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RESEARCH ARTICLE

Rapid Accumulation of Motility-Activating Mutations in Resting Liquid Culture of *Escherichia coli*

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ABSTRACT Expression of motility genes is a potentially beneficial but costly process in bacteria. Interestingly, many isolate strains of *Escherichia coli* possess motility genes but have lost the ability to activate them under conditions in which motility is advantageous, raising the question of how they respond to these situations. Through transcriptome profiling of strains in the *E. coli* single-gene knockout Keio collection, we noticed drastic upregulation of motility genes in many of the deletion strains compared to levels in their weakly motile parent strain (BW25113). We show that this switch to a motile phenotype is not a direct consequence of the genes deleted but is instead due to a variety of secondary mutations that increase the expression of the major motility regulator, FlhDC. Importantly, we find that this switch can be reproduced by growing poorly motile *E. coli* strains in nonshaking liquid medium overnight but not in shaking liquid medium. Individual isolates after the nonshaking overnight incubations acquired distinct mutations upstream of the *flhDC* operon, including different insertion sequence (IS) elements and, to a lesser extent, point mutations. The rapidity with which genetic changes sweep through the populations grown without shaking shows that poorly motile strains can quickly adapt to a motile lifestyle by genetic rewiring.

IMPORTANCE The ability to tune gene expression in times of need outside preordained regulatory networks is an essential evolutionary process that allows organisms to survive and compete. Here, we show that upon overnight incubation in liquid medium without shaking, populations of largely nonmotile *Escherichia coli* bacteria can rapidly accumulate mutants that have constitutive motility. This effect contributes to widespread secondary mutations in the single-gene knockout library, the Keio collection. As a result, 49/71 (69%) of the Keio strains tested exhibited various degrees of motility, whereas their parental strain is poorly motile. These observations highlight the plasticity of gene expression even in the absence of preexisting regulatory programs and should raise awareness of procedures for handling laboratory strains of *E. coli*.

KEYWORDS Keio collection, evolution, flagellar gene regulation, flagellar motility, gene regulation

Motility is a highly effective strategy for bacteria to move within their environment. Although this process is important for cells to survive under nutrient-starved conditions, the cohort of proteins required for this ability are costly to produce and utilize, accounting for up to 2% of energy expenditure in *Escherichia coli* (1, 2). Unsurprisingly, motility genes in many bacteria are highly regulated to ensure both minimal expression when motility is unnecessary and ordered production when motility becomes beneficial. In *E. coli*, integration of regulatory signals primarily takes place at the *flhDC* operon, which encodes the master transcription factor $FlhD_4C_2$ that regulates the flagellar sigma factor σ^{28} gene and many other motility genes (1, 3–6). Like many

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genetic regulatory circuits in *E. coli*, the mechanisms activating and inactivating *flhDC* expression have been well characterized (7–10).

In addition to prewired regulation, genetic mutations can also lead to activation of genes that are otherwise silenced, but the dynamics of such events under changing environments are much less understood. In the case of motility gene expression, a wide array of mutations have been found upstream of *flhDC* in both wild and laboratory isolates of *E. coli* K-12 strains (11–13). In particular, many strains, including several sequenced MG1655 variants, such as CGSC 7740, carry different insertion sequence (IS) elements that perturb the regulation of *flhDC* expression, leading to dramatic differences in the ability to become motile under conditions that are typically used to study motility (11, 14, 15). In contrast, strains without IS elements upstream of *flhDC*, including other isolates of MG1655 like CGSC 6300 and a K-12 derivative, BW25113, have been shown to be poorly motile (14, 16, 17). The loss of motility in these strain raises the question of how they can adapt to detrimental conditions. It was recently found that rare motile isolates can be enriched by growing nonmotile starting cultures on soft agar plates (14, 15), suggesting that phenotypic switching may occur if the appropriate conditions are provided.

The poorly motile *E. coli* strain BW25113 served as the parent strain for the Keio collection, the library of single-gene deletion *E. coli* strains (18). Since its inception 13 years ago, the Keio collection has provided a powerful foundation for a wide array of studies, including functional genomics, experimental evolution, and synthetic biology (19–23). The collection was generated by using lambda red recombination technology to produce single gene deletions of all nonessential genes from the parent BW25113 strain (18). Although the processes of recombination and validation may generate secondary mutations, there have not been systematic reports of any genetic changes in the collection to our knowledge.

Here, we report that a large fraction of the Keio strains tested have dramatically elevated, and likely constitutive, motility gene expression and a motile phenotype compared to that of the parent strain. We have mapped this increase to direct genetic changes near the *flhDC* and *lrhA* loci, the master regulators of motility gene expression. Importantly, this shift from a nonmotile state to a motile one is not due to either the single gene deletions or lambda red recombination. Rather, simply incubating *E. coli* strains in liquid medium without shaking overnight is sufficient to select for mutations that have constitutive expression of motility genes. In contrast, nonmotile cells grown in shaking liquid medium do not undergo selection for motility. These mutations come through various means, but all collectively lead to derepression of the *flhDC* locus and increased motility. Taken together, these results demonstrate a secondary form of heterogeneity in the Keio collection and serve as another example of how rapid genetic changes can sweep through a population of bacteria in the absence of regulation.

RESULTS

Transcriptome profiling of Keio strains shows drastic differences in motility gene expression levels. We first noticed striking disparity in motility gene expression levels while performing a pilot study to map transcriptome responses to single-gene deletions in *E. coli* using the Keio collection. RNA sequencing was carried out for 71 Keio strains obtained directly from the Coli Genetic Stock Center (CGSC) grown with shaking in morpholinepropanesulfonic acid (MOPS) minimal medium (Materials and Methods). Hierarchical clustering of gene expression changes relative to the levels in the parent strain, BW25113, showed a prominent signature of upshift for flagellar σ^{28}-controlled genes in 49 of the 71 strains (Fig. 1A; see also Table S1 in the supplemental material). Among these 49 strains, the increases in motility gene expression account for most of the largest transcriptome differences with the parent strain (Fig. 1B).

