Distributional profiles of homologous open reading frames among bacterial phyla: implications for vertical and lateral transmission

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If open reading frames (ORFs) have been transmitted primarily by vertical descent, the distributional profile of orthologues of each ORF should be congruent with the organismal tree or a subtree thereof. Distributional patterns not reconciled parsimoniously with tree-like descent and loss are prima facie evidence of lateral gene transfer. Herein, a rigorous criterion for recognizing ORF distributions is described and implemented; it does not require the inference of phylogenetic trees, nor does it assume any specific tree. Because lineage-specific differences in rates of sequence change can also generate unexpected distributional patterns, rate artefacts were controlled for by requiring pairwise matches between ORFs to exceed a rigorous *inclusion* threshold, but absence of a match was assessed against a more-permissive *exclusion* threshold. Applying this dual-threshold criterion to cross-domain and cross-phylum distributional patterns for ORFs in 23 bacterial genomes, a relative abundance of ORFs was observed that find a match in exactly seven other bacterial phyla; 94–99% of these ORFs also find matches among the *Archaea* and/or *Eukarya*. In the larger (and some smaller) bacterial genomes, ORFs that find matches in exactly one other bacterial phylum are also relatively abundant, but fewer of these have non-bacterial homologues; most of their matches within the *Bacteria* are to the *Proteobacteria* and/or *Firmicutes*, which cannot be sister lineages to all bacteria. ORFs that are neither distributed universally among the *Bacteria*, nor necessarily shared with topologically adjacent lineages, are preferentially enriched in large bacterial genomes.

Keywords: lateral gene transfer, horizontal gene transfer, genome evolution, comparative genomics

INTRODUCTION

Since Darwin (1859), biologists have accepted that organisms have diversified via a tree-like process of

genealogical descent with modification. This common phylogeny serves as a unifying theme in modern biology, not least in systematics and studies of biodiversity. Although developed originally to explain the diversity of living organisms, the Darwinian paradigm has proven exceptionally powerful in application to the evolution of individual genes and gene families. More recently, it has come to be appreciated that the orderly view of the living world inherent in the Darwinian paradigm is, to some extent, complicated by ‘lateral’ or ‘horizontal’ events (Syvanen, 1994), in which genes are transferred from branch to branch in the phylogenetic tree of organisms. It appears well-established, for instance, that plastids and mitochondria have arisen from endosymbiotic bacteria (Doug-

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More-detailed versions of Tables 1–3 are available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

Abbreviation: LGT, lateral gene transfer.

las, 1998; Gray, 1999; Gray *et al.*, 1999; Andersson & Kurland, 1999) and that plasmids can be spread from one bacterial population to another (Spratt & Maiden, 1999). In these cases, as in others, the underlying lateral nature of the phenomenon was recognized initially from distributions of phenotypes and was only later confirmed by molecular-phylogenetic analyses.

If lateral gene transfer (LGT) is rare, phylogenetic trees inferred from most individual genes should be topologically congruent. In reality, there is much less congruence than a unitary pattern of descent would suggest, and examples are proliferating of distributions most-parsimoniously explained as resulting from LGT (Brown & Doolittle, 1997; Doolittle & Logsdon, 1998; Doolittle, 1999; Jain *et al.*, 1999). With the advent of genomics, attempts have recently been made to estimate the extent of non-tree-like descent in complete microbial genomes. The estimated values are higher than many would have suspected: some 18% of the open reading frames (ORFs) of *Escherichia coli* (Lawrence & Ochman, 1998) and 24% of the ORFs of *Thermotoga maritima* (Nelson *et al.*, 1999). If these values are correct and representative, LGT may be an important determinant of genomic diversity in bacteria (Ochman *et al.*, 2000).

Comparison of phylogenetic trees is, in principle, the most rigorous approach for identifying putative LGT (Syvanen, 1994). But inference of trees is subject to manifold biases and artefacts and, for large datasets, can be computationally challenging; thus, in practice, it can be difficult to determine whether non-congruence is real (i.e. an ORF has moved laterally) or only apparent (i.e. an incorrect gene-tree topology has been inferred). We and others have therefore sought alternative approaches that do not require the inference of trees. These methods fall into two major categories: those based on statistical comparison of an ORF primarily vis-à-vis its own genome (Lawrence & Ochman, 1997, 1998; Karlin *et al.*, 1998a; Lawrence & Roth, 1999; Mrázek & Karlin, 1999; Worning *et al.*, 2000) and those involving a statistical comparison of an ORF with similar ORFs in other genomes. Among the latter, comparisons have been based on dinucleotide and oligonucleotide frequencies (Karlin *et al.*, 1998b), best BLAST matches (Koonin *et al.*, 1997; Makarova *et al.*, 1999; Nelson *et al.*, 1999) and distributions of normalized BLASTP similarity scores (Clarke *et al.*, 2002). At least in principle, it is possible by each approach to identify ORFs whose presence in a particular genome is anomalous or not parsimoniously explicable, given the composition of that genome itself (the query genome) and others (target genomes).

