sediments to the water column (*10*). Fe(II) accumulates in the anoxic waters, and upward-diffusing Fe(II) can be quickly oxidized at the anoxic:oxic interface, causing the precipitation of HFO, which can then scavenge As and other particle-reactive compounds and settle. During reductive dissolution of HFO, sorbed As(V) is generally reduced to As(III) (*30*, *31*). This is consistent with thermodynamic predictions in an Fe-reducing system [50 to 98% of As should be present as As(III) at Fe(II) levels from 50 to 100 μM and pH 6.6 to 6.8]. It is therefore As(III), which is typically found to constitute most porewater As in reducing sediments and soils (*18*, *32*, *33*), that should diffuse into overlying anoxic waters. Direct mobilization of As(V) during HFO reduction has only been observed in laboratory pure culture study with an Fe(III)-reducing bacterium (*4*). At the anoxic:oxic interface, abiotic or biologically mediated (*34*) As(III) oxidation by O_2 may take place. Abiotic oxidation by particulate $Mn^{(IV)}O_2$ is also possible (*35*).

20. D. B. Senn, thesis, Massachusetts Institute of Technology (2001).
21. Materials and methods are available as supporting material on *Science* Online.
22. C. J. Ottley, W. Davison, W. M. Edmunds, *Geochim. Cosmochim. Acta* **61**, 1819 (1997).
23. D. Dzombak, F. M. M. Morel, *Surface Complexation Modeling: Hydrous Ferric Oxide* (Wiley Interscience, New York, 1990).
24. S. W. A. Naqvi *et al.*, *Nature* **408**, 346 (2000).
25. B. T. Nolan, J. D. Stoner, *Environ. Sci. Technol.* **34**, 1156 (2000).
26. D. Postma, C. Boesen, H. Kristiansen, F. Larsen, *Water Resour. Res.* **27**, 2027 (1991).
27. S. Ratering, S. Schnell, *Environ. Microbiol.* **3**, 100 (2001).
28. British Geological Survey, www.bgs.ac.uk/arsenic/bangladesh/datadownload.htm.
29. R. T. Nickson, J. M. McArthur, P. Ravenscroft, W. G. Burgess, K. M. Ahmed, *Appl. Geochem.* **15**, 403 (2000).
30. D. Ahmann, L. R. Krumholz, H. F. Hemond, D. R. Lovley, F. M. M. Morel, *Environ. Sci. Technol.* **31**, 2923 (1998).
31. J. M. Harrington, S. E. Fendorf, R. F. Rosenzweig, *Environ. Sci. Technol.* **32**, 2425 (1998).
32. P. H. Masscheleyn, R. D. Delaune, W. H. Patrick, *Environ. Sci. Technol.* **25**, 1414 (1991).
33. J. N. Moore, W. H. Ficklin, C. Johns, *Environ. Sci. Technol.* **22**, 432 (1988).
34. J. A. Wilkie, J. G. Hering, *Environ. Sci. Technol.* **32**, 657 (1998).
35. M. J. Scott, J. J. Morgan, *Environ. Sci. Technol.* **29**, 1898 (1995).
36. B. Voelker and P. Gschwend provided guidance throughout the study, D. Ahmann and J. Jay aided in developing microcosm procedures, and D. Brabander assisted with field work. This research was funded by National Institute of Environmental Health Sciences (NIEHS) Superfund Basic Research Program grant 5P42ES04675-06.

Supporting Online Material
www.sciencemag.org/cgi/content/full/296/5577/2373/DC1.
Materials and Methods
Fig. S1
Table S1

1 April 2002; accepted 30 May 2002

50 Million Years of Genomic Stasis in Endosymbiotic Bacteria

Ivica Tamas,[1]* Lisa Klasson,[1]* Björn Canbäck,[1] A. Kristina Näslund,[1] Ann-Sofie Eriksson,[1] Jennifer J. Wernegreen,[2] Jonas P. Sandström,[1] Nancy A. Moran,[2] Siv G. E. Andersson[1]†

Comparison of two fully sequenced genomes of *Buchnera aphidicola*, the obligate endosymbionts of aphids, reveals the most extreme genome stability to date: no chromosome rearrangements or gene acquisitions have occurred in the past 50 to 70 million years, despite substantial sequence evolution and the inactivation and loss of individual genes. In contrast, the genomes of their closest free-living relatives, *Escherichia coli* and *Salmonella* spp., are more than 2000-fold more labile in content and gene order. The genomic stasis of *B. aphidicola*, likely attributable to the loss of phages, repeated sequences, and *recA*, indicates that *B. aphidicola* is no longer a source of ecological innovation for its hosts.