To validate the transcriptomic shift, we utilized a green fluorescent protein (GFP) reporter construct driven by the promoter of *fliC,* a major component of the flagellin system, and one of the genes that show the largest increase in expression (24) (Fig. 2A). Plasmids harboring the P_{fliC}-*gfp* reporter were transformed into BW25113, our lab's

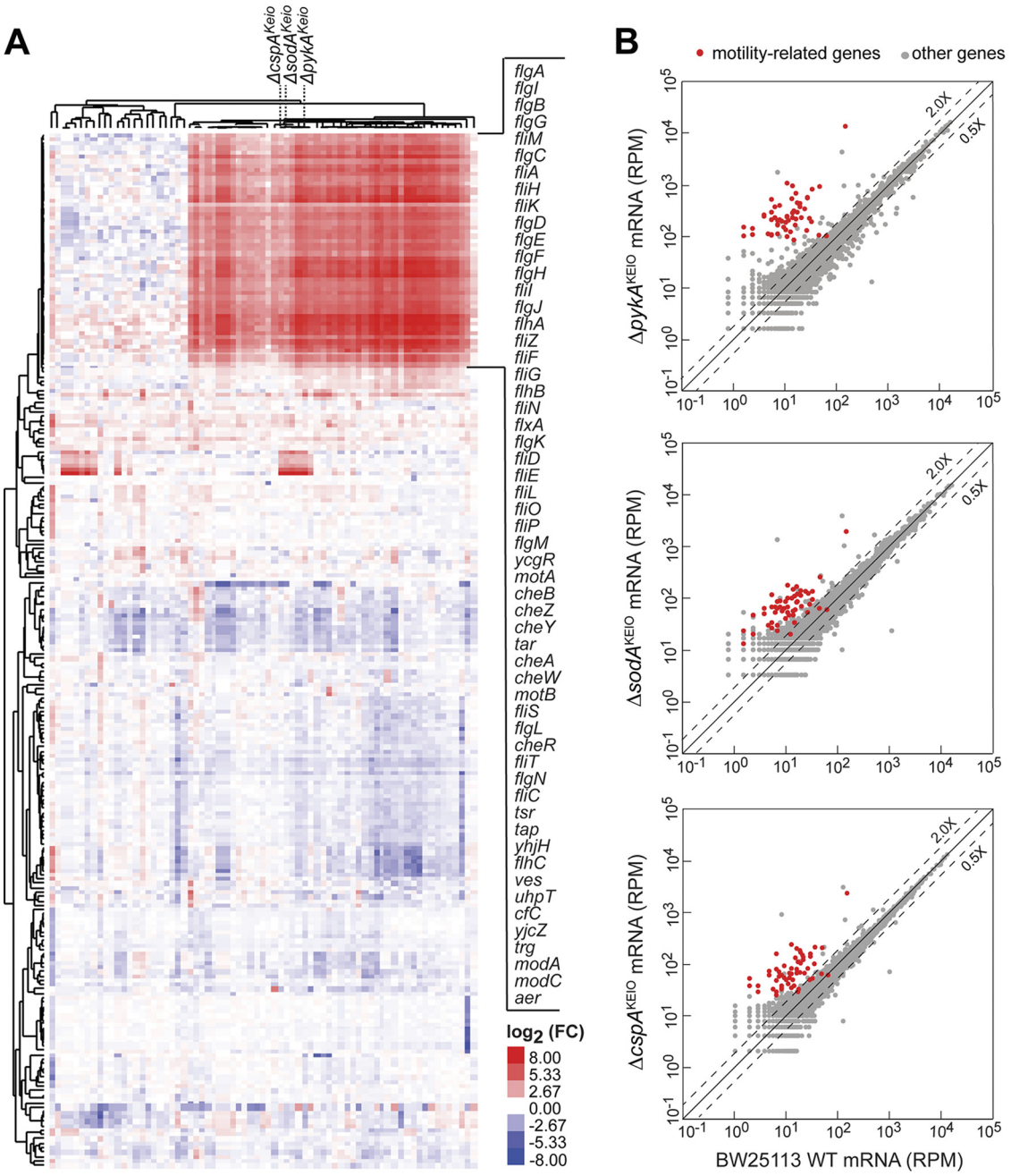


FIG 1 Transcriptome remodeling among Keio strains. (A) Hierarchical clustering of gene expression data from 71 Keio knockout strains. Columns denote strains, and rows denote genes. Data for fold change (FC) between mutants and BW25113 are $\log_2$ transformed and colored according the scale shown (inset). Uncentered Pearson correlation was used as the distance metric, and clustering was performed using complete linkage. Forty-nine strains showed highly upregulated expression of genes regulated by the flagellar sigma factor σ^{28}, as highlighted on the right of the heat map. (B) Transcriptome-wide comparisons between BW25113 (WT) and three representative Keio knockout strains highlighted in panel A. Annotated motility-related genes are shown in red.

