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Comparative Genomic Hybridization Detects Secondary Chromosomal Deletions in *Escherichia coli* K-12 MG1655 Mutants and Highlights Instability in the *flhDC* Region▽

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The use of whole-genome microarrays for monitoring mutagenized or otherwise engineered genetic derivatives is a potentially powerful tool for checking genomic integrity. Using comparative genomic hybridization of a number of unrelated, directed deletion mutants in *Escherichia coli* K-12 MG1655, we identified unintended secondary genomic deletions in the *flhDC* region in Δ*fnr*, Δ*crp*, and Δ*creB* mutants. These deletions were confirmed by PCR and phenotypic tests. Our findings show that nonmotile progeny are found in some MG1655 directed deletion mutants, and studies on the effects of gene knockouts should be viewed with caution when the mutants have not been screened for the presence of secondary deletions or confirmed by other methods.

Many studies on bacterial gene function or regulation are based on the comparison between a wild-type strain and a mutant strain, where the mutant has an inactivated or deleted gene but is otherwise assumed to be isogenic with the wild type. In *Escherichia coli* K-12 and other enteric bacteria, comparative studies between wild-type and mutant strains have been facilitated by the development of rapid methods for generating precise deletions, particularly λ-Red-mediated gene replacement techniques (8, 16, 27), but there has been no simple way of confirming that no unintended deletions had occurred during the mutagenesis procedure. Further, many mutants created in the past by a variety of methods remain in use, sometimes after extensive serial subculture but without verifying their genomic integrity by newer technologies.

Flagellum-mediated motility and its regulation have been well characterized in *E. coli* and other enteric bacteria and are regulated in *E. coli* K-12 by the activator complex encoded by *flhDC* (2, 15). The FlhDC heterotetramer regulates the synthesis and assembly of flagella, and motility is a phenotype that is crucial to fitness in many environments: it is characteristic of the vast majority of *E. coli* wild isolates, and yet it carries a heavy penalty in energy demands. Motility appears to be subject to counterselection in some biological contexts, for example, in the emergence of nonmotile *Shigella* spp. in more than one distinct lineage within the *E. coli-Shigella* phylogenetic cluster (12) and of nonmotile strains of enterohemorrhagic *E. coli* O157. A recent report also indicates that spontaneous *flhDC* mutants in K-12 MG1655 are advantaged in colonization of the mouse intestine (14). In contrast, an enterohemorrhagic *E. coli* strain with a defective *flhC* gene was impaired in colonization of the bovine digestive tract (9). The expression of flagella is important in avian colonization by O157 strains (3), while it is a hindrance in infections of pigs (4). These reports support the concept that the motility regulon may in some circumstances be unstable and subject to selective pressure and may be particularly liable to deletion when cells are stressed. Alternatively, spontaneous deletions of the flagellar regulon may be an advantage to cells under certain conditions. In addition to the flagellar regulon, FlhD is involved in the regulation of 29 operons of known function in *E. coli* K-12, including genes regulated by Aer involved in anaerobic metabolism and the Entner-Doudoroff pathway (22).

Comparative genomic hybridization (CGH) of *E. coli* strains using microarrays is a powerful tool to compare the gross genetic differences between strains and has been used to analyze evolutionary changes (26), determine the relationships between pathogenic *E. coli* and *Shigella* strains (10), characterize probiotic *E. coli* strains (11), and define the genome of the *E. coli* laboratory strain MC4100 in relation to the genome-sequenced K-12 strain MG1655 (19). CGH also has the potential to be a useful tool for characterizing bacterial gene knockout strains and bacterial subpopulations that may arise within cultures because of spontaneous deletions.

We report here the use of CGH to characterize genetically a series of regulatory gene deletion mutants we had made in *E. coli* K-12 MG1655 using the λ-Red method of Datsenko and Wanner (8). In each case the mutants had been verified by PCR using primers that anneal to DNA sequences on either side of the gene that had been replaced by an antibiotic resistance cassette. We describe the use of CGH to validate gene deletion mutants and describe the secondary gross deletions in the flagellar regulon that we detected in some knockout strains, which were confirmed by PCR and motility tests.

