Genome-Wide Experimental Determination of Barriers to Horizontal Gene Transfer
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Supporting Online Material

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Genome-Wide Experimental Determination of Barriers to Horizontal Gene Transfer

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Horizontal gene transfer, in which genetic material is transferred from the genome of one organism to that of another, has been investigated in microbial species mainly through computational sequence analyses. To address the lack of experimental data, we studied the attempted movement of 246,045 genes from 79 prokaryotic genomes into *Escherichia coli* and identified genes that consistently fail to transfer. We studied the mechanisms underlying transfer inhibition by placing coding regions from different species under the control of inducible promoters. Our data suggest that toxicity to the host inhibited transfer regardless of the species of origin and that increased gene dosage and associated increased expression may be a predominant cause for transfer failure. Although these experimental studies examined transfer solely into *E. coli*, a computational analysis of gene-transfer rates across available bacterial and archaeal genomes supports that the barriers observed in our study are general across the tree of life.

The rapidly accumulating sequenced genomes of bacteria and archaea reveal the role of horizontal gene transfer (the nonsexual exchange of genes across hierarchal boundaries) in shaping noneukaryotic genomes (*1*, *2*). Gene exchange has been documented for nearly all types of genes and at all phylogenetic distances (*3*). These observations have sparked debates about whether microbial genes can be used for phylogenetic classification, because the proposed lack of barriers to gene transfer between genomes suggests that a treelike classification of microorganisms might be impossible (*4*, *5*).

Identifying the limitations of gene transfer is hampered because nearly all transfer events have been inferred on the basis of sequence analysis of microbial genomes. Computational approaches, including detection of nucleotide or codon compositional biases and atypical distribution of genes, identify signatures of transfer events predicted to have occurred millions of years ago (*6*). On the basis of such studies, specific categories of genes were suggested as less prone to transfer, and hence potentially useful as phylogenetic markers (*7*, *8*), but the validity of this idea relies nearly exclusively on computational evidence (*1*). The paucity of experimental and quantitative data on horizontal gene transfer, therefore, impedes our ability to understand the extent and limitations of this phenomenon.

Natural gene transfer is largely mediated by naked DNA uptake (transformation), viruses (transduction), and plasmids (conjugation) (*9*). When a microbial genome is being sequenced, multiple copies of the genome are randomly sheared into overlapping fragments of DNA (typically to libraries sized 3 kb and 8 kb), and plasmids containing the cloned fragments are transformed into an *E. coli* cell (*10*). The ends of the cloned fragments are then sequenced, and overlapping sequences are used for genome assembly. Because cloned fragments contain the full set of genes belonging to the sequenced organism, microbial genome sequencing can be viewed as a large-scale experiment in horizontal gene transfer to *E. coli*, where each gene in a given genome undergoes multiple transfer attempts to the host with an extrachromosomal plasmid. In the course of nearly all prokaryotic sequencing projects, a small fraction of the organism's genome fails to clone in *E. coli*, resulting in sequence gaps. The sequence for these gaps is acquired during a clone-independent stage termed "finishing," eventually producing an unbroken sequence of the organism's genome (*11*).

We explored the limits to horizontal transfer by studying the nature of unclonable ("untransferable") genomic regions. Of the 85 finished microbial genomes with accessible original sequence reads, we selected 79 (including 75 bacterial and 4 archaeal) with sufficient clone coverage for detailed analysis (SOM Text and table S1) (*12*). We used the original sequencing data to map the clone positions on these genomes. Overall, this data set included 1,873,649 clones spanning more than 8.9 billion bases of genomic DNA fragments successfully transferred into an *E. coli* host.