MG1655 strain, and four representative Keio strains, three of which exhibited motility gene activation (Δ*pykA*$^{\text{Keio}}$, Δ*sodA*$^{\text{Keio}}$, and Δ*cspA*$^{\text{Keio}}$ strains). While little fluorescence was observed for the parent strain BW25113, the Keio strains with upregulated σ^{28}-controlled genes all showed elevated fluorescence from P_{fliC}-*gfp* when plated on solid LB agar (Fig. 2B). Additionally, the Δ*sodB*$^{\text{Keio}}$ strain did not show any increase in σ^{28}-controlled genes and exhibited no increase in fluorescence over its parent. As another negative control, our lab strain of MG1655 showed low GFP fluorescence, in agreement with our previous transcriptome profiling data (25). During the construction

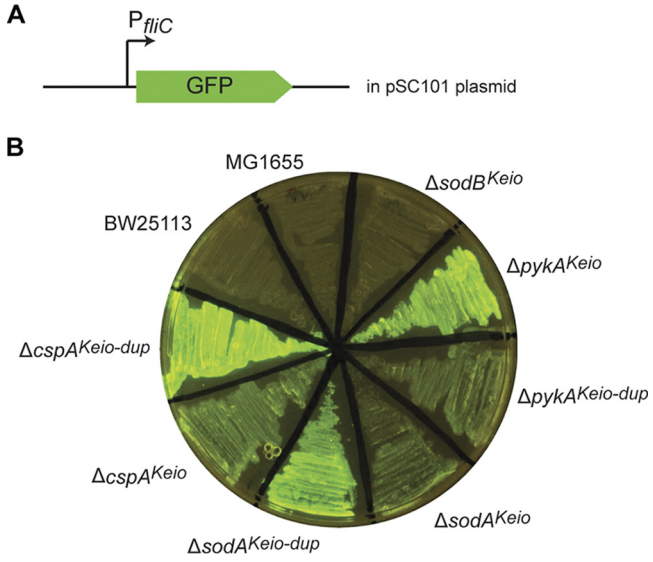


FIG 2 Confirmation of motility gene expression using a GFP reporter driven by the *fliC* promoter. (A) Schematic of the GFP reporter driven by the σ^{28}-dependent *fliC* promoter for motility gene expression. Seventy nucleotides upstream of the coding sequence of *fliC* were cloned in front of *gfp* and inserted into a low-copy-number plasmid, pSC101. (B) Fluorescence image of strains containing the P_{fliC}-*gfp* reporter. Seven Keio strains, along with BW25113 and MG1655, were transformed with the plasmid carrying the reporter construct and streaked on a 1.2% LB agar plate before exposure to UV light. The $\Delta sodB^{Keio}$ strain did not show upregulation of motility genes in RNA-seq experiments and was included as a negative control.

of the Keio collection, two independent clones were saved for each deletion (18). We found that the second (duplicate) clone from the collection ($\Delta pykA^{Keio\text{-}dup}$, $\Delta sodA^{Keio\text{-}dup}$, and $\Delta cspA^{Keio\text{-}dup}$ strains) also showed high, albeit different, levels of fluorescence when they were transformed with the plasmid containing P_{fliC}-*gfp* (Fig. 2B). Taken together, these findings suggest that a shift in motility gene expression is a common signature in a large fraction of the Keio collection even though the strains originate from the poorly motile strain BW25113.

Swimming phenotype reflects increased motility gene expression. We next examined whether the transcriptomic changes led to an observable increase in the cells' motility. Cell cultures in exponential phase were directly inoculated at the center of 0.25% soft-agar plates and grown overnight at 37°C. In comparison to the nonmotile parent BW25113 strain, the selected Keio strains from both duplicates of the collection covered a larger area on the soft-agar plate, demonstrating that these cells were able to swim further outwards from the origin of inoculation (Fig. 3A). The diameter of the swimming cultures varied across strains and was strongly correlated with the expression level of *fliC* as measured by quantitative PCR (qPCR) (Fig. 3B), and all swimming cultures exhibited greater expression than the wild type (WT). Together, these results show that the transcriptional activation of the flagellar genes directly relates to an increase in swimming ability.

Widespread secondary mutations affect the expression of motility regulators. To directly assess whether the gene deletions were sufficient to explain the motility phenotype, we transduced the deletion cassette from selected Keio strains into a wild-type BW25113 background using bacteriophage P1. Comparing the expression levels of *fliC* and *cheA*, another gene of the flagellar regulon, via qPCR, we observed that their expression levels in freshly transduced cells were similar to the levels in BW25113 (Fig. 4A). The lack of motility gene expression and phenotype in freshly transduced cells was further confirmed using the P_{fliC}-*gfp* reporter construct and soft-agar swimming assays, respectively (Fig. 4B and C). These results demonstrate that the widespread activation of the σ^{28} regulon in the Keio collection is not a direct response to gene