The availability of genome sequences for related bacteria is providing exciting insights into evolution, but one limitation has been the lack of identifiable bacterial fossils to provide a time frame for these studies. We have quantified total rates of genomic evolution for *Buchnera aphidicola*, an obligate mutualistic symbiont of aphids, by sequencing the genome of the *B. aphidicola* symbiont of *Schizaphis graminum* (Sg) (*1*) and analyzing its divergence from the published sequence of the *B. aphidicola* symbiont of *Acyrthosiphon pisum* (Ap) (*2*).

This case allows genome evolution to be calibrated reliably with respect to time. Because the symbiont phylogeny mirrors that of its aphid hosts, indicating synchronous diversification, divergence dates reconstructed for ancestral aphids can be extended to the corresponding *B. aphidicola* ancestors. This approach has been used to infer that this endosymbiosis was established at least 150 million years ago (Ma) and that the lineages represented by *B. aphidicola* (Sg) and *B. aphidicola* (Ap) diverged 50 to 70 Ma (*3*, *4*) (Fig. 1A). These are the only fully sequenced organisms that have eliminated *recA*, which is expected to lower the incidence of recombination events (*5*).

The genomes of *B. aphidicola* (Sg) and *B. aphidicola* (Ap) are similar in size [0.64 megabases (Mb)] and are among the smallest of bacterial genomes. Their gene content is also very similar, with 526 genes shared of the 564 and 545 intact genes present in *B. aphidicola* (Ap) and *B. aphidicola* (Sg), respectively (Table 1). A comparison of the aligned genome sequences (*1*) confirms a high degree of divergence at the nucleotide sequence level. On the basis of a divergence date of 50 million years (My), average rates of sequence evolution were estimated at 9.0×10^{-9} synonymous substitutions per site per year and 1.65×10^{-9} nonsynonymous substitutions per site per year. The observed divergence at synonymous sites shows low variance among genes (*1*), suggesting that the synonymous divergence level corresponds to the mutation rate of *B. aphidicola*, which is similar to or slightly higher than the rate estimated in *E. coli* and *Salmonella typhimurium* (*4*, *6*, *7*).

Despite high levels of sequence divergence, the two *B. aphidicola* genomes show complete conservation of genomic architecture (Fig. 1B). No inversions, translocations, duplications, or gene acquisitions have occurred in either lineage since their divergence. Of the 564 protein-coding genes originally annotated in *B. aphidicola* (Ap), only four (*yba*1 to *yba*4) were reported not to have orthologs in *E. coli* (*2*), a closely related free-living species (Fig. 2A). Our analyses suggest that even these genes were present before the establishment of the symbiosis (*1*), providing even stronger evidence that the symbiotic life-style did not in-

Table 1. Comparison of genome features for *B. aphidicola* (Sg) and *B. aphidicola* (Ap).

Feature	*B. aphidicola* (Sg)	*B. aphidicola* (Ap)
Genome size (bp)	641,454	640,681
Genic G + C content (%)	26.2	26.3
Intergenic G + C content (%)	14.8	16.1
Protein coding genes (no.)	545	564
Pseudogenes (no.)	38	13
Avg. gene length (bp)	978	985
Avg. intergenic length (bp)	118	127

[1]Department of Molecular Evolution, Evolutionary Biology Center, University of Uppsala, Uppsala, Sweden. [2]Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: Siv.Andersson@ebc.uu.se

volve the uptake of novel genes. Many of the *fli* and *flg* homologs (loci involved in flagellar biosynthesis and protein export) show unusually high levels of amino acid divergence within *B. aphidicola* relative to the divergence between *E. coli* and *Salmonella typhi* (Fig. 2B). This suggests modification of function of these loci, which is also supported by lack of a flagellum in micrographs of *B. aphidicola* and lack of *fliC*, encoding the filament protein.