We next explored the transfer of the individual genes residing in the 79 analyzed genomes. For each of the 287,884 annotated genes contained in these genomes, we calculated the number of clones fully spanning the gene on the

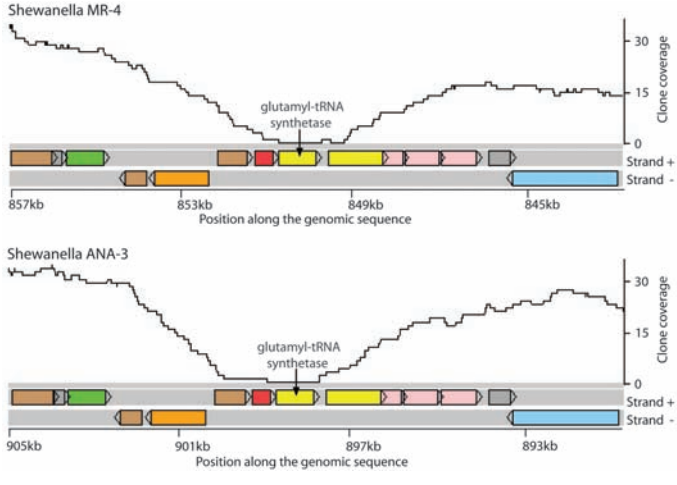

Fig. 1. Coverage plots created on the Artemis genome browser (*20*) of a syntenic 14-kb genomic region in two closely related *Shewanella* bacterial species: (**top**) *S. sp. MR-4*; (**bottom**) *S. sp. ANA-3*. Colored rectangles represent genes, with colors denoting functional categories; arrow direction indicates whether the gene is on the forward or reverse strand. Coverage is measured per nucleotide.

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basis of the mapped clone positions. We considered only genes 1.5 kb or less (246,045 genes, representing 85% of all annotated genes), because larger genes are less likely to be covered to their full length by multiple clones. The average number of clones covering each of these 246,045 genes to its full length was 22.57, indicating that each gene underwent, on average, more than 22 independent transfer attempts to the host.