The approach we introduce herein falls into the latter category, involving cross-genome statistical comparisons of ORFs. Our motivating idea is that at least some of the LGT between major lineages will distribute ORFs in ways not parsimoniously predicted by Darwinian descent-with-modification and that these unexpected profiles can be identified using a comparison

tool such as gapped BLAST (Altschul *et al.*, 1997) applied to conceptually translated protein sequences ('BLASTP'). We assume that there *is* an organismal, or genomic, tree, but we treat its topology as unknown and do not require that ORFs be distributed accordingly. In this communication, we focus on biological domains (kingdoms) and, within the *Bacteria*, on phyla. Putative LGT at a finer scale can be examined by application of these methods at the appropriate level, e.g. bacterial orders or below.

Five factors jointly determine the observable distribution of ORFs: vertical (Darwinian) descent, catastrophic (irreversible) loss in specific lineages, absolute and relative rates of sequence change, and lateral transfer. Each bears a specific relationship to the underlying organismal tree, whatever its topology. Vertical descent and catastrophic loss are intrinsically tree-like, yielding symplesiomorphies (shared presences or absences unchanged from the ancestral state) and synapomorphies (shared derived states). Rapid sequence change that occurs at similar rates in all lineages also follows a tree-like pattern, but erodes our ability to resolve the topology of the corresponding gene trees and, in extreme cases, can largely obliterate evidence of homology. Sequence change can be dissimilar in different lineages in ways that are independent of phylogeny, instead reflecting population-level phenomena or selection. LGT is (by definition) discordant with the organismal tree, although it could conceivably be more frequent among closely related organisms or within a community.

To identify ORFs that have assumed their current distribution by LGT, it is important to recognize only those pairwise similarities best explained as resulting from discrete, all-or-nothing events inconsistent with vertical descent, i.e. to remove from consideration all rapidly evolving ORFs and as many instances of differential, lineage-specific sequence change as can be recognized. By contrast, others (Aravind *et al.*, 1998; Brucoleri *et al.*, 1998; Nelson *et al.*, 1999) have attempted to draw conclusions about vertical and lateral transmission of genes based on the best pairwise match, even if it is only slightly better than other matches. To distinguish all-or-nothing matches from those that fall at one end of a continuum, we impose dual thresholds, an *inclusion threshold* for recognizing a significant pairwise match and an *exclusion threshold* for accepting the absence of a match (Gaasterland & Ragan, 1998a, b). As the former is made more stringent (e.g. more-negative BLASTP expectation) and the latter more lenient (e.g. less-negative BLASTP expectation), the patterns of ORF distribution that emerge can be interpreted with increasing confidence as having arisen exclusively by vertical descent, loss and LGT.

METHODS

Conceptually translated protein sequences corresponding to all ORFs in each of 23 bacterial genomes were

used to query GenBank, using gapped BLAST version 2.0 (Altschul *et al.*, 1997) and masking regions of low sequence complexity. We queried GenBank (release 117.0), not merely the subset of completely sequenced genomes, to maximize the likelihood of finding matches in each biological domain or phylum. We define as bacterial *phyla* the second rank of categories recognized by the US National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>). The exclusion threshold was typically set at an expectation value $e \leq 1.0e-5$ (Gaasterland & Ragan, 1998a, b) to reduce the number of false-positive matches. We examined a series of inclusion thresholds from $e \leq 1.0e-8$ to $e \leq 1.0e-300$, but here present data obtained mostly at inclusion thresholds of $e \leq 1.0e-10$ or $e \leq 1.0e-20$. The latter value, in particular, is sufficiently stringent to require that most or all of each query ORF, not only a conservative domain or region, be matched. As such, we identify these match partners as presumptive homologues. We do not assume (as do e.g. Tatusov *et al.*, 1997) that they are orthologues; elsewhere (G. P. D. Clarke, R. G. Beiko, M. A. Ragan & R. L. Charlebois, unpublished), we implement a reciprocal best-match criterion to identify ORF pairs that are most likely to be orthologues. For automation of searches, implementation of dual thresholds and taxonomic parsing of GenBank, we used software implemented at <http://www.neurogadgets.com> (St Jean & Charlebois, 1998). Lists of ORFs in each of the 23 bacterial genomes are available at <http://www.tigr.org/tdb/mdb/mdb.html> and <http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/bact.html>

RESULTS AND DISCUSSION

Distributional profiles among bacterial genomes

We compared ORFs in 23 completely sequenced bacterial genomes against GenBank, sorted the results taxonomically and then filtered with dual thresholds to find all-or-nothing distributional profiles. As can be seen for the ORFs in *Escherichia coli* K-12 (Table 1), as the inclusion threshold is made increasingly stringent, fewer ORFs are recognized as having all-or-nothing distributional profiles. Opinion differs on how stringent an inclusion threshold should be used for the recognition of a match. In recognizing clusters of orthologous groups, Tatusov *et al.* (1997) set the BLAST inclusion threshold at $e \leq 1.0e-2$ (and did not implement an exclusion threshold, although they additionally required a mutuality of best match within each cluster of orthologous groups). Gaasterland & Ragan (1998a), on the other hand, accepted only those pairwise FASTP expectation scores $\leq 1.0e-5$ so as to minimize the numbers of false positives and, even so, recognized that their weaker matches sometimes targeted paralogues. In this study, the most-permissive thresholds we accept are BLASTP $e \leq 1.0e-10$ for inclusion and BLASTP $e \leq 1.0e-5$ for exclusion. We recognize that, at these thresholds, some homologues

will be overlooked, but believe that they should be brought into the analysis on a case-by-case basis by identification of specific structural features (e.g. sequence motifs), not by a general relaxation of thresholds.