Our comparison highlights the first clear pattern of genome-scale evolution: obligately host-associated bacteria show enhanced stability of genome architecture relative to sequence evolution (Fig. 2), which is also indicated by comparisons of pathogen genomes (*5*). *B. aphidicola* is the most extreme organism analyzed so far, with no rearrangements or gene acquisitions and only a few gene losses during the past 50 My (Fig. 2, C and D). This stasis is remarkable because *E. coli*, *S. typhi*, and *S. typhimurium*, the closest relatives of *B. aphidicola*, have highly labile genomes (Fig. 2, C and D). The ratio of insertions and deletions (indels) and rearrangements per nonsynonymous substitutions is more than 2000-fold higher in modern *E. coli* and *Salmonella* spp.; this represents a massive difference even when normalized for the eightfold difference in genome size.

The *B. aphidicola* (Sg) genome sequence also provides insight into the ecological role of the endosymbionts in the supplementation of the host's phloem sap diet, which is deficient in the 10 essential amino acids required in animal diets (*8*). Both genomes possess 54 genes that produce these amino acids (*1*, *2*). However, five genes, *cysN*, *-D*, *-G*, *-H*, and *-I*, involved in sulphur reduction and biosynthesis of cysteine contain frameshifts and stop codons in *B. aphidicola* (Sg) but are intact in *B. aphidicola* (Ap) (Fig. 1C). *S. graminum* ingests more cys-

