

Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection

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We have systematically made a set of precisely defined, single-gene deletions of all nonessential genes in *Escherichia coli* K-12. Open-reading frame coding regions were replaced with a kanamycin cassette flanked by FLP recognition target sites by using a one-step method for inactivation of chromosomal genes and primers designed to create in-frame deletions upon excision of the resistance cassette. Of 4288 genes targeted, mutants were obtained for 3985. To alleviate problems encountered in high-throughput studies, two independent mutants were saved for every deleted gene. These mutants—the ‘Keio collection’—provide a new resource not only for systematic analyses of unknown gene functions and gene regulatory networks but also for genome-wide testing of mutational effects in a common strain background, *E. coli* K-12 BW25113. We were unable to disrupt 303 genes, including 37 of unknown function, which are candidates for essential genes. Distribution is being handled via GenoBase (<http://ecoli.aist-nara.ac.jp/>).

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Introduction

The increased availability of genome sequences has provided the basis for comprehensive understanding of organisms at the molecular level. Besides sequence data, a large number of experimental and computational resources are required for genome-scale analyses. *Escherichia coli* K-12 has been one of the best-characterized organisms in molecular biology. Yet, many key resources for functional genomics and systems biology studies of *E. coli* are still lacking.

Whole genome sequences are now available for two closely related K-12 strains, MG1655 (Blattner *et al*, 1997) and W3110 (Hayashi *et al*, 2006). Whole-genome comparative sequencing and reconciliation of differences by re-sequencing selected regions from both strains have recently provided the most accurate genome of any organism (accompanying manuscript; Hayashi *et al*, 2006). Of 267 regions that were initially found to have short insertion or deletion (indel) and nucleotide (nt) disparities, only eight sites were found to be true differences. The vast majority (243) were due to errors in the original 4.5-Mb *E. coli* K-12 MG1655 genome (an error rate of less than 1 per 13 000 nt 8 years later); 16 were due to errors in the 2.6 Mb of the W3110 genome reported from 1992 to 1997. Sequence corrections resulted in major changes in the translation of 111

MG1655 open-reading frames (ORFs), mostly due to frame shifting (85), but also due to gene fissions (2), gene fusions (23), and inversion (1; Hayashi *et al*, 2006).

The availability of highly accurate *E. coli* K-12 genomes (Hayashi *et al*, 2006) provided an impetus for the cooperative re-annotation of both MG1655 and W3110 (Riley *et al*, 2006). Sequence corrections also changed many gene boundaries, which led to dropping 31 previously annotated genes and adding 66 new ones. The composite K-12 genome has 4453 genes, encoding 4296 ORFs (including 74 pseudogenes), 156 RNAs, and one annotated feature (*oriC*). Major differences between the MG1655 and W3110 genomes are the 12 additional sites of an insertion sequence (IS) in W3110, and one additional IS site and the defective CPZ-55 phage (seven prophage genes) only in MG1655. Consequently, MG1655 and W3110 have two and 17 extra copies of IS genes, respectively, and MG1655 has 11 and W3110 has 21 unique genes (including seven additional pseudogenes). Thus, on the basis of the 2005 annotation snapshot, MG1655 has a total of 4464 genes and W3110 has 4474 (Hayashi *et al*, 2006). In addition to updating annotations of gene functions, start sites were changed for 682 MG1655 ORFs (Riley *et al*, 2006). An additional 76 ORFs that have been predicted in W3110 have been targeted, for a total of 4550 genes encoding 4390 ORFs (Hayashi *et al*, 2006),

although these have not been recognized as ORFs in the recent K-12 annotation workshops.

An *E. coli* K-12 functional genomics project was initiated in Japan to (1) create new experimental resources, (2) establish new analysis methods, (3) develop new computational approaches, (4) improve databases, and (5) analyze gene function through experimentation by using these resources, methods, approaches, and databases (Mori *et al*, 2000). Newly created experimental resources now include: (a) two *E. coli* K-12 ORFeome plasmid banks of nearly all predicted ORFs, the ASKA clone sets (Kitagawa *et al*, 2005), (b) a large collection of transposon-generated gene-disruption mutants (Mori *et al*, 2000), and (c) mutants individually deleted of all nonessential *E. coli* K-12 genes (this study). Newly established analysis methods have included DNA microarrays (Oshima *et al*, 2002b), proteome analysis tools (Katayama *et al*, 2002), and tagged genes for detecting protein–protein interactions (Arifuzzaman *et al*, 2006). Newly developed computational approaches have included tools for gene clustering and codon usage diversity (Kanaya *et al*, 2001). An improved *E. coli* K-12 GenoBase (version 5.0; <http://ecoli.aist-nara.ac.jp/>) supports data analysis based on these new resources, analysis methods, and computational approaches. These resources and methods have been helpful for assignment of new cellular roles to many genes of unknown or poorly described function (e.g. Oshima *et al*, 2002a).

In a *Saccharomyces cerevisiae* functional genomics project, a nearly complete set of single-gene deletions covering 96% of yeast annotated ORFs was constructed by using a PCR gene replacement method (Giaever *et al*, 2002). The yeast mutants were isolated by direct transformation with PCR products encoding kanamycin resistance and containing 45-nt flanking homologous sequences for adjacent chromosomal regions. Genome-scale disruption of *Bacillus subtilis* genes (Kobayashi *et al*, 2003) was carried out by inactivating each gene with a gene-specific plasmid clone. Comprehensive transposon mutagenesis of *Pseudomonas aeruginosa* was carried out by generating a large set (30 100) of sequence-defined mutants (Jacobs *et al*, 2003).