Distributional profiles similar to that presented in Table 1 for *Escherichia coli* were generated for all 23 bacterial genomes. For clarity of presentation, we find it useful to fix a value for the inclusion threshold (the exclusion threshold was set at $e \leq 1.0e-5$ in almost all our analyses) and to project the three remaining axes (query genome, number of target phyla and number or proportion of ORFs) into two dimensions, connecting points for each genome. This can be done for different subsets of query ORFs: those that also find a match outside the *Bacteria* at a BLASTP expectation value better than the exclusion threshold, those that find no such match, or all query ORFs regardless of match status outside the *Bacteria*. Fig. 1(a) shows the proportions of ORFs, irrespective of match status outside the *Bacteria*, that find a match in exactly one, exactly two and so on bacterial target phyla at respective BLASTP inclusion and exclusion thresholds of $e \leq 1.0e-10$ and $e \leq 1.0e-5$. Very similar patterns are found at a succession of more-stringent inclusion thresholds, until numbers of ORFs in some categories become very small for some genomes.

At BLASTP inclusion $e \leq 1.0e-10$ (Fig. 1), 16 of these genomes appear to be relatively enriched in ORFs that find a match in exactly one other bacterial phylum (the phylum represented by the query ORF itself is not counted, as every ORF necessarily matches itself); we term these '1-matchers'. Twenty of the genomes are relatively enriched in ORFs that find matches in exactly seven phyla other than their own, or '7-matchers'. Of the seven genomes that do not show relative enrichment in 1-matchers, four are among the smallest known (*Mycoplasma genitalium*, 467 ORFs; *Ureaplasma urealyticum*, 611 ORFs; *Chlamydia trachomatis*, 877 ORFs; *Treponema pallidum*, 1031 ORFs). The three bacterial genomes that are not relatively enriched in 7-matchers are among the largest released to date: *Escherichia coli* (4289 ORFs), *Bacillus subtilis* (4099 ORFs) and *Mycobacterium tuberculosis* (3918 ORFs). The two thermophiles, *Aquifex aeolicus* and *Thermotoga maritima*, exhibit relatively more matches at three, four, five and six other phyla than does any mesophile.

Few ORFs find matches in more than seven other phyla, because the eight phyla represented by these 23 genomes together accounted for > 99.0% of all bacterial sequences in GenBank (version 117.0). 7-Matchers are thus broadly distributed among bacteria, finding matches in all matchable bacterial phyla. In an earlier version of this analysis, conducted using 16 bacterial genomes representing seven phyla (against GenBank version 114.0), this higher mode was observed at six other target phyla; the subsequent release of the *Deinococcus radiodurans* genome (the first

Table 1. Numbers of ORFs in the genome of *Escherichia coli* K-12 finding matches in exactly 1 to 10 bacterial phyla other than its own (*Proteobacteria*) at selected BLASTP thresholds

BLASTP inclusion thresholds from $e \leq 1.0e-8$ to $e \leq 1.0e-300$ were used. The exclusion threshold was $e \leq 1.0e-5$. Numbers are shown irrespective of whether these ORFs also find matches in the *Archaea* and/or *Eukarya* at BLASTP $e \leq 1.0e-5$ (left) or excluding ORFs that also find matches outside the *Bacteria* (right). A more-detailed version of this Table is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

Inclusion BLASTP e	Number of non-self phyla matched irrespective of non-bacterial matches										Number of non-self phyla matched (bacteria-only ORFs)									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
$\leq 1.0e-8$	360	293	217	237	200	194	237	98	104	32	185	87	64	25	17	17	15	0	1	0
$\leq 1.0e-10$	297	246	175	203	176	161	207	79	93	30	160	76	50	22	14	14	12	0	1	0
$\leq 1.0e-15$	228	193	113	121	133	111	127	52	64	23	122	58	27	13	9	12	9	0	1	0
$\leq 1.0e-20$	189	150	82	84	95	68	99	36	37	17	98	41	13	9	5	6	5	0	0	0
$\leq 1.0e-25$	158	122	58	64	78	48	77	29	26	12	77	34	9	5	4	4	4	0	0	0
$\leq 1.0e-30$	133	88	39	47	66	36	63	23	23	9	70	29	8	3	3	0	3	0	0	0
$\leq 1.0e-40$	107	65	25	24	41	27	39	17	18	4	53	19	5	1	0	0	2	0	0	0
$\leq 1.0e-50$	87	48	19	11	34	19	24	12	14	3	45	11	5	0	0	0	1	0	0	0
$\leq 1.0e-60$	67	30	18	5	23	11	16	10	11	1	32	6	5	0	0	0	0	0	0	0
$\leq 1.0e-80$	41	16	10	0	8	8	9	7	5	0	18	1	3	0	0	0	0	0	0	0
$\leq 1.0e-100$	23	12	7	0	4	3	2	4	3	0	12	1	2	0	0	0	0	0	0	0
$\leq 1.0e-150$	8	4	3	0	0	0	0	1	1	0	3	1	0	0	0	0	0	0	0	0
$\leq 1.0e-300$	4	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0

representative of the *Thermus/Deinococcus* group) provided the diversity of ORF targets sufficient to increase the higher mode discretely to seven other phyla for each query genome.