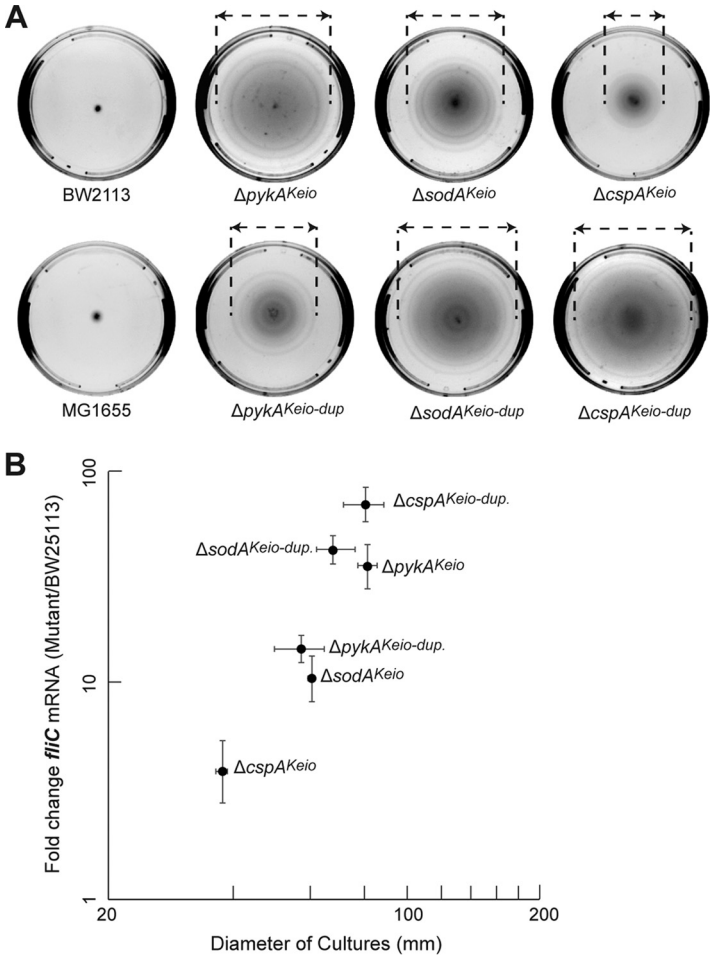


FIG 3 Correlation between motility gene activation and swimming phenotype. (A) Swimming motility assay on soft-agar plates. Plates were imaged after cells were spotted in the center and incubated for 8 h at 37°C. Dashed lines mark the measured diameter of each culture. (B) Correlation between the diameter in the motility assay and *fliC* expression level as measured by qPCR. Vertical error bars indicate the standard deviation for qPCR measurements from three technical replicates. Horizontal error bars indicate the range for diameter measurements from three biological replicates of the swimming assay.

deletion, consistent with the quantitative differences in the swimming assay between two independent mutants (Fig. 3B).

To determine the secondary mutations in the Keio strains that explain increased motility gene expression, we performed whole-genome sequencing on several strains with or without motility gene activation. Overall, there were few differences between the parent strain, BW25113, and the Keio mutants except that all of the motility-activated Keio strains contained either insertion sequence (ISs) or point mutations in the vicinity of the motility regulator genes *lrhA* and *flhD* (Fig. 5A). The only mutation found outside these regions was an intergenic mutation near an rRNA operon in the Δ*pykA*^{Keio} strain, which was also found in several other strains without motility gene activation. In contrast, no changes in either the *flhDC* or *lrhA* regulatory region were observed for a knockout strain that did not show motility gene activation, i.e., the Δ*sodB*^{Keio} strain (Fig. 2B).

We validated these mutations by performing Sanger sequencing on the corresponding genomic regions from each of the knockout strains. Sanger sequencing also confirmed that independent Keio mutants carry distinct secondary mutations in the vicinity of motility regulator genes, including in the coding region of *lrhA* (synonymous or nonsynonymous point mutation) or upstream of *flhDC* (insertion or point mutation) (Fig. 5A and B). Notably, none of the independent clones of the same gene deletion

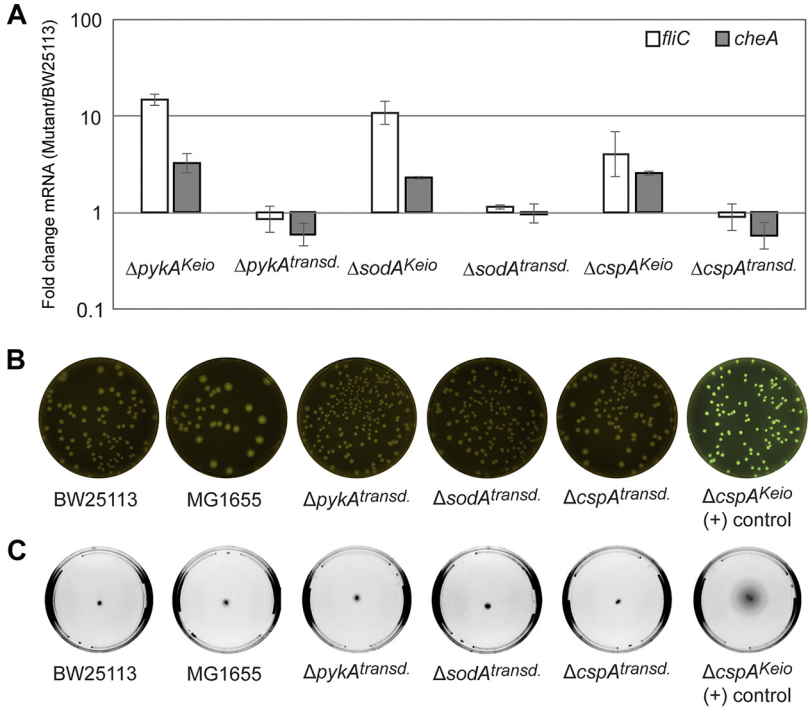


FIG 4 Absence of motility activation after P1 transduction of single-gene deletions. (A) mRNA levels of *fliC* and *cheA* as measured by qPCR for three Keio strains and three freshly transduced (transd) strains. Error bars indicate standard deviations among three technical replicates. (B) Fluorescence images of LB agar plates for freshly transduced deletion strains. The Δ*cspA*^{Keio} strain from the Keio collection was included as a positive control. (C) Swimming motility assay on soft-agar plates for the same strains used in the experiment shown in panel B.

shared identical secondary mutations. One of the strains (Δ*cspA*$^{Keio\text{-}dup}$ strain) even appeared as nonisogenic in our stock, with single colonies exhibiting differences in the sites of IS insertion (Fig. 5C). Overall, these results argue that the pressure to obtain genetic changes at *lrhA* or *flhD* is selected for at some step downstream of the initial gene deletion.