Two groups began projects to construct comprehensive transposon mutant libraries of *E. coli* K-12. In Japan, chromosomal segments in a phage λ library (Kohara *et al*, 1987) were subjected to transposon mutagenesis, after which the mutations were recombined onto the chromosome by homologous recombination (Mori *et al*, 2000; T Miki, personal communication). The other group subjected PCR products encoding ORFs to *in vitro* Tn5 transposition (Goryshin *et al*, 2000), and then recombined the mutations onto the chromosome by λ Red-mediated recombination (Datsenko and Wanner, 2000), which led to the creation of insertion alleles for 1976 ORFs (Kang *et al*, 2004).

Although transposon mutagenesis has yielded large unique collections of valuable mutants, the methodologies for building a comprehensive library are laborious. First, it is necessary to define the insertion sites by PCR or DNA sequencing. Second, rearrangements or genetic duplications can result when recombining mutations onto the chromosome, compounding results, and requiring additional testing. Third, complications resulting from transposon mutagenesis, such as incomplete disruption of the targeted gene and polarity effects on downstream genes, are unavoidable.

While the project for building a transposon library was underway in Japan, a highly efficient method for direct inactivation of chromosomal genes in *E. coli* K-12 was reported (Datsenko and Wanner, 2000). This breakthrough provided a simple and efficient method for gene deletion analogous to the one that has been used in yeast (Baudin *et al*, 1993), except by use of cells carrying an easily curable, low-copy-number plasmid expressing the λ Red recombinase. Advantages are being able to target genes for complete deletion, to design deletions arbitrarily and precisely, and to easily eliminate the antibiotic resistance marker subsequently. Here, we used the λ Red system for the systematic construction of a set *E. coli* K-12 mutants with precisely defined single-gene deletions, called the Keio collection, which upon release of the resistance marker will leave behind an in-frame deletion. For convenience of gene transfer, the Keio collection retains the resistance marker.

Results and discussion

Keio collection mutants

The Keio collection is comprised of 3985 deletions in duplicate (7970 total) of *E. coli* K-12 strain BW25113 (Datsenko and Wanner, 2000), a strain with a well-defined pedigree that has not been subjected to mutagens (Figure 1; Supplementary Table 1). Mutants were directly selected as kanamycin-resistant (Km^R) colonies after electroporation of BW25113 carrying the λ Red expression plasmid pKD46 (Datsenko and Wanner, 2000). To alleviate problems that can arise in high-

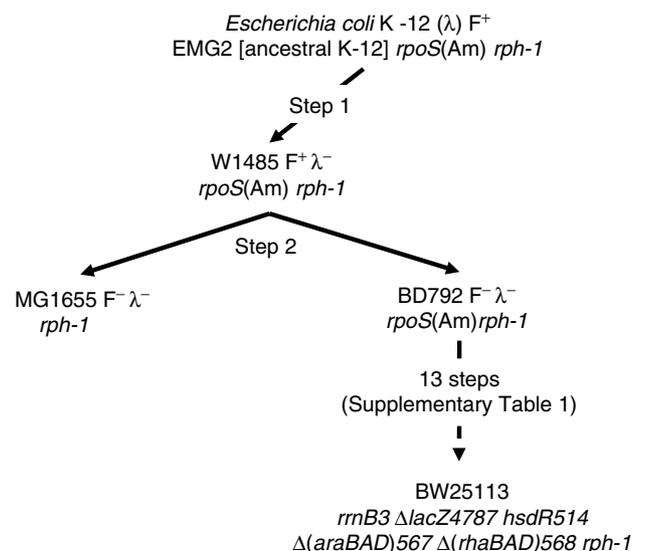


Figure 1 Derivation of *E. coli* K-12 BW25113. Strain BD792, like MG1655, is a two-step descendent of ancestral *E. coli* K-12, EMG2, originally called WG1 (Bachmann, 1996; Hayashi *et al*, 2006; late BJ Bachmann, personal communication). Like its predecessor W1485F⁺ (Hayashi *et al*, 2006), BD792 has the *rpoS*396(Am) allele (codon 33, TAG (Am); unpublished data). Strain BW25113 was derived from BD792 in a series of steps involving generalized transduction and allele replacements, which included introducing the pseudo-reversion *rpoS* (Q33) allele from MG1655 into a predecessor of BW25113 (Supplementary Table 1). The derivation of W3110 is shown in Figure 1 of accompanying manuscript (Hayashi *et al*, 2006).

throughput experiments, resulting from handling errors, crosscontamination, and accumulation of secondary mutations, two independent mutants were saved for each deletion.

Design of in-frame, single-gene deletion mutants

Chromosomal genes were targeted for mutagenesis with PCR products containing a resistance cassette flanked by FLP recognition target (FRT) sites and 50-bp homologies to adjacent chromosomal sequences (Figure 2). To reduce polar effects on downstream gene expression, primers were de-

signed so that excision of the resistance cassette with the FLP recombinase would create an in-frame deletion of the respective chromosomal gene (Figure 3). Primer sequences were based on the highly accurate *E. coli* K-12 genome (Hayashi *et al*, 2006), in which the majority of the corrections to coding regions and start codon re-assignments had been made in accordance with the November 2003 *E. coli* K-12 annotation workshop (Riley *et al*, 2006).

