* **91,** 1609–1613 (1994).
22. Stevens, T. O. & McKinley, J. P. Lithoautotrophic microbial ecosystems in deep basalt aquifers. *Science* **270,** 450–454 (1995).
23. Fry, N. K. *et al.* Population structure of microbial communities associated with two deep, anaerobic, alkaline aquifers. *Appl. Environ. Microbiol.* **63,** 1498–1504 (1997).
24. Anderson, R. T., Chapelle, F. H. & Lovley, D. R. Evidence against hydrogen-based microbial ecosystems in basalt aquifers. *Science* **281,** 976–977 (1998).
25. Chapelle, F. H., Vroblesky, D. A., Woodward, J. C. & Lovley, D. R. Practical considerations for measuring hydrogen concentrations in groundwater. *Environ. Sci. Tech.* **31,** 2873–2877 (1997).
26. Hobbie, J. E., Daley, R. J. & Jasper, S. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* **33,** 1225–1228 (1977).

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Contribution of *Distal-less* to quantitative variation in butterfly eyespots

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The colour patterns decorating butterfly wings provide ideal material to study the reciprocal interactions between evolution and development. They are visually compelling products of selection, often with a clear adaptive value, and are amenable to a detailed developmental characterization[1]. Research on wing-pattern evolution and development has focused on the eyespots of the tropical butterfly *Bicyclus anynana*[2]. There is quantitative variation for several features of eyespot morphology[3–5] but the actual genes contributing to such variation are unknown. On the other hand, studies of gene expression patterns in wing primordia have implicated different developmental pathways in eyespot formation[6–11]. To link these two sets of information we need to identify which genes within the implicated pathways contribute to the quantitative variation accessible to natural selection. Here we begin to bridge this gap by demonstrating linkage between DNA polymorphisms in the candidate gene *Distal-less (Dll)* and eyespot size in *B. anynana*.

The comparison of gene expression patterns across species has been a common approach in evolutionary developmental biology. This approach can identify steps in developmental pathways that have been altered during evolution, but it fails to identify the actual genetic changes that have occurred[12]. Despite the recognized importance of understanding the generation of the variants that can be sorted by natural selection[13], the genes contributing to standing quantitative variation in morphological traits are largely unknown. Recent work has focused on bristle number in *Drosophila melanogaster*[14]. Unfortunately the direct developmental mechanisms through which polymorphisms in candidate genes contribute to variation in bristle number, and the ecological significance of this variation, are difficult to determine. Here we investigate the genetic basis of eyespot size in *B. anynana*, a character of more obvious adaptive significance[2,15] and whose developmental basis can be dissected using manipulative experiments[3].

Studies of gene expression patterns in pre-adult wing primordia have implicated a series of genes in butterfly wing patterning and, in particular, in eyespot formation[6–11]. Genes including *Dll* and genes of the *hedgehog* pathway have circular regions of expression that correspond to the position of eyespot centres[8,9] whose organizing properties have been clearly demonstrated[16,17]. *Dll* is particularly interesting because its expression patterns appear to reveal different stages in eyespot formation and parallel adult variants of eyespot morphology[8]. These results suggest that *Dll* is involved in regulating the formation and diversity of eyespot patterns in butterfly wings[8]. However, although *Dll* expression patterns implicate the Dll protein in eyespot formation, it is unclear how standing variation at this locus contributes to inter-individual variation in eyespot patterns. Here we test whether segregating variation at *Dll* contributes to short-term response to selection on eyespot size.

We used artificial selection to establish lines that differed in the size of the two dorsal forewing eyespots of *B. anynana* (see Methods). After nine generations of selection, the lines show markedly different phenotypes; the 'high' line has large eyespots and the 'low' line, small eyespots (Fig. 1a, b). The rapid and gradual response to selection indicates that there is substantial additive genetic variance for eyespot size. Realized heritabilities of around 0.6–0.7 (Fig. 1a) are comparable to previous estimates for the size of the posterior eyespot[3]. The selected butterflies also show quantitative differences in *Dll* expression: wing discs of the high line have considerably larger areas of *Dll* expression around the location of the centre of the presumptive eyespots. These differences are already apparent in late final instar larvae (Fig. 1c), and more marked in pupal wing discs (Fig. 1d). The observed ontogeny of *Dll* expression is comparable to that described previously[8].

The changes in *Dll* expression might be caused by DNA polymorphisms in *Dll* itself or in upstream regulators of *Dll*. To distinguish between the hypotheses of *cis* versus *trans* polymorphisms affecting *Dll* expression, a series of crosses were made using the high and low selection lines (Fig. 2a). The progeny from a backcross between a hybrid butterfly and each of the parental lines have

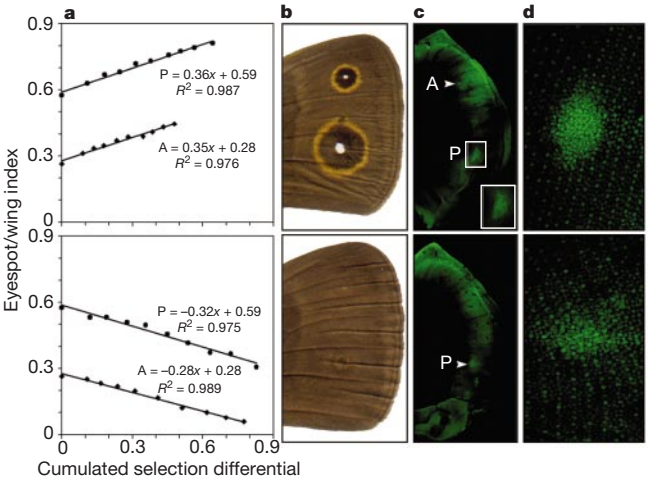

Figure 1 *Bicyclus anynana* selection lines divergent for the size of the dorsal forewing eyespots. **a**, Response to artificial selection for large (high line, top) and small (low line, bottom) eyespots. For each generation, the selection differential is the difference between the mean trait value for all butterflies and that of the selected group[29]. Realized heritabilities are twice (selection has targeted females only)[29] the absolute value of the slopes estimated from least-squares regressions for both eyespots (anterior (A) indicated by diamonds, posterior (P) by circles). **b**–**d**, Eyespot size phenotypes and *Dll* expression in butterflies from the high and low selection lines. Butterflies from the high line have large adult eyespots (**b**, top). They also show enlarged domains of *Dll* expression in the centre of the putative eyespots both in late larval wing discs (**c**, top; inset with higher magnification of central area of posterior (P) eyespot; arrow points at centre of anterior (A) eyespot) and in pupal wings (anterior eyespot centre shown in **d**, top). Butterflies from the low line have small, often absent, adult eyespots (**b**, bottom) and small areas of *Dll* expression in wing primordia (**c**, **d**, bottom). In the larval disc in **c** (bottom) there is no visible *Dll* expression associated with the anterior eyespot; arrow points to the posterior eyespot centre. In the pupal disc there are small domains of *Dll* expression as shown here for the anterior eyespot (**d**, bottom).