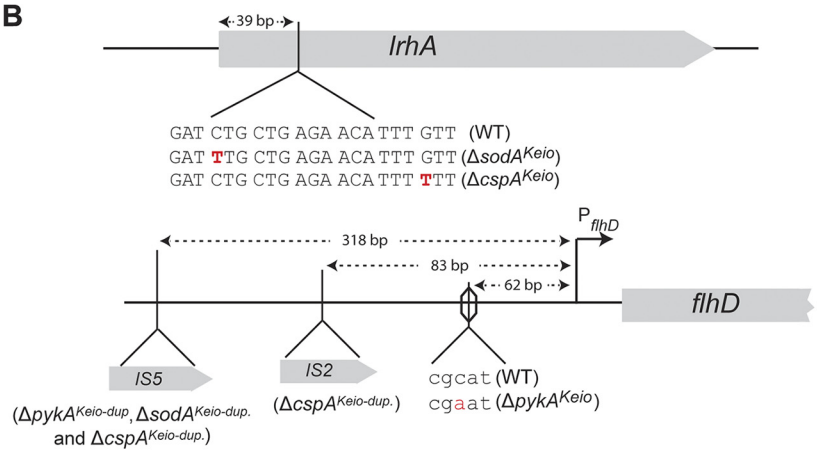
$FlhD_4C_2$ is the transcriptional activator for many motility genes, including *fliA,* which encodes σ^{28} (26). The expression of *flhDC* is repressed by LrhA (27). In our transcriptome profiling (RNA-seq) experiments, strains carrying the *lrhA* mutations (Δ*sodA*^{Keio} and Δ*cspA*^{Keio} strains) had a decreased level of *lrhA* mRNA and an increased level of *flhDC* (Fig. 5D), suggesting that these mutations decrease either the stability of *lrhA* mRNA or its transcription. On the other hand, the point mutation in the cAMP-CAMP receptor protein (CRP) binding site upstream of *flhDC* for the Δ*pykA*^{Keio} strain, which is identical to a mutation found previously in two other strains (12), led to a large increase in *flhDC* expression while having no effect on *lrhA*. In addition, we confirmed previous reports that various insertion sequences upstream of *flhDC* induce its expression but not that of *lrhA* (11–13). Overall, the plethora of secondary mutations observed that induce *flhDC* expression indicate a strong selective pressure for cell motility at some point after the knockout mutants were generated.

Rapid accumulation of motility-activating mutations in resting liquid cultures. To determine how the selection for motility arose, we first sought to re-create the conditions that led to accumulation of secondary mutations. As noted in the protocol of the original paper, during the construction of the Keio collection, independent colonies were isolated, and cultures were incubated at 37°C overnight without shaking (18). We hypothesized that this nonshaking (or resting) condition favors motile cells and therefore selects for mutants that activate *flhDC* expression. To test this hypothesis, we inoculated BW25113 and the strains that were freshly transduced with the deletion cassettes into fresh LB medium and incubated them with or without shaking overnight.

A

	ΔpykA^{Keio}	ΔpykA$^{Keio\text{-}dup.}$	ΔsodA^{Keio}	ΔsodA$^{Keio\text{-}dup.}$	ΔcspA^{Keio}	ΔcspA$^{Keio\text{-}dup.}$
lrhA mutations	None	None	L15L	None	V20K	None
flhD mutations	Point mutation upstream	Insertion upstream	None	Insertion upstream	None	Insertion upstream

B



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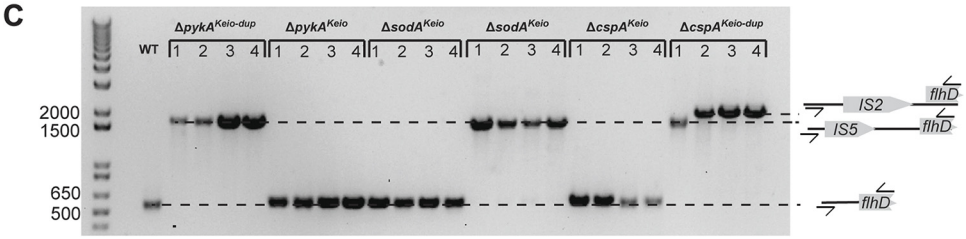

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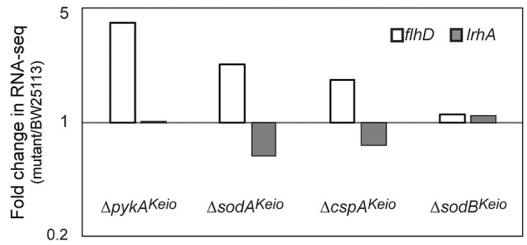

FIG 5 Diverse secondary mutations responsible for motility activation in Keio collection. (A) Mutations in the *lrhA* and *flhD* regions identified by whole-genome sequencing and targeted Sanger sequencing for selected strains in the Keio collection. (B) Schematic showing the locations and the sequence contexts of the mutations. The point mutation in the Δ*pykA*^{Keio} strain is in the cAMP-CRP binding site (hexagon). (C) PCR amplicons for the *flhD* gene confirming the addition of insertion sequences. Four single colonies for each strain were picked. The schematic next to the gel represents the insertions in this region while arrows denote the relative locations of the PCR priming sites. (D) Gene expression changes for *flhD* and *lrhA* from the original RNA-seq experiment on four presented knockout strains. The Δ*sodB*^{Keio} strain did not show any mutations or gene expression changes at either locus.

The following morning, each culture was mixed thoroughly and examined for motility phenotypes by inoculation onto fresh soft-agar plates. After another night at 37°C, cultures for all strain types incubated without shaking the first night showed a noticeable amount of swimming, while the cultures incubated with shaking did not (Fig. 6A). After 5 days of sequential overnight incubation, mixing before daily dilution, and repeated liquid growth, nonshaken cultures showed an even more prominent motility phenotype (Fig. 6A). Similar effects were observed for freshly transduced knockout strains, the parent BW25113, and MG1655.