The targeting PCR products were designed to create in-frame deletions of the 2nd through the 7th codon from the C-terminus, leaving the ORF start codon and translational signal

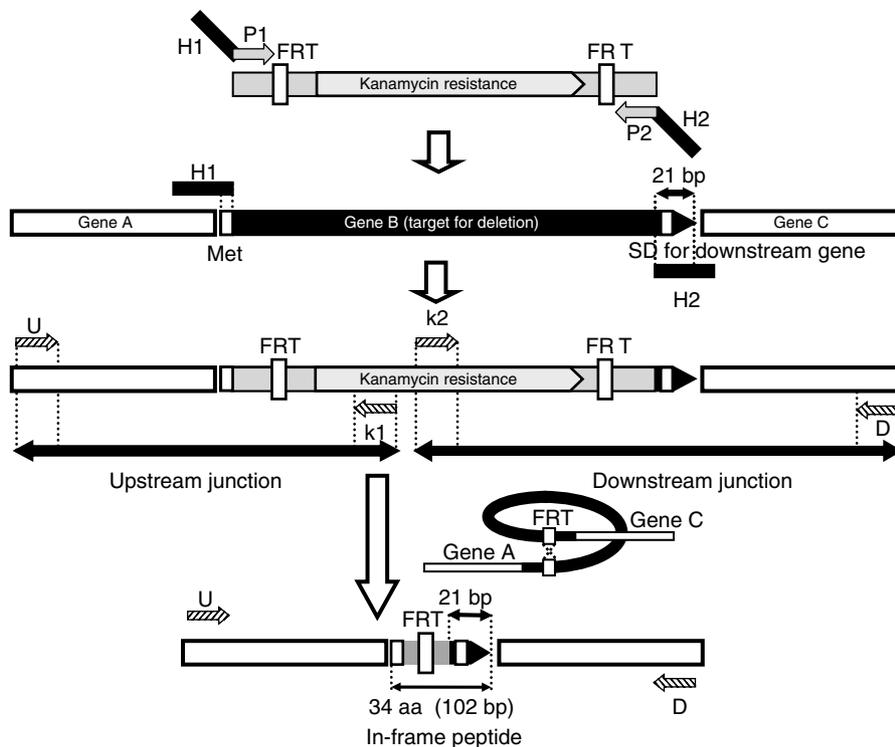


Figure 2 Primer design and construction of single-gene deletion mutants. Gene knockout primers have 20-nt 3' ends for priming upstream (P1) and downstream (P2) of the FRT sites flanking the kanamycin resistance gene in pKD13 and 50-nt 5' ends homologous to upstream (H1) and downstream (H2) chromosomal sequences for targeting the gene deletion (Supplementary Table 2). H1 includes the gene B (target) initiation codon. H2 includes codons for the six C-terminal residues, the stop codon, and 29-nt downstream. The same primer design with respect to gene B was used to target deletions regardless of whether gene B lies in an operon with genes A and C, as shown, or in different chromosomal arrangements. Novel junctions created between the resistance cassette and neighboring upstream (gene A) and downstream (gene C) sequences were verified by PCR with kanamycin (k1 or k2) and locus-specific (U or D) primers. Structures created after excision of the resistance gene are verified by PCR with neighboring gene-specific primers and by direct DNA sequencing of the region encompassing the H1-P1-FRT-P2-H2 scar to verify correct ones, as described elsewhere (Datsenko and Wanner, 2000). SD, Shine-Dalgarno ribosome binding sequence.

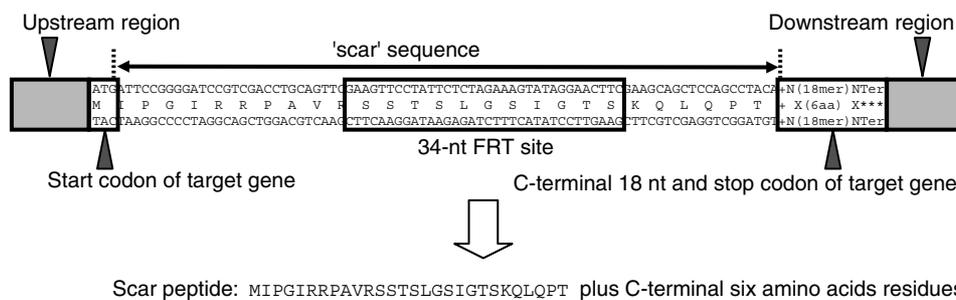


Figure 3 Structure of in-frame deletions. FLP-mediated excision of the FRT-flanked resistance gene is predicted to create a translatable scar sequence in-frame with the gene B target initiation codon and its C-terminal 18-nt coding region. Translation from the authentic gene B SD and start codon is expected to produce a 34-residue scar peptide with an N-terminal Met, 27 scar-specific residues, and six C-terminal, gene B-specific residues.

for a downstream gene intact (Figure 2). However, according to its latest genome annotation, *E. coli* K-12 has 742 overlapping genes, ranging in length from 1 to 260 nt, with the longest being for *ytjP* and *yzfA*. Although the majority are short (1–8 nt), 191 genes overlap by at least 9 nt. Thus, our standard design for construction of in-frame deletions can in some cases simultaneously affect the coding of two overlapping ORFs, which can be especially important when evaluating gene essentiality.

For example, *folC* encodes bifunctional folylpolyglutamate and dihydrofolate synthases and has an 11-nt overlap with the downstream *dedD*, encoding a conserved protein of unknown function. In agreement with an earlier study (Pyne and Bogner, 1992), we found *folC* to be essential (on the basis of the criteria below). Preliminary results suggested that *dedD* was also essential. However, due to the *folC-dedD* gene overlap, it was conceivable that the lethality of a *dedD* deletion was due to alteration of the *folC* C-terminus. To address these kinds of issues, a small number of primers were redesigned to avoid altering two genes simultaneously, by taking into account gene overlaps. Indeed, *dedD* was successfully deleted with a PCR product that was synthesized with an N-terminal primer that was redesigned to prevent altering the *folC* coding region. Primer extensions are given in Supplementary Table 2.