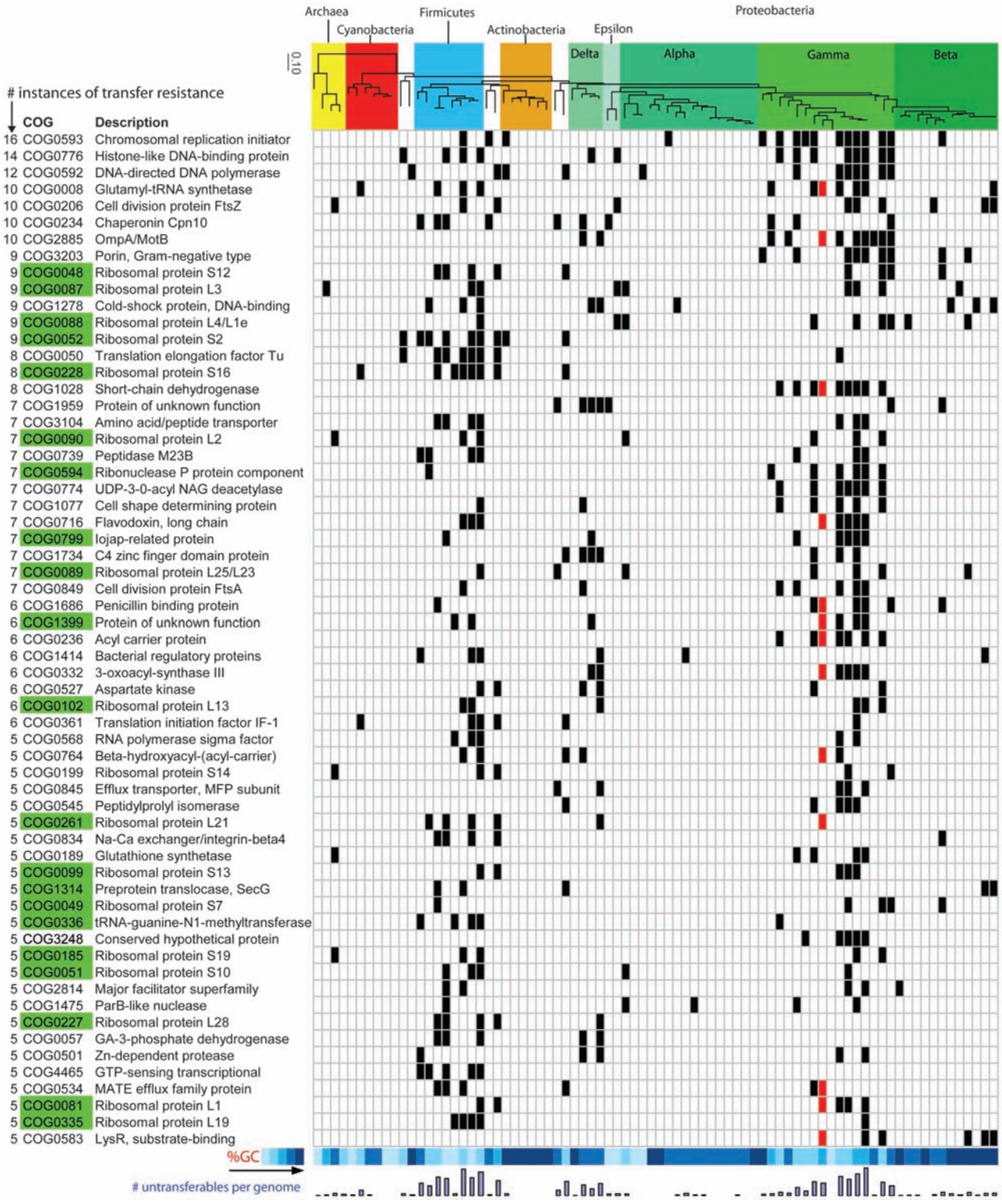

Fig. 2. Genes that cannot be cloned into *E. coli* from five or more genomes. Rows are genes, according to their COG classification (*21*). Columns represent the 79 microbial genomes analyzed, arranged by their phylogenetic relationships as determined by a Maximum Likelihood tree analysis of 16S rRNA sequences (*12*). Unclonable genes are denoted by black boxes. The leftmost column indicates the number of genomes from which the gene was not able to be transferred. Universally single-copy genes are highlighted in green. *E. coli* (Gammaproteobacteria) genes that could not be cloned into the *E. coli* sequencing strain even when originating from an *E. coli HS* genome are marked red. Percentage of GC for each of the genomes is color coded at the bottom of the figure. Darker colors indicate a higher GC content. The histogram below depicts the number of unclonable genes per genome (table S1).

We used the clone coverage distribution to identify genes unclonable into the *E. coli* host. To exclude the possibility that cloning biases are random or human-introduced, we compared clone coverage among genomes of closely related species. These genomes presented relatively similar coverage patterns, with the same sets of orthologous genes from several different organisms absent from sequenced clones, supporting the idea that clone deficiency is largely gene-dependent. Comparison of four *Shewanella* species offers an example for the high reproducibility of clone deficiency: 73 of 99 (74%) *Shewanella sp. MR4* genes found to be uncloned into *E. coli* were also unclonable when transferred from at least one of the three other *Shewanella* species examined (Fig. 1).

Of the genes inspected, we recorded 1402 instances (642 different genes) in which an annotated gene was not fully represented in any single clone, and marked these as untransferable to *E. coli* [with an estimated false positive prediction rate of 0.9% to 1.3% (*12*)]. In 1064 (76%) of these events, the same gene was unclonable to *E. coli* from two or more different genomes. Sixty-one genes (477 events, 34% of total events) could not be cloned from five or more different genomes into *E. coli* (Fig. 2). The high transfer failure rate for certain gene families across several genomes further suggests that specific genes, rather than the experimental protocol or random biases, may cause this lack of horizontal transfer.