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TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study[a]

E. coli strain, plasmid, or oligonucleotide	Sequence (5′–3′)	Source or reference
Strains		
MG1655 (CGSC7740)		5
MG1655 (Δ*crp*)		28
MG1655 (Δ*crp* Δ*flhDC*)		This study
MG1655 (Δ*fnr*)		7
MG1655 (Δ*fnr* Δ*flhDC*)		This study
MG1655 (Cet2)		Cariss and Avison, unpublished
MG1655 (Cet2 Δ*creB*)		This study
MG1655 (Cet2 Δ*creB* Δ*flhDC*)		This study
MG1655 (Δ*rpoS*)		18
MG1655 (Δ*gadA*)		This study
MG1655 (Δ*fur*)		This study
Plasmids		
pKD46		8
pKD3		8
pKD4		8
Oligonucleotides		
Mutagenesis		
CRP P1	GCTCTGGAGAAAGCTTATAACAGAGGATAACCGCGCGTGTAG GCTGGAGCTGCTTC	28
CRP P2	TGGCGCGCTACCAGGTAACGCGCCACTCCGACGGGACATATG AATATCCTCCTTAG	28
K12-FNR1	AAATTGACAAATATCAATTACGGCTTGAGCAGACCTTGTAGG CTGGAGCTGCTTCG	7
K12-FNR2	TGATATGACAGAAGGATAGTGAGTTATGCGGAAAAACATATG AATATCCTCCTTAG	7
K12-*creB*1	CCTGTCATGCCGTGGCGGCAATAACAGAGGCGATTTGTGTAG GCTGGAGCTGCTTC	
K12-*creB*2	AAAAATAGCCCAGCAACAACCGCATGCCGATACGCACATATG AATATCCTCCTTAG	
K12-*rpo*S2	GGCCAGCCTCGCTTGAGACTGGCCTTTCTGACAGATGCTTACT TAGTGTAGGCTGGAGCTGCTTCGAA−3′	
K12-*rpo*S1	GGCTTTTGCTTGAATGTTCCGTCAAGGGATCACGGGTAGGAG CCACCTTCATATGAATATCCTCCTTAGTTCCT	
K12-*gad*A2	GTTTTTTTTTAAAGGCTGGGCATTCGGTTTTTACAACGTTATGTT ATCAGTGTAGGCTGGAGCTGCTTCG−3′	
K12-*gadA*1	CTTCCATTGCGGATAAATCCTACTTTTTTATTGCCTTCAAATAA ATTTAAGGAGTTCGAACATATGAATATCCTCCTTAGTTCC	
K12-*fur*1	ATGAAGTGAACCGCTTAGTAACAGGACAGATTCCGCTGTAGG CTGGAGCTGCTTCG	
K12-*fur*2	CCAACCCGCAGGTTGGCTTTTCTCGTTCAGGCTGGCCATATGA ATATCCTCCTTAG	
Screening		
CRP screen 1	GGATGCTACAGTAATACATTGATG	28
CRP screen 2	GACCGAATCGTAATTCGCCAAG	28
FNR screen 1	TGGTTATTGCGCCATGAAGG	7
FNR screen 2	TGGTTGGTCGTCCTGGTTAG	7
creB screen 1	ACGGCAAAGCTCAGGGCGAG	
creB screen 2	GCAACGTTGCGGTGTCGATC	
rpoS screen 1	GCCTGCACAAAATTCCAC	
rpoS screen 2	CGGATTCTTAATTACCTGG	
gadA screen1	GTTTGGGCGATTTTTATTACG	
gadA screen2	GCACTGTAATTTCCATTAGCG	
yecM forward	GCGGGAGTATACTGCAAATG	
yecM reverse	CGTTAGTGCACATTCCATGC	
yecT forward	GCGGGAGTATACTGCAAATG	
yecT reverse	CGTTAGTGCACATTCCATGC	
flhB forward	CGACAAACGCACACTATGCT	
flhB reverse	AGCAACACCATGATCGACAA	
cheZ forward	GGCTATGTGGTGAAGCCATT	
cheZ reverse	AGCATAGTGTGCGTTTGTCG	
tap forward	TCACCAATAAACCGCAAACA	
tap reverse	CATGTCGCGTTTATGGTCAG	