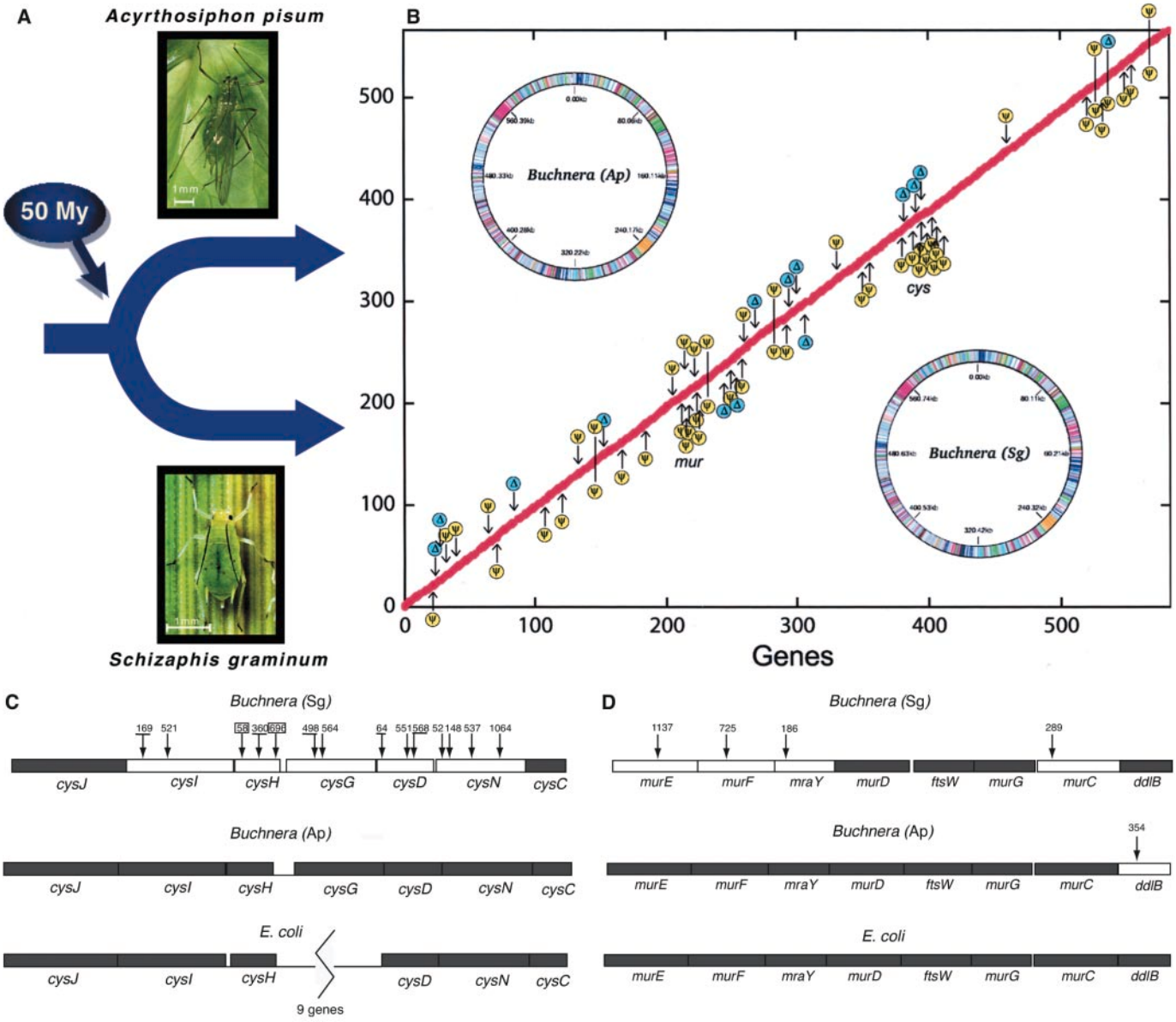

Fig. 1. Comparison of gene order structures and location of pseudogenes in *B. aphidicola* (Ap) and *B. aphidicola* (Sg). (**A**) During the past 50 My *A. pisum* and *S. graminum* have diverged 2-fold in size and 10-fold in body weight. (**B**) The positions of pseudogenes in the *B. aphidicola* (Ap) and *B. aphidicola* (Sg) genomes are indicated with arrows above and below the line, respectively. The axes are the ranked position of each ortholog along the chromosome, with the zero position corresponding to the putative origin of replication. Symbols and colors differentiate pseudogenes (ψ; yellow) and genes uniquely present in one species (Δ; blue). (**C**) and (**D**) Representation of pseudogenes in the cysteine and murein biosynthetic operons, respectively, in *B. aphidicola* (Sg). The nine extra genes in *E. coli* are not related to cysteine biosynthesis. Filled and open boxes represent gene and pseudogene sequences, respectively. Numbers show the position of mutations (black numbers, −1 frameshifts; numbers in boxes, +1 frameshifts; underlined numbers, stop codons).

teine than does *A. pisum* due to differences in phloem composition of their respective food plants (grasses versus legumes) (*9*) as well as the phytotoxic effects of feeding by *S. graminum* (*8*). This enrichment of the host diet renders superfluous the capacity for sulphur assimilation by *B. aphidicola* (Sg), and inactivation of the underlying genes exemplifies evolution of the symbiont in response to host environmental conditions.

In total, we identified 13 and 38 pseudogenes (*1*) in *B. aphidicola* (Ap) and *B. aphidicola* (Sg), respectively; half are located in biosynthetic operons with defects in several contiguous genes (Fig. 1). Another 14 genes, each showing homology to some *E. coli* gene, are present in one *B. aphidicola* genome but are missing entirely from the other. In numerous instances, the intergenic region in one species is as long as the corresponding gene in the other species (*1*). These regions probably consist of genes degraded beyond recognition due to the juxtaposition of several gene remnants and/or due to early inactivation followed by extensive nucleotide substitution.

Among new pseudogenes in *B. aphidicola* (Sg), five are associated with DNA repair processes, including base excision repair. The mutational spectrum in the weakly degraded genes in *B. aphidicola* (Sg) is dominated by deletions of 1 to 2 nucleotides per event, consistent with a reduced capacity for repairing errors caused by bulky residues and/or photoproducts. The remarkably high copy number of the *B. aphidicola* chromosome (*10*) may result from the loss of other genes involved in the metabolism of DNA, such as *seqA* and *datA* that coordinate replication in the cell cycle of related bacteria (*11*).

Overall, we estimate that coding capacity in *B. aphidicola* has been lost at a rate of one