Construction and verification of deletion mutants

Our standard protocol usually yielded 10–1000 Km^R colonies when cells were incubated aerobically at 37°C on Luria broth (LB) agar containing 30 µg/ml kanamycin. The most critical step was preparation of highly electrocompetent cells (> 10⁹ transformants per 1 µg of plasmid DNA under standard conditions). Mutants were isolated in batches, in which each batch included a PCR product for disruption of *yhqQ* as a positive control as well as a no PCR product negative control. The latter usually gave only 10–100 tiny colonies. From every

gene deletion experiment, four or eight Km^R colonies were chosen and checked for ones with the correct structure by PCR using a combination of locus- and kanamycin-specific primers (Figure 2), as described elsewhere (Datsenko and Wanner, 2000). Mutants were scored as correct if two or more colonies had the expected structure based on PCR tests for both junction fragments.

Keio collection deletions

Of 4288 genes targeted, deletions were obtained for 3985 ORFs (Supplementary Table 3). Based on finding mutants with the predicted structure, these 3985 genes are (probably) nonessential, while the 303 genes (including 37 genes of unknown function), for which no mutants were found, are candidates for essential genes (Figure 4; Table I). Our ORF deletions include 3912 genes annotated in both *E. coli* K-12 MG1655 and W3110 and 73 previously annotated genes (Supplementary Table 4). The 3912 composite K-12 ORF deletions include 2157 characterized genes and 1755 genes of uncharacterized or unknown function. ORFs not targeted include 79 IS genes, four genes for small toxic polypeptides (*ldrA*, *ldrB*, *ldrC*, and *ldrD*), and seven genes already disrupted in BW25113 (*araBAD*, *lacZ*, and *rhaBAD*; Datsenko and Wanner, 2000). No in-frame deletion was targeted to 12 ORFs whose coding region was changed at the March 2005 annotation workshop (Riley et al, 2006) after completion of the Keio collection (Supplementary Table 5). RNA genes were also not targeted.

Evaluation of gene essentiality

Several causes can contribute to finding too many or too few nonessential genes. One way to evaluate gene essentiality is to examine our knockout efficiency (Table II), that is, the percent of the Km^R colonies with the correct structure. For nearly 50% of the targeted ORFs, all Km^R colonies had the expected

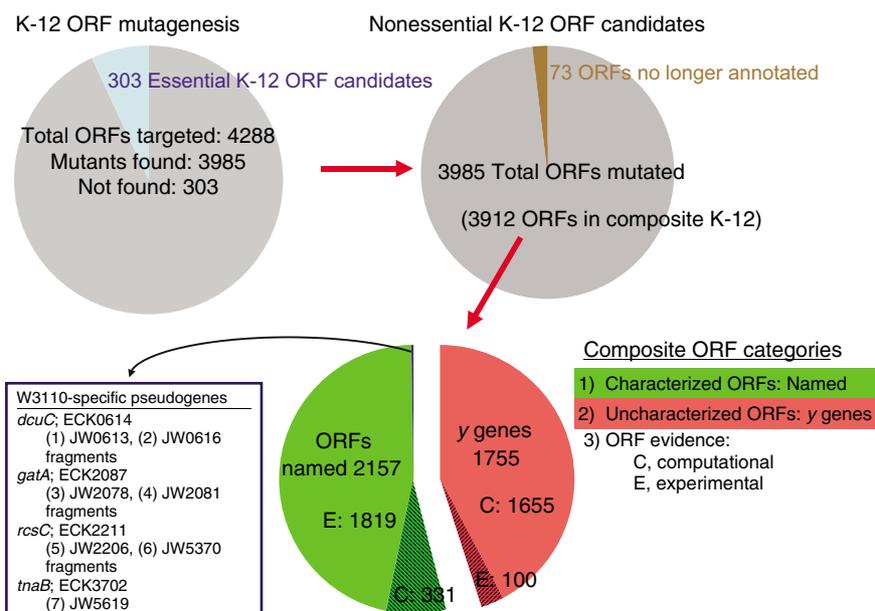


Figure 4 Mutagenesis of *E. coli* K-12 ORFs. See text.

structure for the correct deletion; for 93% of the ORFs, at least 50% were correct; and, with one exception, for all Keio mutants, at least 25% were correct. The exceptional case, *secM*, has a translational arrest sequence within its C-terminus that is required for expression of the downstream *secA*, encoding an essential preprotein translocase SecA subunit (Murakami et al, 2004; Nakatogawa et al, 2005). Thus, it is reasonable to suggest that the sole *secM* mutant arose because it acquired a suppressor allowing *secA* expression. Essential gene candidates are given in Supplementary Table 6.

The ability to select directly for knockout mutants may have led to other mutants with suppressors. For example, the same mutagenesis strategy has been used elsewhere to create a deletion of *mreB* (Kruse et al, 2003), an essential gene, in which case, the mutant was later shown to carry a suppressor (Kruse et al, 2005). Yet, we repeatedly failed to recover a $\Delta mreB$ mutant, even when using the identical primers and host. We also confirmed the absence of *mreB* coding sequences in their $\Delta mreB$ mutant, thus ruling out the possibility of a duplicate *mreB* sequence (data not shown). Clearly, *secM* and *mreB* are examples of 'quasi-essential' genes, for suppressors allow viability of mutants with the respective deletions. By definition, deletions of truly essential genes cannot be mutationally suppressed.

Table I Mutant summary

Class	ORFs
Total	4390
Targeted ^a	4288
Nonessential	3985
Essential	303
Not targeted ^b	102

ORF=open-reading frame.

^aAll targeted ORFs are in given Supplementary Table 2.

^bORFs not targeted are given in the text.

Table II Knockout efficiency^a

Percent correct ^b	ORFs	Essentiality score ^c		
		< -1	-1 to +1	≥ +1
100	1946	1916	30	0
87.5	729	719	10	0
75	499	487	12	0
62.5	316	307	9	0
50	219	211	8	0
37.5	116	112	4	0
25	160	149	11	0
12.5	1	0	1	0
0	302	0	88	214
Total	4288	3901	173	214

ORF=open-reading frame.