Continued on following page

TABLE 1—*Continued*

E. coli strain, plasmid, or oligonucleotide	Sequence (5′–3′)	Source or reference
tar forward	AAATGAACCCGATGATCTGC	
tar reverse	AAATGCCCAAAAAGACATGC	
cheW forward	ATTTCTGCTGCGACCATTCT	
cheW reverse	GCCGCGAAATTGAAATAAAA	
cheA forward	CATCAGCCTGCTGGTACTGA	
cheA reverse	GTTCGCAATCCGTGTTACCT	
motA forward	AACATTCCAGCAGCGGTAAC	
motA reverse	TCAGCCACATCACCAGAAAA	
motB forward	GCTCTATTCCAGCGAACGTC	
motB reverse	GCTGAAGCCAAAAGTTCCTG	
motA forward	AACATTCCAGCAGCGGTAAC	
motA reverse	TCAGCCACATCACCAGAAAA	
flhC forward	GGTTAAGCTGGCAGAAACCA	
flhC reverse	CCACTGTTGACCATGACAGG	
flhD forward	CCAAAAAGGTGGTTCTGCTT	
flhD reverse	AACCAGTCGGTTGAGAATGG	
insB_5 forward	GCTCCAGTGGCTTCTGTTTC	
insB_5 reverse	TCGACGCAACTGTACTCGTC	
insA_5 forward	GCTTGCTAGCGTAGCGAAAA	
insA_5 reverse	CGTCTTCCGGAGACTGTCAT	
yecG forward	GATTGCGTCGTTTCTCCATT	
yecG reverse	GGCAGGAGTGCTTCATTAGC	
otsA forward	GGATGTCTGGAGCTGGCTTG	
otsA reverse	CAAGTGAGGTCGATGTGCTG	
otsB forward	CCATAACGGTTGGCTGTTC	
otsB reverse	TCCTCATTCCCTGTTTCACC	

[a] The strains, plasmids, and PCR primers used in this study are listed. Regions underlined in the mutagenesis primers correspond to homology to genomic DNA sequences flanking genes that were deleted; nonunderlined regions are homologous to the pKD3 and pKD4 antibiotic resistance cassettes (8).

MATERIALS AND METHODS

Gene knockouts in *E. coli* K-12 MG1655 (CGSC 7740). The genome sequenced strain of MG1655, CGSC7740 (5) from the *E. coli* Stock Center (http://cgsc.biology.yale.edu/cgsc.html), was used throughout the present study. Gene knockouts in *fnr* (fumarate and nitrate reductase regulator) (7), *crp* (cyclic AMP receptor protein) (28), *rpoS* (general stress response sigma factor), *creB* (carbon source responsive response regulator) (1), *fur* (ferric uptake regulator), and *gadA* (glutamate decarboxylase) were made in MG1655 by using the λ-Red method of Datsenko and Wanner (8), wherein genes were replaced by chloramphenicol or kanamycin resistance cassettes from pKD3 or pKD4, respectively (8). Chloramphenicol- or kanamycin-resistant colonies were screened by PCR for replacement of the wild-type chromosomal gene by the antibiotic resistance cassette by using primers that flanked the gene that was replaced. Colonies were picked into 100 μl of sterile distilled H_2O and heated to 100°C for 5 min. Cell lysates were centrifuged for 2 min at 13,000 × *g*, and 1 μl of the supernatant was added to 45 μl of ABgene 1.1x Reddymix PCR mix (ABgene, Epsom, United Kingdom) containing appropriate screening primers at a final amount of 20 pmol. The reaction volume was made up to 50 μl with sterile distilled water. The PCR cycling conditions used were 94°C for 4 min, followed by 30 cycles of 94°C for 1 min and 57°C for 1 min, followed by 72°C for 2 min. A final cycle of 72°C for 10 min completed any partial extension reactions. The deletion strains constructed and oligonucleotide primers used for mutagenesis and screening for gene loss are shown in Table 1.