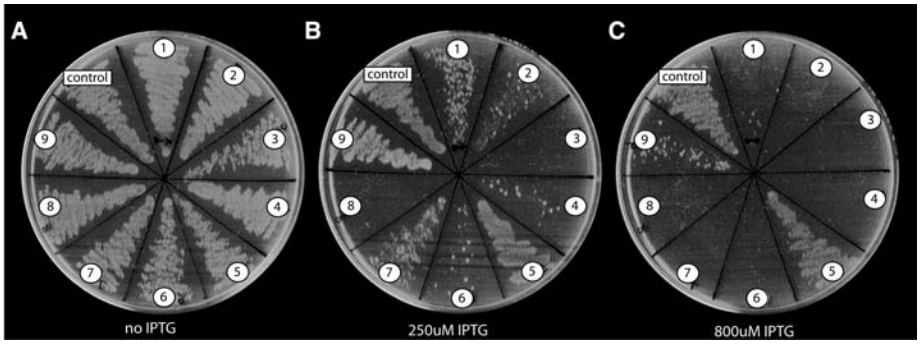

Fig. 3. Toxicity results for the first nine genes tested (table S2) and a control gene (Beta-galactosidase from *E. coli*). The coding regions of predicted unclonable genes were cloned into the pET11 vector under the control of a T7 promoter, transformed into *E. coli* BL21(DE)pLysS cells, and streaked on LB plates. Cells grown (**A**) without the expression-inducer IPTG, (**B**) with 250 μM IPTG, and (**C**) with 800 μM IPTG. 1, Replication initiator DnaA from *Shewanella denitrificans*; 2, Histone-like DNA binding from *Psychrobacter cryohalolentis*; 3, DNA polymerase III, beta subunit from *Deinococcus geothermalis*; 4, Cell division protein FtsZ from *P. cryohalolentis*; 5, Chaperonin Cpn10 from *Nitrosococcus oceani*; 6, OmpA/MotB from *N. oceani*; 7, Ribosomal protein S12 from *Rhodoferax ferrireducens*; 8, Ribosomal protein L4/L1e from *Burkholderia sp. strain 383*; 9, Ribosomal protein L3 from *P. cryohalolentis*.

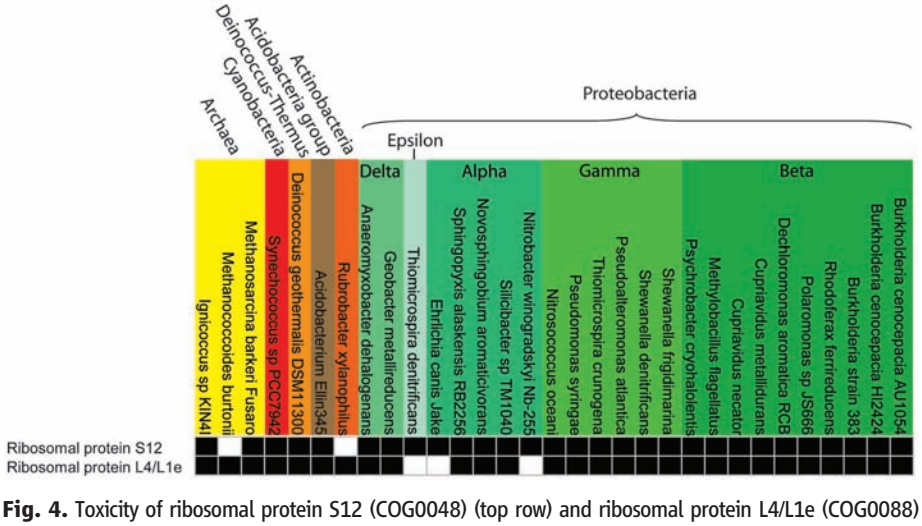

Fig. 4. Toxicity of ribosomal protein S12 (COG0048) (top row) and ribosomal protein L4/L1e (COG0088) (bottom row) from 31 microbial genomes. Columns represent species, arranged by phylogenetic classification, with different colors representing different groups (names indicated above). ORFs were cloned into the pET11 vector adjacent to a T7 promoter and transformed into *E. coli* BL21(DE)pLysS cells. Colony growth was tested without gene expression and after induction of expression with various concentrations of IPTG. Black boxes indicate growth inhibition after activation of expression; white boxes indicate that no growth inhibition was observed (details in table S3).