^aData are in given Supplementary Table 3.

^bPercent of the four or eight Km^R colonies shown to have the correct structure by PCR as described in text is given.

^cThe number of ORFs with different essentiality scores is given. Scores less than -1 or greater than +1 mean that the gene is nonessential (< -1) or essential (> +1) with no inconsistency with previous studies. Scores between -1 and +1 mean some inconsistency exists.

In addition to suppressors, a functional redundancy or duplication can hide gene essentiality. It is difficult to assess functional redundancy without further experimentation. However, gene duplications can explain why we recovered mutants with deletions of some genes, like *ileS* and *glyS*, encoding isoleucyl-tRNA and glycine (β -subunit) tRNA synthetases, which are essential. In these cases, the mutants carry intact copies of the respective deleted gene elsewhere (R D'Ari and K Nakahihashi, personal communication), presumably resulting from gene duplications. Nevertheless, because the vast majority of mutants were recovered at a high frequency (Table II), neither suppressors nor duplications seem to be major concerns. Genetic duplications resulting from gene amplification have been well documented in bacteria; however, the frequency is low; under ordinary conditions, about one in 400 genes is on average duplicated in a culture (Anderson and Roth, 1977). If we assume similar values, then no more than about 10 of our mutants is likely to have a gene duplication. Even though about 1.5% of the yeast mutants were eliminated due to duplications (Giaever et al, 2002), most studies on gene essentiality fail to consider this issue.

Special cases

A few discrepancies exist between our results and those of earlier studies. For example, we were able to delete *hlpA*, encoding a periplasmic chaperone for outer membrane proteins, which had been reported to be essential (Dicker and Seetharam, 1992). This can be explained by the location of *hlpA* immediately upstream of *lpxD*, encoding an essential UDP-3-O-(3-hydroxymyristoyl)-glucosamine *N*-acyltransferase. A polar effect of the *hlpA* disruption on *lpxD* expression was likely responsible for the earlier evidence of gene essentiality. Mutants described here are initially nonpolar because downstream genes can be expressed from the resistance gene promoter (Figure 2), and from the upstream native promoter upon elimination of the resistance cassette.

A number of factors can cause a nonessential gene to appear to be essential. The absence of diaminopimelic acid from standard laboratory media is surely why no mutants requiring this supplement (*dapA*, *B*, or *E*) were recovered. Likewise, our inability to recover particular mutants in central metabolic pathways, for example, *gapA*, is due to our use of media on which mutants lacking (nonessential) glycolysis genes fail to grow (Fraenkel, 1996), which can be due to the accumulation of toxic intermediates.

Occasional technical problems can also interfere with the isolation of deletions. In a few instances, PCR products failed to target a gene due to the presence of IS elements at sites that were previously unrecognized. Such deletions were successfully made when the primer(s) was redesigned to take the IS element into account. Primer quality is also important. In rare cases, we failed to isolate a deletion for no apparent explanation, yet we were able to do so with a new batch of primers.

Toxin-antitoxin (TA) systems

Deletion of a single gene can lead to aberrant behavior in certain gene contexts. Well-studied examples are the prokar-

ytic TA stress response loci (Gerdes *et al*, 2005). For example, RelE and MazF are toxins that cleave mRNA in response to a nutritional stress. Under nonstress conditions, a specific antitoxin (RelB or MazE) prevents cleavage, allowing normal growth. *E. coli* K-12 encodes six such TA systems, three belonging to the RelE (toxin)/RelB (antitoxin) family, RelE/RelB, YafQ/DinJ, and YoeB/YefM; two belonging to the MazF/MazE family, ChpA(MazF)/ChpR(MazE) and ChpB/ChpS; and one belonging to the TA-like system, HipA/HipB. Our failure to find deletions of *yefM*, *chpR*, or *chpS* is likely because they encode TA system antitoxins.

Categories of essential genes

ORFs can be classified into clusters of orthologous groups (COGs) belonging to different functional categories (Figure 5; Supplementary Table 7). It is natural for multidomain proteins to be comprised of more than one COG. Some COGs also belong to more than one functional class. Consequently, the 4390 ORFs in *E. coli* K-12 strain W3110 correspond to 4011 COGs (and 1214 with no COG assignment), while the 303 essential ORF candidates correspond to 315 COGs (and 26 with no COG assignment). The fraction of essential genes varies widely with the COG classification. The greatest fractions are for COGs with roles in translation, ribosomal structure, and biogenesis. The vast majority of essential genes belong to COGs with roles in cell division, lipid metabolism, translation, transcription, and cell envelope biogenesis. For example, our results showed that *rpoE* and *rpoH*, encoding RNA polymerase

heat-shock sigma factors E and H, respectively, are essential, in agreement with earlier studies (Zhou *et al*, 1988; Hiratsu *et al*, 1995). Our data also showed that we were able to disrupt genes for five ribosomal proteins (S6, S20, L1, L11, and L33), which had been previously shown to be nonessential (Dabbs, 1991). Discrepancies for 11 others may have resulted from use of different growth conditions or strain.

Comparison with other *E. coli* gene essentiality studies

Genetic footprinting (Gerdes *et al*, 2003; Tong *et al*, 2004) revealed 620 genes to be essential for robust aerobic growth of *E. coli* K-12. Yet, only 67% (205 genes) overlap with the predicted essential genes in this study. Striking differences can be attributed to the use of different mutagenesis strategies (transposon insertion versus deletion), different growth conditions (broth versus agar), or the approach for discriminating essential versus nonessential genes. Because genetic footprinting measures cell populations, a mutation causing slow growth can lead to under-representation of the mutant and hence false classification of many genes as essential. In contrast, we sought deletion mutants as survivors without regard to growth rate. Supplementary Table 6 has a comparison of our results with those from genetic footprinting (Gerdes *et al*, 2003), the PEC database (Hashimoto *et al*, 2005), and transposon mutagenesis (Kang *et al*, 2004), in which an 'essentiality score' is computed for all 303 essential gene candidates from our study.