Similar distributional profiles are observed over a wide range of inclusion thresholds (alternative sections through the large graphic described above). At very stringent thresholds, however, numbers of ORFs eventually decrease to the point where patterns are obscured (results not shown).

When ORFs that find matches at BLASTP $e \leq 1.0e-5$ outside the *Bacteria* are excluded from consideration, the signal at ≥ 7 other phyla almost disappears; at $e \leq 1.0e-20$ (Fig. 1b), the decrease is 94–99% across these 23 genomes (weighted mean 96.7%). Thus, ORFs that are broadly distributed within the *Bacteria* (matching all matchable phyla) overwhelmingly have homologues among the *Archaea* and/or *Eukarya*. We hypothesize that most of these ORFs have assumed this multi-domain distribution by descent from an ancestor that predated the divergence of the *Bacteria*. This does not, however, exclude the possibility (not tested here) that some of these ORFs may subsequently have undergone lateral transfer *within* individual domains, e.g. within the *Bacteria*. For each of these 23 genomes, these broadly distributed ORFs are fewer in number (range 71–212, weighted mean 144) than, and do not strongly match the functional profile of, the 256-gene set proposed to be minimal for cellular life (Mushegian & Koonin, 1996).

Similarly, 74–94% (weighted mean 90.0%) of bacterial ORFs that find a match in exactly three, four, five or six other bacterial phyla also find a match in the *Archaea* and/or *Eukarya* (Fig. 1a versus Fig. 1b). We hypothesize that many of these patterns have been generated by catastrophic loss, in individual lineages, of ancient ORFs (e.g. during genomic streamlining: Andersson & Kurland, 1998; Andersson & Andersson, 1999). A contribution from promiscuous lateral transfer cannot be excluded, but cannot be parsimoniously assumed in the absence of additional evidence (e.g. from a well-supported phylogenetic tree).

1-Matchers: candidates for LGT?

In contrast to the 7-matchers discussed above, ORFs that find matches in exactly one other bacterial genome show a much higher frequency of matching *only* bacterial ORFs. Over these 23 bacterial genomes, 40.1% of the 1-matchers at BLASTP $\leq 1.0e-20$ have no homologue detectable, at BLASTP $e \leq 1.0e-5$, outside the *Bacteria*. For the distributional profiles of these ORFs to be parsimoniously explicable as the result of vertical descent on a well-resolved tree, each must match a target in the topologically nearest lineage for which sufficient data are available. Keeping in mind that our approach does not assume any specific tree, four lines of evidence indicate that this is not what is observed for most of these genomes.

Fig. 1. In each of 23 bacterial genomes, the percentage of ORFs that find matches in exactly one, exactly two, etc. other bacterial phyla (second-level NCBI categories: see Methods) at respective inclusion and exclusion thresholds BLASTP $e \leq 1.0e-10$ and $1.0e-5$, when ORFs that also find matches at $e \leq 1.0e-5$ in the Archaea and/or Eukarya are (a) included or (b) disqualified from consideration. Thus, ORFs contributing to (a) may be exclusively bacterial or more broadly distributed, whereas those in (b) are exclusively bacterial.

Firstly, the most-matched target phylum is always either the *Firmicutes* or *Proteobacteria* (Table 2). In any resolved tree (irrespective of its precise topology), all phyla cannot have the same sister-group; thus, the most-matched target cannot, for most of these query genomes, be topologically adjacent. Were genealogy

irrelevant for these bacterial 1-matchers, the expected distribution of matches should be a function only of the relative numbers of potential targets (protein sequences in GenBank) among the other phyla; we term this the ‘stochastic model’. Alternatively, if genealogy were an important determinant of how

Table 2. Phyla targeted by 1-matchers from 23 bacterial genomes (grouped by phylum) at BLASTP inclusion threshold $e \leq 1.0e-20$ and exclusion threshold $e \leq 1.0e-5$

Phyla are abbreviated as: Aqu, *Aquificales*; Chly, *Chlamydiales*; Cyan, *Cyanobacteriales*; Cytp, *Cytophagales*; Firm, *Firmicutes*; GNS, green non-sulfur bacteria; GSB, green sulfur bacteria (includes *Cytophaga-Flavobacterium-Bacteroides*); Prot, *Proteobacteria*; Spir, *Spirochaetales*; Sym, *Symbiobacteria*; Ther, *Thermotogales*; Th/D, *Thermus/Deinococcus* group (includes *Chloroflexus/Deinococcus* group); Other, other (inconsistent taxonomic labelling for which aliases were not available at the time of analysis, but since corrected). ×, Phylum of the query genome. Numbers shown are for matches in bacterial phyla other than that of the query genome, irrespective of whether these ORFs also find a match outside the *Bacteria*. A more-detailed version of this Table, showing match distributions for each of the bacterial genomes, is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