Genomic DNA preparations. Cultures (20 ml) of MG1655 wild-type and mutant strains were grown in LB broth (23) with appropriate antibiotic selection at 37°C to an optical density at 600 nm (OD_{600}) of 1.7 to 1.8. Ten milliliters of the cultures were harvested by centrifugation at 6,000 × *g* for 10 min. Total DNA was prepared by using the QIAGEN Genomic DNA buffer set (QIAGEN, Crawley, United Kingdom) and Genomic Tip 500/G columns according to the manufacturer's protocols, except that the total DNA precipitated by isopropanol was washed three times with 70% (vol/vol) of cold ethanol to remove any residual salt before the material was dried and resuspended in QIAGEN EB buffer. Total DNA was sheared by repeated passage through a 19G sterile needle and quantified by using a Nanodrop ND1000 microspectrophotometer (Nanodrop Technologies, Inc.). DNA purity was tested by digestion using the NaCl-sensitive restriction endonuclease HindIII.

CGH microarray experiments. Microarrays were printed using an Operon Array Ready 70-mer *E. coli* oligonucleotide set version 1.0 (Operon Biotechnologies, Cologne, Germany) and processed as previously described (7). Total DNA from each strain (5 μg) was labeled in a 50-μl reaction mixture with either Fluorolink Cy3 or Cy5 d-CTP (GE-Amersham, Little Chalfont, United Kingdom) in a reaction containing 60 ng of random hexamers (Invitrogen, Paisley, United Kingdom)/μl; 0.1 mM dA, dG, and dT NTPs; 0.04 mM dCTP (Bioline, London, United Kingdom); 50 U of Klenow exo$^-$ fragment of DNA polymerase I; and 10× EcoPol buffer (New England Biolabs, Hitchin, United Kingdom). Total DNA was mixed with random hexamers and sterile filtered high-pressure liquid chromatography-grade water (VWR, Lutterworth, United Kingdom) to a volume of 41.5 μl and then heated to 95°C for 5 min before rapid cooling on ice and brief centrifugation at 13,000 × *g* in a microcentrifuge. The remaining components were added, and the reaction was incubated overnight in the dark at 37°C. Cy dye-labeled total DNA was purified by using a QIAGEN QIAquick PCR purification kit according to the manufacturer's protocol and eluted in sterile high-pressure liquid chromatography-grade water before quantification of the DNA concentration and Cy dye incorporation using a Nanodrop microspectrophotometer.

Next, 80 pmol of Cy3-labeled total DNA from wild-type MG1655 was cohybridized with 80 pmol of Cy5-labeled total DNA from a mutant to the oligonucleotide arrays. Microarray slides were prehybridized, hybridized, washed, and scanned as previously described (7). The signal intensity from each printed feature on the array was quantified by using Genepix v5.0 software (Molecular Devices Corp. Sunnyvale, CA), and microarray data were analyzed by using Genespring software (v6.1; Agilent Instruments, South Queensferry, West Lothian, United Kingdom). Intensity-dependent LOWESS normalization was used to transform raw signal data in order to eliminate Cy dye bias, and spots with an intensity value lower than the cutoff value for the error model were filtered out. Transcriptomics experiments and analysis were carried out as previously described (7).

Motility tests. The Δ*fnr*, Δ*crp*, and Δ*creB* mutants identified by CGH as having deletions in the flagellar regulon were tested for motility by using soft agar motility tests with point inoculation of a colony from an overnight plate culture of the strains into LB agar plates containing 0.35% (wt/vol) agar. The motility

test plates were incubated at 37°C for 24 h, and the zone of bacterial growth was measured.

PCR confirmation of CGH data. Secondary gross deletions identified by microarray CGH of the MG1655 mutants were confirmed by PCR of each individual gene, as described above. The primers shown in Table 1 were used to amplify the genomic DNA flanking the gross gene deletion in the Δ*fnr*, Δ*crp*, and Δ*creB* secondary deletion strains.

Data deposition. The CGH data from these experiments is deposited under accession number GSE7695, and transcriptomics data for the nonmotile Δ*fnr* mutant are available under accession number GSE3591, in the gene expression omnibus (GEO) at the National Center for Biotechnology Information (http:www.ncbi.nlm.nih.gov/geo).