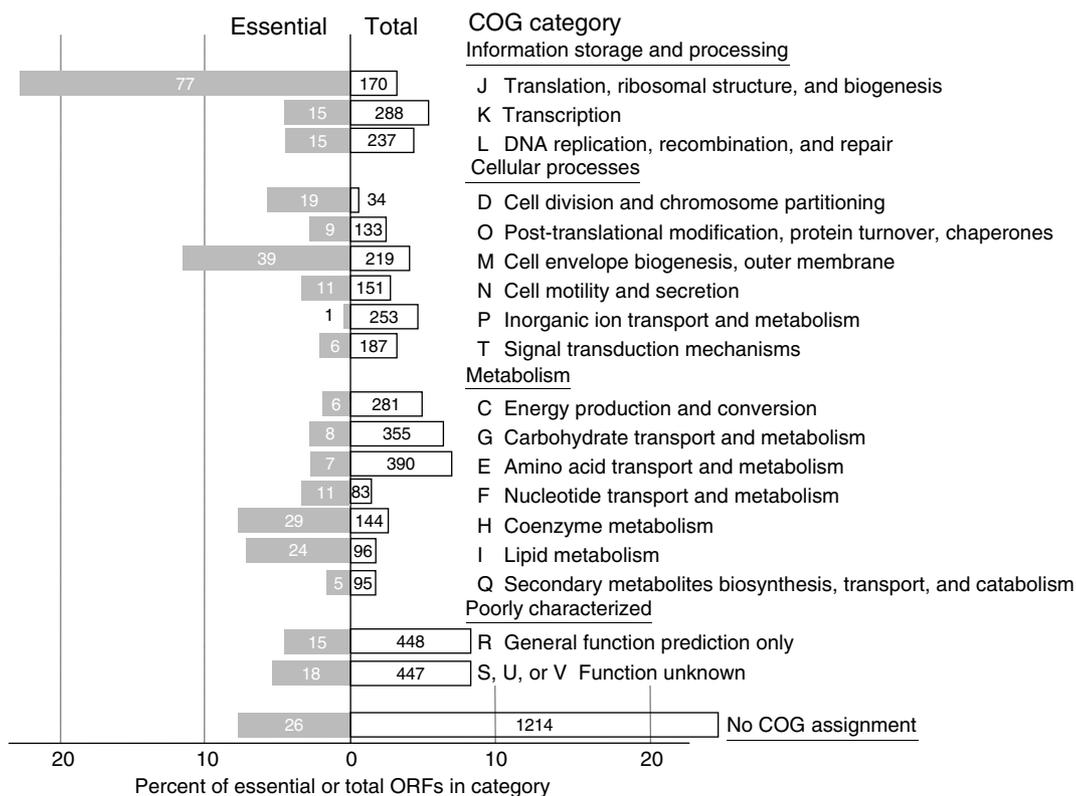


Figure 5 COG classification of K-12 genes. See Supplementary Table 7.

We also examined the conservation of the K-12 essential genes in genomes of other organisms in the Microbial Genome Database (<http://mbgd.genome.ad.jp/>; Uchiyama, 2003). Comparison with three other *E. coli* genomes revealed that more than 90% (282) of the essential genes are universally present. About one-half (147) are conserved among 20 different Enterobacteriaceae genomes. One-third (85) are conserved among 74 Proteobacteria and less than 15% (42) are conserved among 171 bacteria (Supplementary Tables 6 and 8).

Comparison with gene essentiality in other free-living bacteria

B. subtilis has a 4.2-Mb genome and 271 essential genes (Kobayashi *et al*, 2003). About one-half (150) of the orthologous genes are also essential in *E. coli*. Another 67 genes that are essential in *E. coli* are not essential in *B. subtilis*, while 86 *E. coli* essential genes have no *B. subtilis* ortholog. Details are given in Supplementary Table 6.

Profiling contributions of individual genes during growth on rich and minimal media

All mutants were profiled for growth yield in both rich (LB) and minimal glucose MOPS media (Figure 6). Growth data in Figure 6 are summarized according to COG category in Table III. Complete information is in Supplementary Table 3. Many

factors can contribute to how efficiently cells convert nutrients into biomass. The vast majority showed no differences from wild type. Mutants in circled area 1 gave higher yield in minimal than rich; those in area 2 gave similar yields in both media; and those in area 3 gave higher yields in rich than minimal. No correlation with mutant class was seen for those in areas 1 or 2. As expected, the majority in area 3 has defects in biosynthesis, for example, for amino acids, purines, pyrimidines, and vitamins. Curiously, a subset of these auxotrophs showed modest growth after 48 h, suggesting that suppressors arose. The trivial explanation of crosscontamination is unlikely because similar results were obtained in replica cultures. A few mutants with deletions of genes of unknown function also grew well in rich but not in minimal, which may provide a handle on determination of their function. Some grew after 24 h but showed no growth after 48 h, suggesting lysis, for example, *ddlB* (D-alanine:D-alanine ligase), *csGC* (predicted curli production protein), *rsxC* (predicted 4Fe-4S ferredoxin-type protein), and others. Many grew poorly on both rich and minimal media, for example, *priA* (primosome factor), *atp* (ATP synthase components), and *cyaA* (adenylate cyclase). Nevertheless, the majority showed no striking growth defect.