Whereas gene transfer in the wild is believed to be mediated by the transfer of single as well as multiple copies of the DNA, the cloning vectors used in most small-insert sequencing libraries exist in 20 to 100 copies per cell (*13*, *14*). We examined the impact of single- versus multiple-copy transfers by studying the subset of 35 sequenced genomes where, in addition to the small-insert libraries, large fragments (35 kb) of the microbial genome were propagated in fosmids, which typically exist in a single copy per *E. coli* cell (*15*) (table S1). In 124 out of 483 (26%) uncloned genes in these genomes, the genes were also covered by zero (22%) or statistically fewer (4%) fosmids than expected (fig. S1) (*12*). The consistency of functional results obtained with multicopy plasmids and with single-copy fosmids suggests that a considerable portion of the observed lack of transfer is not solely due to high copy number and that the barriers described in this study are gene copy–number independent.

We selected 40 genes that resisted transfer from two or more genomes into *E. coli* and were able to clone the coding regions of 39 of these genes into an expression vector system that strongly suppresses the expression of the cloned gene in the absence of the expression inducer isopropyl-β-D-thiogalactopyranoside (IPTG) (table S2 and SOM Text). In the absence of inducer, bacterial growth was observed. However, upon induction of expression, 32 of the 39 genes (82%) inhibited *E. coli* growth, indicating that the products of these genes are toxic to the host (Fig. 3 and table S2) which explains the lack of transfer observed in the genome sequencing data.

Although we identified genes that were transfer-resistant from a wide range of prokaryotes, no single gene was untransferable among all genomes examined (reflected by the absence of a horizontal line of black squares running across the complete list of organisms in Fig. 2). This was coupled with the observation that the resistance to transfer of genes tended to be similar among closely related organisms (Fig. 2). A possible explanation is that promoters (usually found adjacent to the gene and hence transferred with it) from some species may be recognized by the host *E. coli* transcriptional machinery and may drive the expression of the foreign gene leading to growth inhibition, whereas promoters of other species are not active in the *E. coli* cell. Indeed, sequences from Firmicutes were previously shown to drive strong expression when tested as promoters in *E. coli* (*16*), which is consistent with Firmicutes having high numbers of transfer resistant genes (Fig. 2). GC-rich genomes tended to have fewer untransferable genes, again consistent with observations that promoters recognized by *E. coli* are GC-poor (*17*). Therefore, we predicted that some of the genes cataloged as nontoxic would be toxic if their promoters were active in *E. coli*.

To test this, we examined two relatively transfer-resistant genes, ribosomal protein L4/L1e (COG0088) and ribosomal protein S12 (COG0048). Each of these genes did not transfer

in 9 of 79 genomes (Fig. 2). We isolated the coding sequences of these genes from 31 microorganisms for which genomic DNA was readily obtainable, including 26 organisms in which transfer resistance had not been observed on the basis of genome sequencing, and cloned them into the inducible expression system described above. Clones holding these genes grew normally in the absence of inducer. However, growth inhibition was observed in 53 of 62 (85%) clones when expression of the cloned gene was induced by low IPTG concentrations (100 μM to 600 μM) and in 57 of 62 clones (92%) in higher (800 μM) IPTG (Fig. 4 and table S3). Such a high frequency of growth inhibition was not observed in a survey of 15 randomly selected putative negative control genes, of which 2 of 15 (13%) and 7 of 15 (47%) inhibited growth in low and high IPTG, respectively (SOM Text and table S4). These results suggest that some of the genes we identified are almost universally toxic when expressed, suggesting that they face a near absolute, phylum-independent barrier to horizontal transfer into *E. coli*. We expect that the small number of putative negative control genes found to be toxic are not true negatives due to the possibility that they possess endogenous promoters inactive in the conditions in which the sequencing *E. coli* strain was grown. In these cases, such toxic genes would escape our detection method.