RESULTS AND DISCUSSION

CGH of the MG1655 *fnr* mutants. Three putative *fnr* deletion mutants were characterized by PCR using FNR primers A and B (Table 1). PCR confirmed that *fnr* had been replaced by the kanamycin resistance cassette from pKD4 in all three mutant strains (data not shown). The Δ*fnr* mutants were each characterized by CGH against the MG1655 (CGSC7740) parental strain total DNA. The operon 70-mer oligonucleotide array set we used was designed to contain one oligonucleotide per gene and lacks the coverage to detect small changes in the genome, but it showed that in two of the mutants *fnr* and a cluster of eight other genes at a different location on the genome were deleted (Fig. 1), suggesting that these were possible siblings, while in the third mutant only *fnr* was missing. CGH data showed that the unintended deletion was contiguous and included the *insB5* component of IS*1*, the *flhDC* master regulators of the flagella biosynthesis operon, *motA*, *motB*, *cheA*, *cheW*, and *tar*, indicating that regulation of the flagellar regulon had been lost. Soft agar motility tests of the MG1655 Δ*fnr* mutants carrying the motility gene deletion confirmed that the strains were nonmotile, whereas wild-type MG1655 and the Δ*fnr* mutant with no other deletions in it were motile (Fig. 2A).

CGH and motility test data were confirmed by transcriptomics experiments (GEO accession number GSE3591), which showed that expression of the flagellar regulon was ablated in the mutant, and by attempting PCR amplification of the region identified by CGH as being deleted and of genes upstream and downstream of this region (Fig. 1A and B). PCR was used to attempt to amplify *yecM*, *yecT*, *flhB*, *cheZ*, *cheY*, *tap*, *tar*, *cheA*, *cheW*, *motA*, *motB*, *flhC*, *flhD*, *insA5*, *insB5*, *yecG*, *otsA*, and *otsB* from the chromosome of wild-type MG1655 and from the Δ*fnr* mutant using primers detailed in Table 1. PCR products for each of the genes detailed above were detected in the wild-type MG1655 strain, as would be expected, but not in the Δ*fnr* mutants where CGH had identified a deletion in the flagellum genes (Fig. 1A and B). In the Δ*fnr* strains with the CGH detected secondary mutations, *insB5*, *flhD*, *flhC*, *motA*, *motB*, *cheA*, *cheW*, and *tar* did not amplify as determined by PCR, confirming the CGH results. The Δ*fnr* mutant where only *fnr* had been deleted was used in transcriptomics experiments reported elsewhere (7).

Screening other gene knockouts in MG1655. CGH of Δ*fur*, Δ*rpoS*, and Δ*gadA* mutants made by using the Datsenko and Wanner method (8) showed that only the required gene had been deleted. However, in two other mutants we made, the Δ*crp* and Δ*creB*mutants, CGH identified gross unintended secondary deletions. One of two Δ*crp* mutants had only *crp* deleted, while the other also contained additional deletions similar to those in the nonmotile *fnr* mutants, despite being made in different experiments and by different researchers. The secondary deletions in this Δ*crp* mutant were also centered on a contiguous tract of the chromosome from *otsA* to *tar* inclusive, amounting to 9.52 kb in total (Fig. 1A). PCR confirmed the CGH results except that CGH of the *crp* mutant indicated that *insA5* and *insB5* were present in the mutant, but PCR showed that they were absent. This inconsistency is probably due to cross-hybridization between multiple copies of Is*1* found in the MG1655 genome and the *insA5* and *insB5* oligonucleotides in the operon array, although it is unclear why this phenomenon was not seen in the *creB* and *fnr* mutants. Neither of the Δ*crp* mutants (Fig. 2B) was motile, a finding in agreement with previous reports that CRP mutants are nonmotile (25), and this shows that in certain cases motility tests alone cannot be used to determine the loss of flagellar genes. The Δ*crp* mutant strain that did not contain secondary deletions was used for experiments published elsewhere (28).