Use and distribution of the Keio collection

Several complete sets of the Keio collection as well as thousands of individual mutants have already been distributed

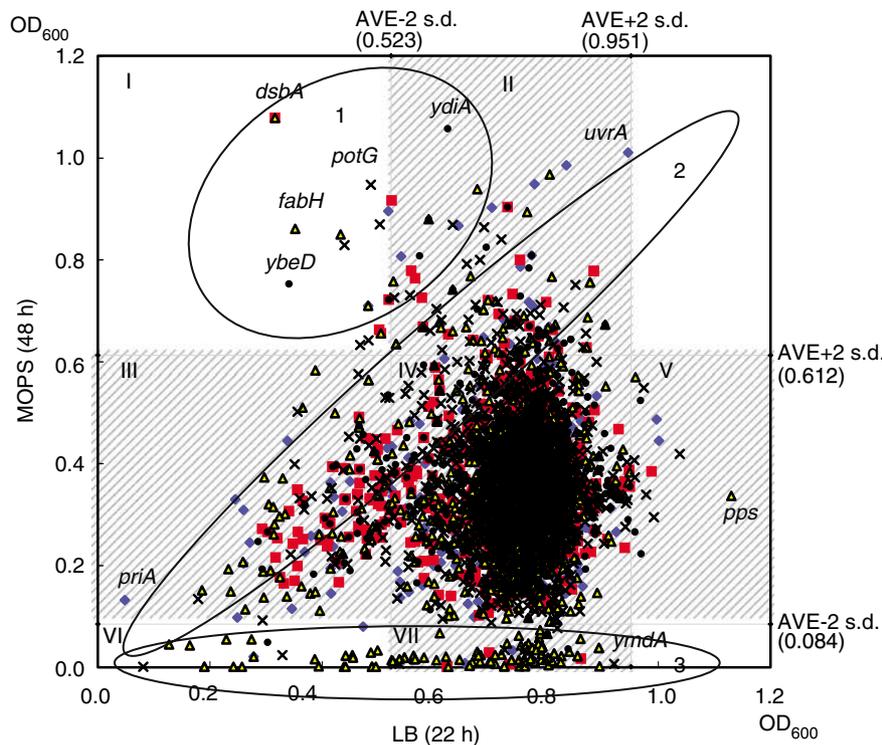


Figure 6 Profiling gene contribution for growth. Mutants of all 3985 genes in the Keio collection were grown 22 h in LB and 24 and 48 h in 0.4% glucose MOPS 2 mM Pi medium (Wanner, 1994). Maximum cell density values are plotted. Circled areas 1, 2, and 3 are discussed in text. Grayed areas show $2 \times$ s.d. Groups labeled I–VII differ by more than $2 \times$ s.d. Summary data are given in Table III. Additional time-course data are given in Supplementary Figure 1. Data are in given Supplementary Table 3. Major COG categories: blue diamond, information storage and processing; red square, cellular processes; yellow triangle, metabolism; black filled circle, poorly characterized; and black cross, no COG assignment.

Table III Summary of growth data for Keio collection according to COG category

COG category	Medium LB MOPS	Group ^a						
		I <0.52 0.61 <	II 0.52–0.95 0.61 <	III <0.52 0.08–0.61	IV 0.52–0.95 0.08–0.61	V 0.95 < 0.08–0.61	VI <0.52 <0.08	VII 0.52–0.95 <0.08
Information storage and processing	J	0	1	8	84	0	0	0
	K	1	12	8	248	1	1	2
	L	1	6	9	127	1	1	1
	D	0	0	0	15	0	0	0
	O	2	2	11	110	0	0	0
Cellular processes	M	0	3	12	163	0	0	1
	N	2	5	33	100	0	0	0
	P	0	11	4	234	1	0	2
	T	0	5	9	165	0	0	0
	C	1	12	26	229	0	2	4
	G	2	15	13	311	1	0	0
Metabolism	E	0	12	7	305	1	5	53
	F	0	1	3	47	0	12	9
	H	1	2	5	88	0	2	17
	I	1	2	0	69	0	0	0
	Q	0	1	0	88	0	0	1
Poorly characterized	R	0	15	11	403	0	1	2
	S	1	7	8	259	3	0	0
	U	1	4	15	88	0	0	0
	V	0	2	0	41	0	0	0
No COG		5	34	36	1091	6	2	7

COG=clusters of orthologous groups; LB=Luria broth; MOPS=0.4% glucose MOPS 2 mM Pi medium (Wanner, 1994).
^aGroups are shown in Figure 6. Number of mutants in each COG category are given.

worldwide. Distribution is being handled via GenoBase (<http://ecoli.aist-nara.ac.jp/>) together with supporting data and other key resources, including the ASKA (A complete Set of *E. coli* K-12 ORF Archive) clone sets (Kitagawa *et al*, 2005). Several studies have already reported use of these mutants. For example, single-gene deletion mutants of the Keio collection were utilized for the study of uncharacterized gene function (Melnick *et al*, 2004) and the analysis of metabolism (Jiao *et al*, 2003; Hua *et al*, 2003, 2004; Yang *et al*, 2003; Zhao *et al*, 2004a, b). The use of subsets of Keio collection mutants has substantiated the value of systematic approaches for the understanding of cellular systems (Tenorio *et al*, 2003; Ito *et al*, 2005; Perrenoud and Sauer, 2005).

Summary

We have undertaken a large-scale project for systematic construction of a set of precisely defined single-gene, knockout mutants of all nonessential genes in *E. coli* K-12. These mutants were designed to create in-frame (nonpolar) deletions upon elimination of the resistance cassette. Our analysis of these mutants has provided new key information on *E. coli* biology. First, the vast majority of the genes that were independently disrupted at least twice are probably nonessential, at least under the conditions of selection. Second, those genes that we repeatedly failed to disrupt are candidates for essential *E. coli* genes. Lastly, by comparing the effects of these mutations in the same *E. coli* K-12 genetic background,

we profiled the contribution of these genes to growth on synthetic minimal and rich medium.