Query phylum	Target phylum													Total
	Aqui	Chly	Cyan	Cytp	Firm	GNS	GSB	Prot	Spir	Sym	Ther	Th/D	Other	
Aqui	×		1		5			8			18	1		
Chly		×			17			36				4		
Cyan	1		×		24		1	32			6	9		
Firm	5	2	21	3	×			210			15	30	1	
Prot	26	24	70	3	337	1	1	×	15	1	7	27		
Spir		2	1	1	5			10	×		1			
Ther	14		4		12			9	2		×	3		
Th/D		1	7		38			20			2	×	3	
All	46	29	104	7	438	1	2	325	17	1	49	74	4	1097

Table 3. Numbers of matches from 1-matchers as distributed among major target phyla, at BLASTP inclusion threshold $e \leq 1.0e-20$ and exclusion threshold $e \leq 1.0e-5$

See legend to Table 2 for definition of abbreviations. Values expected under the stochastic model (see text) and observed values for query ORFs in the 23 bacterial genomes, grouped by phyla. A more-detailed version of this Table, showing data for each of the bacterial genomes, is available in IJSEM Online (<http://ijs.sgmjournals.org/>).

Phylum	Requiring match outside bacteria								Having no match outside bacteria							
	Firm		Prot		Cyan		All others		Firm		Prot		Cyan		All others	
	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs
Aqui	8.5	3	11.8	5	1.3	1	3.4	16	2.7	2	3.8	3	0.4	0	1.1	3
Chly	13.5	11	18.7	28	2.1	0	4.7	0	6.2	6	8.7	8	1.0	0	2.1	4
Cyan	13.4	13	18.7	16	—	—	5.9	9	12.3	11	17.2	16	—	—	5.5	8
Firm	—	—	121.5	134	13.9	13	38.7	29	—	—	77.5	76	8.8	8	24.7	27
Prot	192.8	204	—	—	30.6	42	85.5	62	127.9	133	—	—	20.3	30	56.8	42
Spir	3.5	4	4.8	4	0.6	0	1.1	2	3.5	1	4.8	6	0.6	1	1.1	2
Ther	10.2	6	14.2	7	1.6	2	4.0	15	4.8	6	6.6	2	0.8	2	1.8	4
Th/D	11.0	18	15.4	8	1.8	3	3.8	3	13.5	20	18.7	12	2.1	4	4.7	3
All	253	259	205	202	52	61	147	136	171	179	137	123	34	45	98	93

frequently a potential target phylum is matched, we should observe profiles that deviate from those expected under the stochastic model. In fact, observed numbers of matches are remarkably close to expectations under the stochastic model over all these 23 genomes and in most (but not all) individual phyla and genomes where numbers of matches are sufficient to constitute a fair test (Table 3). The few apparent deviations from expectation are discussed below. The

stochastic model as presented here is only approximate, as the populations of both target and query ORFs are non-random due, for example, to the occurrence and differential extent of gene families in the various genomes, the over-representation in GenBank of well-studied genes and many other biases. Even so, we find no compelling evidence, for 1-matchers representing most of these eight query phyla, that the profile of their matches among bacterial phyla

Table 4. Frequencies at which major target phyla are matched by 1-matchers that find matches in the *Archaea* and/or *Eukarya* at $e \leq 1.0e-5$ and by *Bacteria*-only 1-matchers

See legend to Table 2 for definitions of abbreviations.

Target phylum	Requiring match outside bacteria		Having no match outside bacteria	
	1.0e-20	1.0e-10	1.0e-20	1.0e-10
Firm	259/657 = 0.394	382/1008 = 0.379	179/440 = 0.407	334/873 = 0.383
Prot	202/657 = 0.307	317/1008 = 0.314	123/440 = 0.280	227/873 = 0.260
Cyan	61/657 = 0.093	85/1008 = 0.084	45/440 = 0.102	83/873 = 0.095
Th/D	37/657 = 0.056	66/1008 = 0.065	36/440 = 0.082	85/873 = 0.097
All others	98/657 = 0.149	158/1008 = 0.157	57/440 = 0.130	144/873 = 0.165
Firm + Prot	461/657 = 0.702	699/1008 = 0.693	302/440 = 0.686	561/873 = 0.643

reflects anything other than numbers of target sequences available.