A Δ*creB* mutant, in a Cet2 (*creC* point mutant [see reference 1]) background (which was constructed independently by S. J. L. Cariss and M. B. Avison in Bristol [unpublished data]) was also shown by CGH to contain a secondary genome deletion. Although this secondary deletion was in approximately the same region of the chromosome as in the Δ*crp* and Δ*fnr* mutants, it was more extensive, covering the region between *insB5* and *yecT*/*argS* (approximately 15.5 to 17.2 kb) (Fig. 1A), and PCR confirmed the CGH result (Fig. 1B). In the Δ*creB* mutant, the antibiotic resistance cassette replacing *creB* had been excised using FLP recombinase to negate polar effects on expression of the downstream *creC* observed if the cassette remained. *flhD* PCR analysis of mutants retained at each stage of the *creB* deletion procedure showed that deletion of the flagellar regulator had occurred during excision of the antibiotic resistance cassette rather than during λ-Red-mediated recombination (data not shown). This finding was in contrast to the Δ*fnr* and Δ*crp* secondary deletion mutants which had both lost *flhDC* and contiguous genes at some point during λ-Red-mediated gene replacement: the antibiotic resistance cassette had not been excised from either of these strains. Another Δ*creB* mutant that had not lost the *flh* region was made in the MG1655 Cet2 mutant background. Although the Cet2 mutant and this Δ*creB* derivative were both reduced in motility compared to wild-type MG1655, motility was totally abolished in the Δ*creB* mutant with the secondary deletion in the *flhDC* region (Fig. 2C).

The similarity of the secondary deletions in the Δ*fnr*, Δ*crp*, and Δ*creB* mutants may be a feature of an unstable genomic region in MG1655 centered around IS*1* upstream of *flhD* (2) rather than a problem specifically associated with the mutagenesis technique we used and could be due to a low level of spontaneous deletions in the *flhDC* region in MG1655 cultures. The deletions we detected are highly similar to those reported by Leatham et al., who found that selection of spontaneous nonmotile MG1655 *flhDC* deletion mutants occurred under nutrient-limited conditions in the mouse intestine, and these mutants grew faster than wild-type MG1655 on several sole carbon sources, including D-gluconate, L-fucose, D-glucuronate, and D-mannose (14). All of the flagellar regulon mutants we detected were in directed mutants where genes in-