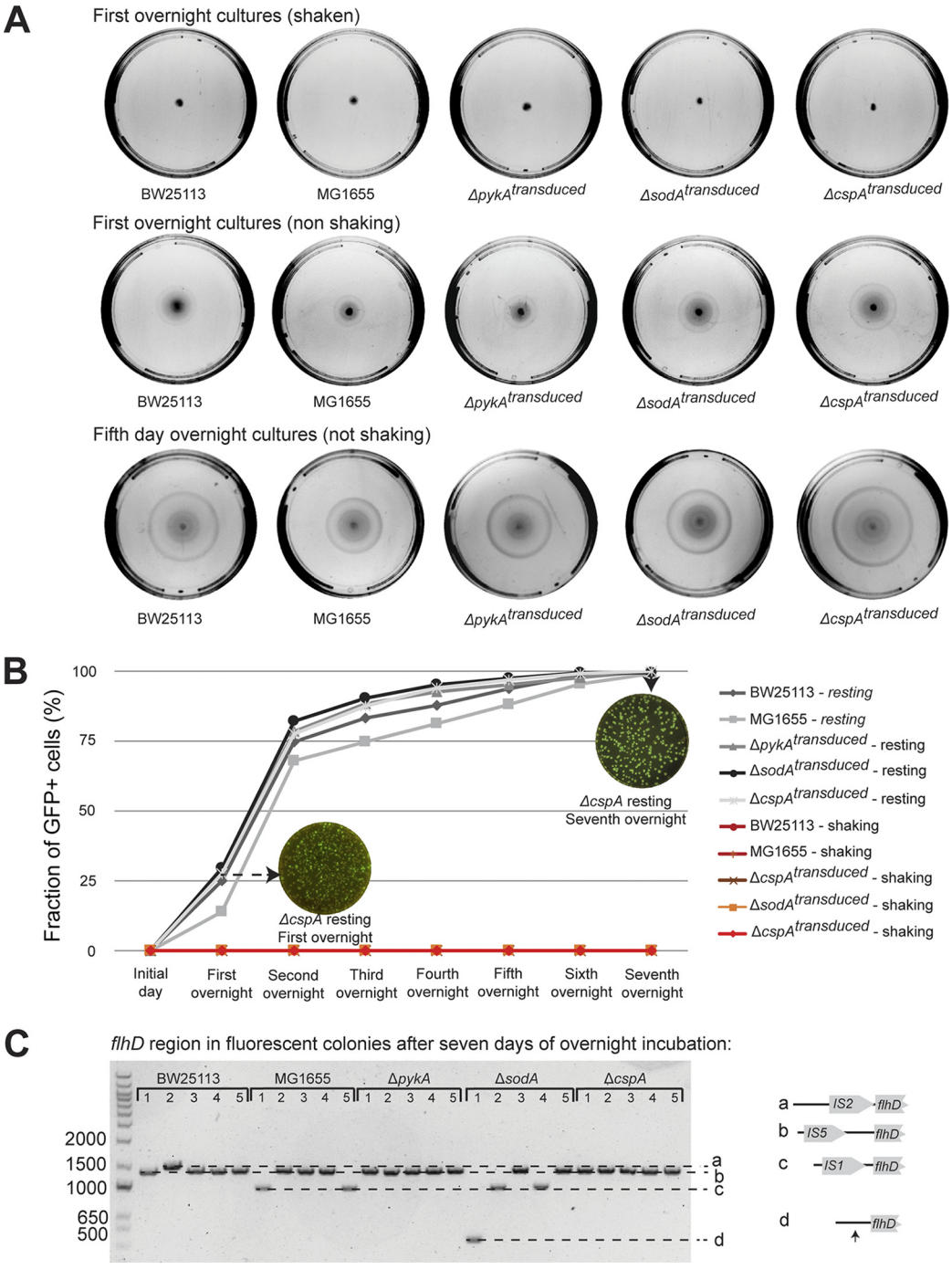

FIG 6 Rapid accumulation of motility-activating mutations in resting liquid culture. (A) Soft-agar swimming assay plates for cells cultured with or without shaking. The first two rows are from single-night overnight cultures, and the final row is from the fifth day of successive overnight cultures. (B) Fraction of motility-activated colonies as a function of time. The P_{fliC}-*gfp* construct was transformed into freshly transduced knockout cells. After every overnight incubation, a fraction of cells was diluted, plated, and counted. Insets show representative fluorescence images of hard-agar LB plates for the Δ*cspA*$^{\text{transduced}}$ strain after the first day and the seventh day of incubation without shaking. (C) PCR amplicons for the *flhD* neighborhood of fluorescent colonies showing a diverse mixture of insertions and a point mutation in each culture. The schematic on the right represents the insertions found by sequencing. The arrow corresponds to a point mutation.

To quantify and compare the rates of mutation accumulation in different backgrounds, we used the P_{fliC}-*gfp* reporter construct to examine individual colonies after incubation. We incubated the freshly transduced deletion strains containing the reporter overnight in LB medium under either shaking or resting conditions. After each

overnight incubation, an aliquot was plated on LB agar to count the fraction of fluorescent cells, and another aliquot was diluted into liquid culture to start a new round of overnight incubation. We continued this experiment for 7 days. In order to ensure that we were not artificially selecting for motile cells while plating, the overnight culture was mixed vigorously for 1 min prior to plating.

After the first overnight incubation, the fraction of fluorescent colonies from nonshaken cultures accumulated to >25% for all BW25113-derived strains and to about 18% for MG1655 (Fig. 6B). Over subsequent days, BW25113 and freshly transduced single-gene deletion strains followed similar trajectories, with the entire population becoming motile by the 7th overnight. In contrast, we did not detect any fluorescent colonies from any of the shaken cultures, indicating that the frequency of motile mutants was below our detection limit (0.8%) in these populations.