Secondly, matches among the *Firmicutes* or *Proteobacteria* are not found more frequently by *Bacteria*-only 1-matchers than by 1-matchers that have homologues detectable among *Archaea* and/or *Eukarya* at BLASTP $e \leq 1.0e-5$. At inclusion threshold $1.0e-20$, the frequencies with which the *Bacteria*-only and the more widely distributed 1-matchers find matches in these two target phyla are $302/440 = 0.69$ and $461/657 = 0.70$, respectively, while, at inclusion threshold $1.0e-10$, they are respectively $561/873 = 0.64$ and $699/1008 = 0.69$ (Table 4). Thus, the frequency at which the *Firmicutes* or *Proteobacteria* are the only bacterial phylum matched is no greater (and indeed slightly less) among *Bacteria*-only ORFs (which could, in principle, have a specific sister-group relationship with one of these two phyla) than among the more-numerous ORFs that, by virtue of having homologues outside the *Bacteria*, cannot have an exclusive sister-group relationship with any single bacterial phylum. (This argument is weakened to the extent that these non-*Bacterial* homologues might themselves have arisen by cross-domain LGT, but such exceptions would only strengthen the overall case for LGT. The most-basal branch within the *Bacteria* constitutes a special case.) *Bacteria*-only and ancient 1-matchers likewise show similar frequencies of finding matches in the *Cyanobacteria* (Table 4). *Bacteria*-only 1-matchers find matches more frequently among the *Thermus/Deinococcus* group than do putatively ancient 1-matchers, but absolute values are small and, in the absence of a realistic statistical model, it cannot be demonstrated that the increased frequency is significant. Thus, where numbers are adequate to constitute a fair test, we find no evidence for a specific genealogical (vertical) component in the match frequencies of *Bacteria*-only ORFs vis-à-vis those of ORFs that have non-bacterial homologues.

Thirdly, 1-matchers in the larger bacterial genomes, and in the better-represented phyla, typically find targets among most or all bacterial phyla adequately represented in GenBank (Table 2). Given sufficient

numbers of query ORFs, even the topologically most-distant phyla are therefore matched (although, because no specific tree is assumed, we do not know for each query genome which target phylum is most distant). Finally, analyses below the level of bacterial phylum reveal an even greater diversity of distributional pattern (results not shown).

Although, as described in the first point above, a simple stochastic model explains many aspects of the distributional profiles of these genomes (Table 3), some deviations are apparent. Most notably, ORFs of *Aquifex aeolicus* find a match in the *Thermotogales* much more frequently than expected under the stochastic model (for *Bacteria*-only ORFs at BLASTP inclusion $e \leq 1.0e-20$ and exclusion $e \leq 1.0e-5$, 0.15 matches expected, 3 observed; for ORFs with non-bacterial matches at $e \leq 1.0e-5$, 0.5 matches expected, 15 observed). Conversely, ORFs of *Thermotoga maritima* find a match in the *Aquificales* more frequently than expected (for *Bacteria*-only ORFs at the same thresholds, 0.2 matches expected, 3 observed; for ORFs with non-bacterial matches at $e \leq 1.0e-5$, 0.5 matches expected, 11 observed). The excess of ORFs over expectation is most easily interpreted as a reciprocal sister-group effect between *Aquifex aeolicus* and *Thermotoga maritima*. Although the absolute values are small, the excess appears to be somewhat more pronounced among ORFs that also have non-bacterial homologues, as would be expected if the *Aquificales* and *Thermotogales* together constitute the most-basal branch among bacteria whose genomes have been sequenced (Aravind *et al.*, 1998, 1999; Kyrpides & Olsen, 1999; Logsdon & Faguy, 1999).

Other deviations from expectation apparent from Table 3 include: among the ORFs of *Deinococcus radiodurans*, an excess of matches against the *Firmicutes* at the expense of *Proteobacteria*; among several but not all proteobacteria, an excess of matches against the *Firmicutes* and *Cyanobacteria* at the expense of other phyla; and among all firmicutes, a slight but consistent excess of matches against the *Proteobacteria* at the expense of phyla other than the *Cyanobacteria*. Nonetheless, given the obvious deficiencies (above) of

Table 5. Numbers of ORFs in each of 23 bacterial genomes that have presumed homologues (operationally defined as matches at BLASTP inclusion $e \leq 1.0e-10$ and exclusion $e \leq 1.0e-5$) only in its own bacterial phylum and in *Archaea* and/or *Eukarya*

Query genome	Matches			
	<i>Archaea</i>	<i>Eukarya</i>	<i>Archaea</i> or <i>Eukarya</i>	<i>Archaea</i> and <i>Eukarya</i>
<i>Aquifex aeolicus</i>	28	2	28	2
<i>Bacillus subtilis</i>	17	11	26	2
<i>Borrelia burgdorferi</i>	2	6	8	0
<i>Campylobacter jejuni</i>	7	9	15	1
<i>Chlamydia muridarum</i>	0	10	10	0
<i>Chlamydia trachomatis</i>	0	9	9	0
<i>Chlamydophila pneumoniae</i> CWL029	0	9	9	0
<i>Chlamydophila pneumoniae</i> AR39	0	9	9	0
<i>Deinococcus radiodurans</i>	14	8	20	2
<i>Escherichia coli</i>	22	25	43	4
<i>Haemophilus influenzae</i>	9	14	22	1
<i>Helicobacter pylori</i> 26695	4	15	19	0
<i>Helicobacter pylori</i> J99	6	12	18	0
<i>Mycobacterium tuberculosis</i>	25	23	47	1
<i>Mycoplasma genitalium</i>	2	2	4	0
<i>Mycoplasma pneumoniae</i>	0	6	6	0
<i>Neisseria meningitidis</i> MD58	5	15	20	0
<i>Neisseria meningitidis</i> Z2491	5	15	20	0
<i>Rickettsia prowazekii</i>	0	14	14	0
<i>Synechocystis</i> sp. PCC6803	13	96	108	1
<i>Thermotoga maritima</i>	39	5	42	2
<i>Treponema pallidum</i>	2	3	5	0
<i>Ureaplasma urealyticum</i>	1	0	1	0