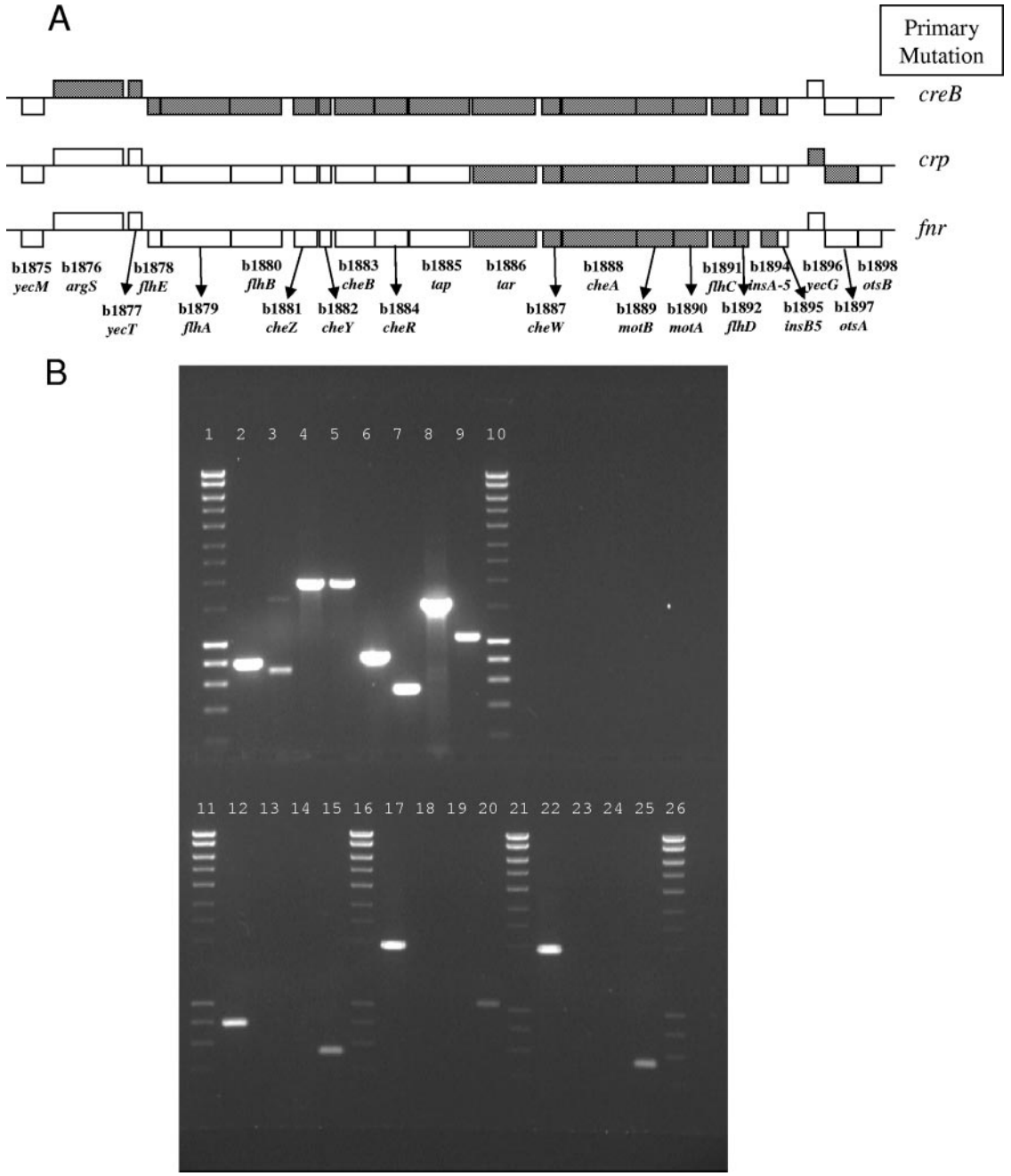

FIG. 1. (A) Graphical representation of CGH data showing gene loss in the *flhDC* region for the nonmotile Δ*fnr*, Δ*crp*, and Δ*creB* mutants. Genes are denoted by their "b number" and name. Genes are denoted by boxes. Genes encoded on the top strand of the genome are shown above the line; those on the bottom strand are shown below the line. Gray-shaded boxes indicate a loss of signal in the mutant strain as determined by the CGH array. (B) Demonstration PCRs confirming gene presence or loss in the *flhDC* region for each mutant. No PCR product indicates a loss of all or part of the gene in the nonmotile mutants. Top row of the gel (MG1655; CGSC7740): lanes 1 and 10, DNA marker (ABgene Hyperladder I); lane 2, *yecM*; lane 3, *yecT*; lane 4, *tap*; lane 5, *tar*; lane 6, *insB5*; lane 7, *insA5*; lane 8, *otsA*; lane 9, *otsB*. Bottom row of the gel: lanes 11, 16, 21, and 26, DNA marker (ABgene Hyperladder I); lanes 12 to 15, nonmotile MG1655 Δ*creB* mutant; lane 12, *yecM*; lane 13, *yecT*; lane 14, *insB5*; lane 15, *insA5*; lanes 17 to 20, nonmotile MG1655 Δ*crp* mutant; lane 17, *tap*; lane 18, *tar*; lane 19, *otsA*; lane 20, *otsB*; lanes 22 to 25, nonmotile MG1655 Δ*fnr* mutant; lane 22, *tap*; lane 23, *tar*; lane 24, *insB5*; lane 25, *insA5*. For each mutant, a PCR for each deleted gene, as well as for genes flanking the deletion, was carried out to confirm the CGH data (data not shown).

volved in central metabolism control had been deleted and occurred before or during the λ-Red mutagenesis procedure (Δ*fnr* and Δ*crp*) or after it (Δ*creB*). Loss of motility may be an advantage to cells that are metabolically challenged by the loss of important metabolic regulators, since there is an energy penalty in flagellar production (14), and reports of additional regulation of metabolism by *flhD* (14, 22) have shown that the loss of this gene allows *E. coli* K-12 MG1655 cells to utilize a