The Keio collection should provide not only a basic resource for systematic functional genomics but also experimental data source for systems biology approaches. The mutants can serve as fundamental tools for a number of reverse genetics approaches, permitting analysis of the consequences of the complete loss of gene function, in contrast to forward genetics approaches in which mutant phenotypes are associated with a corresponding gene(s). By providing this resource to the research community, the authors hope to contribute to worldwide efforts directed towards a comprehensive understanding of the *E. coli* K-12 model cell. Because many *E. coli* gene products are well conserved in nature, the Keio collection is likely to be useful not only for studying *E. coli* and other bacteria but also for examining properties of genes from a wide range of living organisms.

Materials and methods

Bacteria and plasmids

E. coli BW25141 (*rrnB3* *DElacZ4787* *DEphoBR580* *hsdR514* *DE(araBAD)567* *DE(rhaBAD)568* *galU95* *DEendA9::FRT* *DEuidA3::pir(wt)* *recA1* *rph-1*) was used for maintenance of the template plasmid pKD13 (GenBank™ Accession number AY048744). pKD46 (GenBank™ Accession number AY048746; Datsenko and Wanner, 2000) was made by PCR amplification of the Red recombinase genes from phage λ and cloning into pKD16, a derivative of INT-ts (Haldimann and Wanner, 2001) carrying *araC* and *araBp* from pBAD18 (Guzman *et al*, 1995).

Media, chemicals, and other reagents

Cells were routinely grown in LB medium containing 1% Bacto Tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl with or without antibiotics at 50 µg/ml for ampicillin (Wako, Osaka, Japan) and 30 µg/ml for kanamycin (Wako, Osaka, Japan). Glucose, L-arabinose, and other chemicals were from Wako (Osaka, Japan). *DpnI* was from New England Biolabs (MA, USA); *Taq* polymerase, *TaKaRa Ex Taq*, and agarose, SeaKem GTG Agarose from Takara Shuzo Inc. E-Gel 96 systems were from Invitrogen. MOPS medium was prepared as described elsewhere (Wanner, 1994).

PCR primers

With a few exceptions, N-terminal deletion primers had a 50-nt 5' extension including the gene initiation codon (H1) and the 20-nt sequence 5'-ATTCCGGGGATCCGTCGACC-3' (P1), and C-terminal deletion primers consisted of 21 nt for the C-terminal region, the termination codon and 29-nt downstream (H2), and the 20-nt sequence 5'-TGTAGGCTGGAGCTGCTCG-3' (P2; Figure 2). All extensions are in given Supplementary Table 2.

Generation of PCR fragments

PCR reactions were carried out in 96-well microplates in 50 µl reactions containing 2.5 U of *TaKaRa Ex Taq* polymerase, 1 pg pKD13 DNA, 1.0 µM of each primer, and 200 µM dNTPs. Reactions were run for 30 cycles: 94°C for 30 s, 59°C for 30 s, 72°C for 2 min, plus an additional 2 min at 72°C. PCR products were digested with *DpnI*, ethanol precipitated, resuspended in 6 µl H₂O, and analyzed by 1% agarose gel electrophoresis using 0.5 × Tris-acetate buffer or the E-Gel 96 system.

Electroporation and mutant selection

E. coli K-12 BW25113 carrying the Red helper plasmid pKD46 was grown in 100 ml SOB medium with ampicillin and 1 mM L-arabinose at 30°C to an OD₆₀₀ of 0.3, and electroporation-competent cells were prepared as described elsewhere (Sambrook et al, 1998). A measure of 50 µl of competent cells was mixed with 400 ng of the PCR fragment in an ice-cold 0.2 cm cuvette (Bio-Rad Inc.). Cells were electroporated at 2.5 kV with 25 mF and 200 Ω, immediately followed by the addition of 1 ml of SOC medium (2% Bacto Tryptone (Difco), 0.5% yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) with 1 mM L-arabinose. After incubation for 2 h at 37°C, one-tenth portion was spread onto agar plate to select Km^R transformants at 37°C.

PCR verification of deletions

Two PCR reactions were carried out to test for correct chromosomal structures. Eight independent colonies were transferred into 150-µl LB medium with kanamycin in 96-well microplates and incubated overnight at 37°C without shaking. A measure of 1 µl of each culture was separately examined in 20-µl PCR reactions following 2-min 'hot start' at 95°C. PCR verification with kanamycin-specific primers k1 and k2 and locus-specific primers U and D (Figure 2) was carried out as described previously (Datsenko and Wanner, 2000). PCR products were analyzed by 1% agarose gel electrophoresis as above.

Storage of mutants

Mutants were stored at -80°C in 96-well microplates containing 150-µl LB medium with kanamycin and 15% glycerol.

Growth tests

Mutants were tested for growth in 200-µl LB medium with kanamycin as a rich medium in 96-well microplates inoculated directly with 96 inoculation pins (Genetix Limited, UK) and incubated for 22 h

at 37°C without shaking. Absorbance at 600 nm was measured after mixing for 5 s in a 96-well plate reader (Molecular Dynamics). Mutants were transferred with 96-inoculation pins (Genetix Limited, UK) from LB into 200-µl 0.4% glucose MOPS medium with 2 mM Pi (Wanner, 1994) and kanamycin as minimal medium and incubated for 24 and 48 h at 37°C without shaking. Absorbances were similarly measured.

Supplementary information

Supplementary information is available at *Molecular Systems Biology* website (www.nature.com/msb).

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