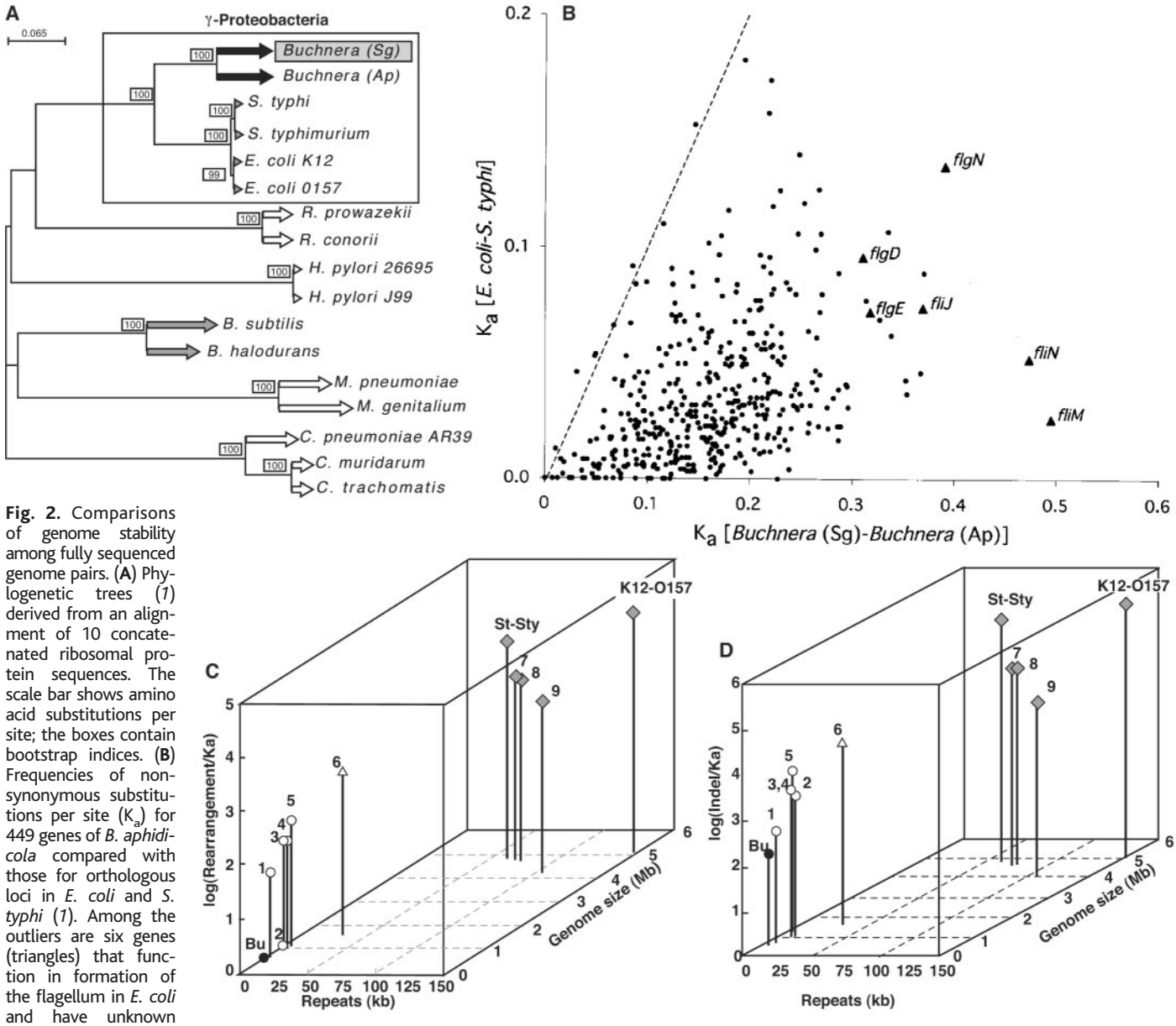

Fig. 2. Comparisons of genome stability among fully sequenced genome pairs. (**A**) Phylogenetic trees (*1*) derived from an alignment of 10 concatenated ribosomal protein sequences. The scale bar shows amino acid substitutions per site; the boxes contain bootstrap indices. (**B**) Frequencies of nonsynonymous substitutions per site (K_a) for 449 genes of *B. aphidicola* compared with those for orthologous loci in *E. coli* and *S. typhi* (*1*). Among the outliers are six genes (triangles) that function in formation of the flagellum in *E. coli* and have unknown functions in *B. aphidicola*. (**C** and **D**) Genome structure divergence (*18*) normalized to nonsynonymous substitutions averaged over 56 genes (*1*) in comparison to repeat content (*1*) and genome size in *B. aphidicola* (black circles) and other obligate host-associated bacteria (open circles), facultative intracellular parasites (open triangles), and free-living bacteria (gray squares). Abbreviations and numbers are as follows: *Bu*, *Buchnera* (Ap)-*Buchnera* (Sg); K12-0157, *E. coli* K-12-*E. coli* 0157; *St-Sty*, *S. typhi*-*S. typhimurium*; 1, *M. genitalium*-*M. pneumoniae*; 2, *C. trachomatis*-*C. muridarum*; 3, *C. muridarum*-*C. pneumoniae* AR39; 4, *C. trachomatis*-*C. pneumoniae* AR39; 5, *R. prowazekii*-*R. conorii*; 6, *H. pylori* 26695-*H. pylori* J99; 7, *E. coli* K12-*S. typhimurium*; 8, *E. coli* K12-*S. typhi*; 9, *B. subtilis*-*B. halodurans*.