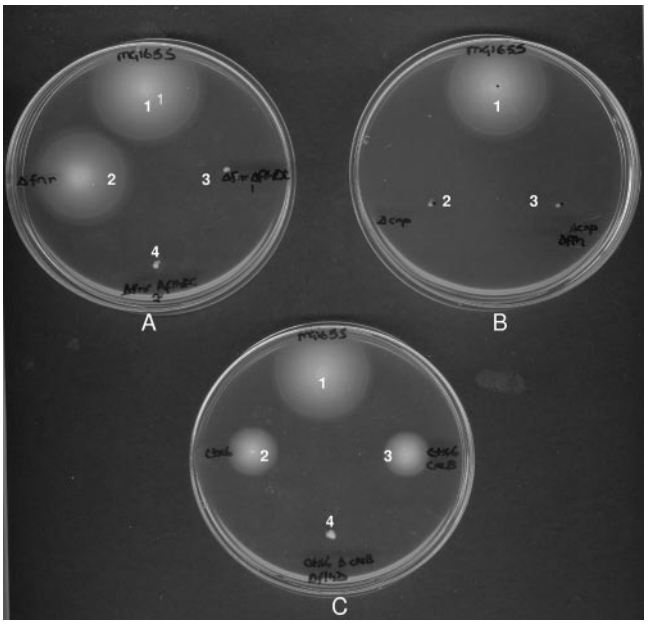

FIG. 2. Motility tests of wild-type MG1655 and deletion mutants where CGH had confirmed that *flhDC* were deleted. (A) Spots: 1, MG1655 (CGSC7740; wild type); 2, MG1655 (Δ*fnr*); 3, MG1655 (Δ*fnr* Δ*flhDC* clone 1); 4, MG1655 (Δ*fnr* Δ*flhDC* clone 2). (B) Spots: 1, MG1655 (CGSC7740) (wild type); 2, MG1655 (Δ*crp* clone 1); 3, MG1655 (Δ*crp* Δ*flhDC* clone 1). (C) Spots: 1, MG1655 (CGSC7740, wild type); 2, MG1655 (Cet2); 3, MG1655 (Cet2 Δ*creB*); 4, MG1655 (Cet2 Δ*creB* Δ*flhDC*).

wider range of carbon sources than the wild-type strain can. The secondary gene deletions we detected could be spontaneous, but there is evidence that the λ-Red mutagenesis procedure causes unintentional genomic deletions or mutations (6, 17, 20, 21), and other gene deletion methods such as suicide vector-driven allelic exchange mutagenesis have resulted in a high frequency of secondary mutations in extraintestinal pathogenic *E. coli* strains (13), so there is a real need to validate mutants as thoroughly as possible. A commonly used strategy to avoid problems of unintended deletions or mutations in *E. coli* K-12 has been to transduce mutations into a wild-type strain using bacteriophage P1, but unless the original mutant genome has no other mutations in the region that will be transduced, this can result in the transduction of undetected secondary mutations into the new host, such as when a secondary mutation in the *astC* gene was cotransduced with a *ynjB* lesion and was detected by using phenotype arrays (6).

Use of single gene deletion mutants has been crucial to understanding gene function in *E. coli* and other bacterial strains because, provided there are no polar or other secondary effects, any phenotypic, proteomic, or transcriptomic differences in the mutants compared to the wild-type are specifically attributable to the targeted deletion (13). CGH shows that confirmation of a mutation by PCR screening or Southern hybridization is not sufficient for the validation of recombineered deletion mutants because both methods are used to detect whether the directed mutation has occurred and not whether there have been any other genomic changes. Oligonucleotide microarray CGH of deletion mutants is a powerful tool for detecting secondary gross deletions and has the advantage that it interrogates the whole genome, with a higher resolution, than do macrorestriction profiles and pulsed-field gel electrophoresis (13). Oligonucleotide arrays, like PCR arrays, are designed based on the genome sequence of the strain, but unlike PCR arrays oligonucleotide arrays are not dependent on amplifying the targets for printing on the array from the parental strain, which itself may contain unknown deletions (24). Oligonucleotide microarray CGH is therefore also a useful tool for confirming that the parental strain has not accumulated gross deletions during storage or growth. Although the oligonucleotide array we used lacks the coverage to detect small changes in the genome, higher-resolution "tiling" arrays, or complete genome resequencing, should allow researchers to identify small deletions or point mutations that are not detectable using the present lower-resolution methods.

We confirm here that there is genomic instability in the *flhDC* region of the MG1655 genome (14) and show that there were significant secondary deletions in several mutants we constructed, which if undetected could have led to incorrect assignment of regulator function in subsequent experiments.

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