the simple stochastic model presented here, we find it remarkable that predictions are so similar to the observed values for the majority of pairwise comparisons.

We have argued, so far, that, because no sister-group signal is apparent for many of the 1-matchers, the distribution of their homologues among bacterial phyla must therefore have involved LGT. This conclusion seems inescapable if the underlying (organismal or genomic) tree is well-resolved. If, however, the underlying tree actually resembles a starburst (i.e. if its internal internodes are much shorter than its terminal branches), there might intrinsically be very little sister-group signal between phyla. Indeed, in published whole-genome trees (Fitz-Gibbon & House, 1999; Snel *et al.*, 1999), the basal (phylum-level) branches within each division are typically poorly resolved. However, many gene trees are well-resolved at the level of bacterial phyla (e.g. Brown & Doolittle, 1997), suggesting that the starburst-like shape of whole-genome trees is itself artefactual. Until the matter is further clarified, an underlying starburst-like tree remains, at least formally, as an alternative explanation for the lack of sister-group signal among 1-matchers.

Profiles and the endosymbiotic origins of organelles

At the time our analyses were conducted, (nearly) complete sets of genomic ORFs were available only for *Saccharomyces cerevisiae* and *Caenorhabditis elegans* among eukaryotes. Given the paucity of genomic sequencing so far among protists, and in view of the importance of using complete sets of genomic ORFs as targets (above), it is premature to attempt a rigorous profile-based analysis of vertical and horizontal patterns of ORF relationship linking bacteria and eukaryotes. Nonetheless, our analyses did reveal an unambiguous signal specifically linking *Synechocystis* sp. (as a representative of the *Cyanobacteria*) with eukaryotes. At BLASTP inclusion $e \leq 1.0e-10$ and exclusion $e \leq 1.0e-5$, we observe 96 *Synechocystis* ORFs that find a match in the *Eukarya* but not in any bacterial phylum other than its own; only one of these ORFs also finds a match at these threshold values in the *Archaea* (Table 5). Among the 95 ORFs specifically shared between cyanobacteria and eukaryotes are genes encoding components of photosystems I and II, phycobilisome subunits, proteins involved in photosynthesis-related electron transport and haem- and chlorophyll-binding proteins, as well as numerous hypothetical proteins (details at <http://www.neurogadgets.com>). Thus, our

profile analysis has recognized and identified the (lateral) origin of plastids from a cyanobacterial precursor.

The corresponding signal for mitochondrial origin is much less easily discernable by this approach. Among proteobacteria, only the α -proteobacterium *Rickettsia prowazekii* finds targets among the *Eukarya* (but not in the *Archaea*, nor in the *Bacteria* other than the *Proteobacteria*) that are annotated as being involved in mitochondrial energetics (succinate dehydrogenase cytochrome b_{560} ; ubiquinone biosynthesis; cytochromes c and c_1 ; cytochrome- c oxidase). Our analysis alone cannot readily distinguish whether these data indicate the specific α -proteobacterial origin of mitochondria or shared retention of ancestral sequence (cf. Ragan & Gaasterland, 1998).

Profiles in relation to genome size

The smaller bacterial genomes contain proportionally many more 7-matchers than 1-matchers (Fig. 2a, b). As noted above, most of these 7-matchers have homologues outside the *Bacteria* (Fig. 2a). Particularly to the extent that these non-bacterial homologues are distributed broadly within the *Archaea* and/or *Eukarya*, the default hypothesis must be that these gene families arose before the divergence of the *Bacteria* from the other organismal domains. Conversely, ORFs with homologues in exactly one other bacterial phylum, particularly those lacking homologues outside the *Bacteria* detectable even at BLASTP $e \leq 1.0e-5$ (Fig. 2b), are both relatively and absolutely more abundant in the larger bacterial genomes than in small ones. We have argued (above) that there is little evidence of a genealogical (vertical) component in the distributional profiles of *Bacteria*-only 1-matchers. Thus, large bacterial genomes have become (or remain) larger, in part, by preferential accumulation (or retention or amplification) of ORFs that are neither ancient, nor preferentially shared with the topologically adjacent lineage – i.e. laterally transferred ORFs.