Analysis of individual fluorescent colonies revealed a large array of mutations in each resting culture. We sequenced the *flhD* region for 25 total colonies (five from each of the 7th-day cultures) and found mutations in all of them. The mutations included an IS*5* insertion, an IS*2* insertion, an IS*1* insertion, and a point mutation close to the coding region of *flhD*. All but one plate had more than one distinct mutation, indicating diverse composition after the 7th day. These findings suggest that the resting condition uniquely contributes to the accumulation of motile mutants and further highlights that the single-gene deletions do not appear to play a role in the frequency of this accumulation.

DISCUSSION

In this study, we found that a large fraction (69% of 71 strains tested) of the *E. coli* strains in the single-gene deletion Keio collection have upregulated the expression of their motility genes. This switch to a motile state is not due to the deleted genes but, rather, to secondary mutations accumulated after growth in resting liquid cultures. Nonshaking liquid cultures that start with nonmotile strains such as BW25113 or MG1655 begin to show subpopulations of motile mutants after just a single overnight incubation. These motile mutants are accompanied by a variety of genetic changes that culminate in the increased expression of the master regulator of motility, FlhDC. Importantly, this genotype and phenotype switch was not observed for the same strains grown in liquid culture with shaking. Moreover, the fractions of motile mutants were similar between the knockout strains and the wild-type parent strain BW25113, suggesting that the accumulation of secondary mutations is not dependent on the three genes that are deleted. Therefore, the resting condition is sufficient to cause accumulation of motility-activating mutations in *E. coli*.

The nature of these mutations demonstrates another example of how bacterial cells adapt their transcriptome to changing environments by rapidly rewiring regulatory networks. The most common way we observed this rewiring occurring in our data was through the presence of IS elements upstream of the *flhDC* operon (Fig. 5A to C and Fig. 6C). IS elements are abundant in the *E. coli* K-12 genome and are frequently associated with mutation events (3.5×10^{-4} insertions per genome per generation and 4.5×10^{-5} recombinations per genome per generation), especially in certain regions of the genome, such as the *flhDC* neighborhood (12, 13, 28). It remains to be fully determined whether the rate of IS-mediated gene activation is subject to environmental control.

The possibility that stress conditions trigger directed rearrangement of insertion elements, and specifically at *flhDC,* has been discussed in the literature (14, 15, 29, 30). Two potential models exist to explain how the mutations arose. In the first model (Larmarckian), the resting condition itself leads to the formation of secondary mutations by triggering the transposition of insertion sequences or increasing the mutation rate. The Larmarckian model has been suggested in previous work on mutations causing *flhDC* activation on soft-agar plates (14, 15). Although it was shown that the accumulation of mutations requires functional flagella, no direct molecular mechanisms leading to the increased transposition or mutation rate at *flhDC* or *lrhA* have been

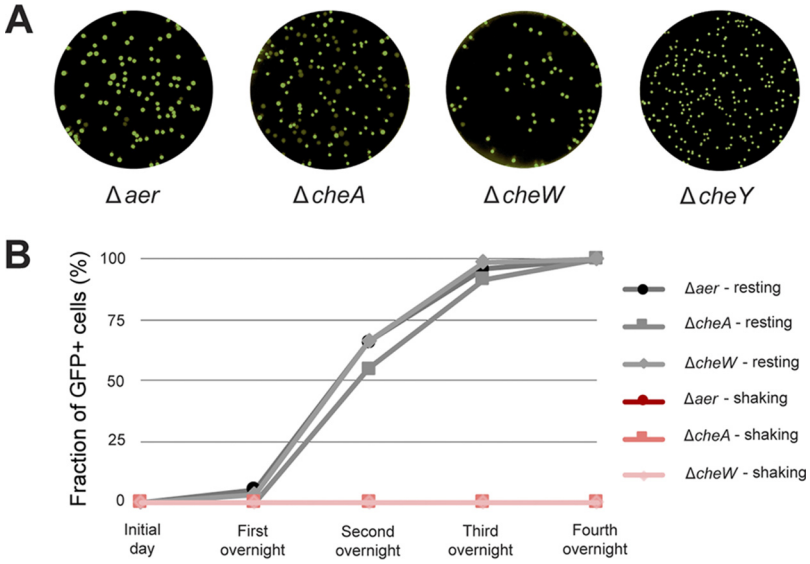

FIG 7 Motility gene activation in strains deficient for aerotaxis and chemotaxis. (A) Transformation results for Keio Δ*aer*, Δ*cheA*, Δ*cheW*, and Δ*cheY* deletion strains expressing the P_{fliC}-*gfp* construct. Transformants for P_{fliC}-*gfp* were selected for on 100 μg/ml ampicillin-LB agar selection plates, and plates were imaged under blue light. (B) Fraction of motility-activated colonies as a function of time. Nonfluorescent colonies of Keio Δ*aer*, Δ*cheA*, and Δ*cheW* strains transformed with P_{fliC}-*gfp* were isolated and grown overnight in shaking or resting medium. After every overnight incubation, a fraction of cells were diluted, plated, and counted. Another fraction was back-diluted into fresh shaking or resting medium to continue the experiment.

uncovered. In the second model (Darwinian), mutants are either preexisting in the cultures at low frequencies or spontaneously arise under the resting condition. Importantly, our data do not distinguish between these two possible scenarios.

In a nonshaking culture, it is plausible that aerotaxis or chemotaxis provides a selective advantage by allowing motile cells to reach the liquid-air interface and is required for the increase in the motility phenotype in the population (31, 32). Interestingly, we do not find this to be likely as Keio knockout strains for *aer*, *cheA*, *cheW*, and *cheY*, regulators for aerotaxis and chemotaxis, show a large fraction of GFP-positive cells when strains are transformed with the P_{fliC}-*gfp* construct (100% for the Δ*cheY* strain) (Fig. 7A). We isolated nonfluorescent colonies of Δ*aer,* Δ*cheA,* and Δ*cheW* cells with the P_{fliC}-*gfp* construct and found that after a single overnight in resting liquid culture, individual isolates began to express GFP. Over sequential overnights in resting culture, the populations became dominated by GFP-positive cells, similar to results for all previously tested strains (Fig. 7B). Therefore, it remains to be determined what the exact origin of the fitness advantage is and what pathways are required for imparting this advantage.