complete gene elimination per 5 to 10 My during the divergence of these two *B. aphidicola* species. The evidence for reduced effectiveness of selection and lability at the nucleotide sequence level (*12*) makes the stability of gene inventory and gene arrangements in *B. aphidicola* all the more striking.

These seemingly contradictory patterns can be explained by two kinds of losses during genome reduction of obligate host-associated bacteria (*13*). First, eliminated sequences include elements that normally mediate genome dynamics and gene mobility, such as phages, plasmids, repeated sequences, and transposons. Indeed, a survey of the repeat contents in microbial genomes has revealed a decreased density of repeated sequences in obligate intracellular bacteria with genomes in the 1 Mb range (*14*). Here, *B. aphidicola* is the extreme, containing no prophages, a single rRNA operon, and no repeated sequences longer than 30 base pairs (bp). Second, gene losses also include loci that facilitate recombination and incorporation of foreign DNA. Here, the two *B. aphidicola* genomes are also distinct in their lack of *recA* and *recF*; the absence of the corresponding gene functions is expected to lower the incidence of genome rearrangements (*5*, *15*).

If the mutational input of rearrangements is extremely low due to these losses, the frequency of such events that are beneficial or selectively neutral will approach zero, resulting in genomic stasis during lineage evolution. Also, selection on gene content and gene order may be unusually restrictive in small symbiont genomes, further reducing the fixation rate of rearrangements. It is unlikely that sequestering resulting from the symbiotic lifestyle prevents gene uptake, because other bacteria regularly coinfect aphids (*16*).

This leads to the testable hypothesis that the reduction of genome size caused by transitions to obligate host-associated lifestyles is ultimately halted by a corresponding increase in genome stability because of the loss of genetic elements that mediate recombination events. Scaling genome divergence by nucleotide substitutions of orthologous genes reveals a dramatic positive relation between the frequencies of rearrangements and indels and the genomic content of repeats (Fig. 2, C and D). This relation is expected because the number of recombination sites is $n(n-1)/2$, where n represents the number of identical repeats per genome. Thus, the number of possible genome variants that can be generated will decrease rapidly as the repeat content and genome size are reduced. The result is a correlation between genome rearrangements and lifestyle, because obligate host-associated bacteria tend to have smaller genomes with lower content of repeats and less efficient recombination systems than free-living bacteria.

Reconstruction of the ancestor shared with *E. coli* shows that the *B. aphidicola* lineage eliminated at least 2000 genes and underwent multiple chromosomal inversions before the divergence of *B. aphidicola* (Sg) and *B. aphidicola* (Ap) (*17*). This degree of reduction would have required over 10^{10} years if gene disappearance in the early *B. aphidicola* lineage occurred at the rate (14 genes per 50 My) estimated for the period in which these two genomes diverged. Thus, more rapid genomic changes must have been characteristic of the early stages of *B. aphidicola* evolution. This may be attributable to both more repeats and a greater proportion of expendable genes in the ancestor of *B. aphidicola*, allowing deletions of multigene fragments (*17*).

Although the original acquisition of a bacterial symbiont enabled aphids and other sap-feeding insects to exploit food resources that would be otherwise nutritionally unsuitable, the dependence on *B. aphidicola* has not conferred continued evolutionary plasticity in nutritional capabilities and diet breadth. Rather, our study has shown that *B. aphidicola* remains stable in genome content and architecture and has even lost pathways that may affect the ecological range of the aphids. This stability, particularly the complete absence of gene acquisition, implies effectively invariant or diminishing biosynthetic capabilities of the symbionts over periods that span many evolutionary shifts in the diet and life cycles of hosts. Within the clade of aphids, including *S. graminum* and *A. pisum*, there are about 3000 different species living on a wide range of monocots, dicots, and even ferns; yet the corresponding lineages of *B. aphidicola* have not obtained new genes or novel capabilities. Thus, the ecological diversification of aphids cannot be attributed to the genetic diversity of *B. aphidicola*.