The distributional patterns explained least parsimoniously by vertical descent are those in which a bacterial ORF finds a match (i) only outside the *Bacteria* or (ii) in only one bacterial phylum other than its own, regardless of match status outside the *Bacteria*. (These patterns will be less unparsimonious for the deepest-branching bacterial lineage, but here we do not assume any specific bacterial tree and therefore cannot identify basal phyla.) Among these 23 genomes, 0.4% (*Mycoplasma genitalium*) to 4.9% (*Escherichia coli*) of ORFs (weighted mean, 3.3%) exhibit one of these patterns at inclusion threshold $1.0e-20$ and exclusion threshold $1.0e-5$. These values rise to 1.9% (*Mycoplasma genitalium*) to 8.0% (*Escherichia coli*), with weighted mean 5.8%, when the inclusion threshold is relaxed to $1.0e-10$. For these profiles to have been generated primarily by vertical descent, one must assume unparsimoniously that catastrophic ORF loss

Fig. 2. Relationships between genome size (number of annotated ORFs) and percentage of ORFs finding matches in exactly one (◆) or exactly seven (■) other bacterial phyla at respective inclusion and exclusion thresholds BLASTP $e \leq 1.0e-10$ and $1.0e-5$. (a) ORFs are required to find matches in the *Archaea* and/or *Eukarya*; (b) ORFs finding matches outside the *Bacteria* are disqualified from consideration. Points are connected to aid visual inspection.

and/or extreme increases in the rate of sequence divergence have occurred on multiple occasions across different lineages.

These are probably conservative estimates of the proportions of ORFs in these 23 bacterial genomes that show distributional patterns explained most parsimoniously as having been generated by cross-domain and cross-phylum LGT. If LGT is this frequent, it must also have contributed to the proportions of 2-matchers, 3-matchers, etc. Reducing the stringency of our inclusion and exclusion thresholds greatly increases the numbers of ORFs in many categories, including 1-matchers, only some of which can be attributed to false positives. On the other hand, vertical descent and multiple losses may have generated some of the unexpected distributions, even if it is unparsimonious to assume this a priori in the absence of

specific justification on a case-by-case basis. It is, unfortunately, exactly these 1-matchers for which classical phylogenetic analysis (sequence alignment and inference of trees) will prove most problematic, as other bacterial homologues (if any) will necessarily be difficult to identify and align.

Conclusions

LGT generates an underlying topological incongruence among different gene trees and between gene and organismal trees. Incongruence sometimes first becomes apparent during the inference of phylogenetic trees. The challenge then is to determine whether the observed incongruence is adequately supported by the data and, if so, whether and how the unexpected result might be explicable in the context of vertical transmission. To do this, one typically obtains sequences of homologues in related organismal lineages and then applies techniques of molecular phylogenetics, e.g. alternative methods of inference, parametric and non-parametric bootstrapping, likelihood-ratio tests or more-sophisticated models of sequence change. If the incongruence is well-supported and cannot be reconciled with vertical transmission, it may be regarded as a *prima facie* example of LGT. In other cases, however, we first observe an aberrant or unexpected pattern of ORF distribution. Particularly where this involves the sporadic distribution of ORFs among divergent taxa, additional sequencing and phylogenetic inference may not be particularly helpful, and alternative approaches may provide better insight into the history of these ORFs.

Herein, we have employed a dual-threshold criterion to identify the more clear-cut instances where ORF distribution is not explained parsimoniously by vertical transmission. We have shown that ORFs that are widely distributed among the *Bacteria* overwhelmingly also have homologues outside the *Bacteria*, i.e. are probably ancient; in the absence of evidence to the contrary (e.g. from well-supported but discordant phylogenetic trees), it may be assumed that many of these ORFs have assumed their present-day distribution by tree-like vertical descent. By contrast, distributional profiles of bacterial ORFs that find a match in only one other bacterial phylum are, with a few exceptions, adequately explained by a simple stochastic model that does not take genealogy (vertical transmission) into account; many of these ORFs may therefore have assumed their present-day distribution by LGT. We also identified bacterial ORFs that find no match partners among other bacterial phyla, but have presumed homologues in *Archaea* and/or *Eukarya*. In the case of *Synechocystis* sp., transmission has been from bacterium to eukaryote during the endosymbiotic origin of chloroplasts.

Heretofore, most attention has attended LGT at the micro-scale (transmission of antibiotic resistance) and at the mega-scale (origins of organelles). The analysis herein suggests that LGT has occurred between many

phyla of bacteria. Preliminary analyses show profiles suggestive of LGT at the level of bacterial orders or families as well (M. A. Ragan & R. L. Charlebois, unpublished), although we await further genomic-sequence data to conduct this analysis with satisfactory resolution.

We believe that it is important to have initiated this analysis of distributional profiles using relatively conservative inclusion and exclusion thresholds. As more-sophisticated approaches are developed to identify more-divergent homologues, it is likely that estimates of potential LGT will increase. Using the conservative approach outlined above, we find a smaller extent of cross-domain LGT than has previously been estimated (Jain *et al.*, 1999; Nelson *et al.*, 1999; Worning *et al.*, 2000). Nonetheless, our results confirm that bacterial genomes have been shaped, in part, by processes very different from those that Darwin would have imagined.

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