We compared the clusters of orthologous groups (COG) functions of the 61 genes identified as highly unclonable (those untransferable from five or more genomes) to the COG functions of all genes in our data set. These genes were significantly enriched in genes involved in ribosomal structure and translation ($P < 2 \times 10^{-9}$, Fisher's exact test corrected for multiple testing) (fig. S2). This observation is consistent with previous computational analyses that suggested that genes involved in translation tend to be underrepresented in genes postulated to have undergone horizontal transfer (*7*, *8*). The toxicity of ribosomal proteins observed here possibly stems from an incompatibility with the *E. coli* molecular machinery, as they have multiple interactions within the ribosome (*7*). We found that ribosomal proteins that resisted transfer from a large number of genomes also had more surface area in contact with the rRNA ($P = 0.023$, Spearmans test) (fig. S3).

An additional possible mechanism for explaining some of the observed transfer resistance is intolerance of the host to increased dosage of the transferred gene in addition to the endogenous homolog. To test this hypothesis, we examined data from the *E. coli HS* (*18*) genome project, in which clones containing fragments of the *E. coli HS* genome were transferred into a standard *E. coli* sequencing strain (*DH10B*). Despite the near identity between the transferred genes and the host genes, 43 *E. coli HS* genes (all of them conserved in *E. coli K12* in >98% identity) could not be cloned into the host *E. coli*. Therefore, this subset of genes cannot be tolerated in high dosage. Thirty-four (80%) of these 43 genes were also untransferable to *E. coli* from at least one additional foreign genome (Fig. 2), suggesting that their lack of transfer was also due to dosage intolerance. Moreover, 32% of the genes that were untransferable to *E. coli* from five or more genomes were universal single-copy genes, never duplicated in any of the genomes we tested (compared with 3% universal single-copy genes out of the entire gene population), providing additional support that an increased dosage and the associated increased expression of these genes is likely detrimental to most microbes (Fig. 2). The tendency of transfer-resistant genes to universally exist in a single copy provides further support that the barriers described in this study are gene copy–number independent.

Although our analysis of the experimental data from 246,045 genes transferred to *E. coli* suggests that there is a specific set of genes that are unclonable regardless of their genome of origin, it does so for a single recipient organism, the *E. coli* host. To explore whether these results are general, and whether these genes are untransferable to other recipient species, we used a tree-based computational method to predict gene transfer in 191 sequenced genomes across the entire tree of life (*19*) (SOM Text). We found a strong correlation ($P = 0.008$, Wilcoxon Mann-Whitney Test) between genes that we experimentally characterized as unclonable to *E. coli* and single-copy genes that were computationally predicted to be less transferred across the tree of life (fig. S4). These results suggest that the genes we experimentally characterized in a single host are generally transfer-resistant among most bacteria and archaea, and would be expected to be predominately vertically transmitted in prokaryotes.

Our experiments in horizontal transfer used plasmids as the vessel of transfer, imitating the conjugation process. Transfer through transduction and naked DNA uptake were not examined, but because the detected transfer barriers are caused by post-transfer gene toxicity, the vessel of transfer is not expected to play an important role for the effect of these barriers. In addition, homologous recombination between the transferred gene and its endogenous homolog might circumvent the toxicity imposed by expression of the transferred gene, thus enabling transfer. Our observation that the genes we experimentally characterized as unclonable to *E. coli* do not demonstrate transfer among most microorganisms suggests that this scenario had occurred only very rarely, if ever.

Instead, our results suggest that there are universal gene-transfer barriers, regardless of whether transfer occurs among closely or distantly related microorganisms, and that these barriers may be associated with toxicity of the transferred gene to the host. The number of untransferable genes identified in this study probably reflects a lower limit, because the genes we studied were physically forced into the host, plasmid maintenance was aggressively selected for with antibiotics, and additional natural barriers were not taken into account. In addition, transfer-resistant genes larger than 1.5 kb, as well as toxic genes whose promoters are not active in *E. coli*, escaped our detection. Our observation that many unclonable genes are universally found as a single copy (never duplicated in any sequenced bacteria) suggests that the increased expression of these genes inhibits growth in a wide range of bacteria. Accordingly, molecules that would increase the expression of any of these genes might function as broad-range antibiotics.

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