References and Notes

1. The complete annotated genome sequence, methods, and all supporting online material are available at *Science* Online at www.sciencemag.org/cgi/content/full/296/5577/2376/DC1. The GenBank accession number is AE013218.
2. S. Shigenobou, H. Watanabe, H. Masahira, Y. Sakaki, H. Ishikawa, *Nature* **407**, 81 (2000).
3. M. A. Munson, P. Baumann, M. A. Clark, L. Baumann, N. A. Moran, *J. Bact.* **173**, 6321 (1991).
4. M. A. Clark, N. A. Moran, P. Baumann, *Mol. Biol. Evol.* **16**, 1586 (1999).
5. M. Suyama, P. Bork, *Trends Genet.* **17**, 10 (2001).
6. E. U. Brynnel, C. G. Kurland, N. A. Moran, S. G. E. Andersson, *Mol. Biol. Evol.* **15**, 574 (1998).
7. O. G. Berg, M. Martelius, *J. Mol. Evol.* **41**, 449 (1995).
8. J. Sandström, A. Telang, N. A. Moran, *J. Insect Physiol.* **91**, 33 (2000).
9. C. Girousse, J.-L. Bonnemain, S. Delrot, R. Bournoville, *Plant Physiol. Biochem.* **29**, 41 (1991).
10. K. Komaki, H. Ishikawa, *J. Mol. Evol.* **48**, 717 (1999).
11. E. Boye, A. Lobner Olesen, K. Skarstad, *EMBO Rep.* **1**, 479 (2000).
12. N. A. Moran, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2873 (1996).
13. S. G. E. Andersson, C. G. Kurland, *Trends Microbiol.* **6**, 263 (1998).
14. A. C. Frank, H. Amiri, S. G. E. Andersson, *Genetica*, **115**, 1 (2002).
15. J. Courcelle, A. K. Ganesan, P. C. Hanawalt, *BioEssays* **23**, 463 (2001).
16. J. P. Sandström, J. A. Russel, J. P. White, N. A. Moran. *Mol. Ecol.* **10**, 217 (2001).
17. N. A. Moran, A. Mira, *Genome Biol.* **54**, 1 (2001).
18. D. Dalevi, N. Eriksen, K. Eriksson, S. G. E. Andersson. *J. Mol. Evol.*, **55**, 23 (2002).

19. Supported by the NSF grant no. DEB-9978518.

26 February 2002; accepted 20 May 2002

A Tropical Rainforest in Colorado 1.4 Million Years After the Cretaceous-Tertiary Boundary

Kirk R. Johnson* and Beth Ellis

An extremely diverse lower Paleocene (64.1 million years ago) fossil leaf site from Castle Rock, Colorado, contains fossil litter that is similar to the litter of extant equatorial rainforests. The presence of a high-diversity tropical rainforest is unexpected, because other Paleocene floras are species-poor, a feature generally attributed to the Cretaceous-Tertiary (K-T) extinction. The site occurs on the margin of the Denver Basin in synorogenic sedimentary rocks associated with the rise of the Laramide Front Range. Orographic conditions caused by local topography, combined with equable climate, appear to have allowed for the establishment of rainforests within 1.4 million years of the K-T boundary.

The Cretaceous-Tertiary (K-T) boundary in North America is characterized by the extinctions of plant (*1*, *2*), insect (*3*), and vertebrate (*4*) species and the restructuring of terrestrial ecosystems because of the loss of large-bodied herbivores and overall biodiversity. Extensive paleobotanical sampling over the past 150 years has produced hundreds of Paleocene floras with low numbers of species per site (*5*–*9*) that exhibit the foliar physiognomy of temperate deciduous forests (*10*). This suggested that floral recovery from the K-T event may have taken up to 10 million years. In marked contrast to this

Department of Earth Sciences, Denver Museum of Nature & Science, Denver, CO 80205, USA.

*To whom correspondence should be addressed. E-mail: kjohnson@dmns.org