Finally, an important implication of this work is that common laboratory practices can rapidly lead to undesired mutations in *E. coli*. The propensity to accumulate motility-activating mutations during nonshaking overnight incubation could contribute to the widespread secondary mutations that we identified in the Keio collection as well as to the diversity of motility phenotypes in the MG1655 strains from different laboratories (11). Whereas the 71 Keio strains that we profiled were obtained directly from the CGSC, independent and subsequent distributions in and between laboratories may have led to additional secondary mutations that did not arise from the initial library construction. Because of these genomic changes, it is possible that phenotypes observed in Keio knockout strains are not due solely to the genes deleted but are compounded with the motility activation. We therefore urge caution in direct comparisons between Keio strains without P1 transduction since this motility activation adds an additional layer of heterogeneity to the collection. Taken together, these results should inform proper laboratory practices as even a single night of incubation of

resting cultures can lead to robust and unwanted genetic changes that are not immediately obvious.

MATERIALS AND METHODS

Strains. The Keio strains for RNA-seq, including the three strains that we screened individually, denoted as the Δ*pykA*^{Keio}, Δ*sodA*^{Keio}, and Δ*cspA*^{Keio} strains, were obtained directly from the Yale *E. coli* Genetic Stock Center. These strains were also the parent strains for the transduced Δ*pykA*^{transduced}, Δ*sodA*^{transduced}, and Δ*cspA*^{transduced} strains. The duplicate strains as well as the Keio Δ*aer*, Δ*cheA*, Δ*cheW*, and Δ*cheY* deletion strains were obtained from the Baker lab at Massachusetts Institute of Technology (MIT).

RNA sequencing. MOPS minimal medium (M2106; Teknova) was used as the liquid medium for all gene expression analysis (RNA-seq and qPCR). Overnight cultures of the Δ*pykA*^{Keio}, Δ*sodA*^{Keio}, and Δ*cspA*^{Keio} strains were back-diluted 1,000-fold into 10 ml of fresh MOPS minimal medium and grown to an optical density at 600 nm (OD_{600}) of 0.3. Five milliliters of cells was harvested and mixed with equal parts of ice-cold methanol, spun down for 10 min, and decanted before freezing at −80°C. Cells were then lysed with 10 mg/ml lysozyme, and RNA was extracted using an RNeasy column (no. 74104; Qiagen). Purified RNA was rRNA depleted with a MICROBExpress bacterial mRNA enrichment kit (AM1905; Thermo Fisher) according to the manufacturer's instructions. The remaining RNA was fragmented (AM8740; Thermo Fisher), dephosphorylated, polyadenylated, and reverse transcribed using Superscript III and custom oligo(dT) reverse transcription primers. The resulting cDNA was ligated with Illumina sequencing adapters and PCR amplified. Single-end (50 bp) sequencing was performed on an Illumina HiSeq 2000 system.

Hierarchical clustering. We used Cluster, version 3.0, software to perform complete linkage hierarchical clustering with uncentered Pearson correlation on the RNA-seq data. For preprocessing, we first selected genes with count values of over 100 under at least one condition. We further filtered data for genes that showed a minimum 2-fold change under at least one of the conditions. This method filtered out 4,065 genes. We then logarithmically transformed (base 2) fold change values in the expression of the remaining 235 genes between the 71 Keio mutants and BW25113. Java TreeView software was used to visualize the cluster.

GFP reporter construct. Using Gibson assembly, the promoter region of *fliC* and the coding region of green fluorescent protein were fused and cloned into pSC101 (33). The promoter region of *fliC* is identified as a 70-bp-long region upstream of the *fliC* coding sequence. The sequence and plasmid are available through Addgene (pGL001).

Overnight cultivation with and without shaking of strains with a GFP reporter construct. Two independent 10-ml LB cultures were started in 125-ml flasks for each strain transformed with the GFP reporter construct. One set was shaken at 250 rpm on a benchtop shaker, and the other set was kept on a shelf without shaking at 37°C for 16 to 20 h. At the end of the 16- to 20-h duration, 1 ml was taken from each culture, diluted, and plated on 100 μg/ml ampicillin-LB selection plates. Plates were imaged under blue light, and the fraction of colonies that showed any fluorescence was determined by counting for each plate. After initial overnight incubation (16 to 20 h), liquid cultures were diluted 1:200 to continue with a new day of overnight cultures.

qPCR. qPCR experiments were run on a Roche LC480 machine to measure RNA levels of the *fliC* flagellar gene and the *cheA* chemotaxis gene for all strains used in this study. Strain growth and RNA collection were completed as described in "RNA sequencing." Random hexamer reverse transcription with Moloney murine leukemia virus (M-MuLV) (M0253; NEB) was performed according to the manufacturer's specifications. The standard deviation (SD) of the fold change was calculated by propagating the SD from each measurement. *hcaT* was used as a loading control for each sample (34). The primer sequences used for qPCR were as follows: *fliC*, GTTCTATCGAGCGTCTGTCTTC (forward) and ATACCGTC GTTGGCGTTAC (reverse); *cheA*, CAAGAACAGCTCGACGCTTA (forward) and GTTTCGCCTTTCGCTTCTAATG (reverse); *hcaT*, GCTGGTGATGATTGGCTTTA (forward) and GTAATCAAGCGGGAACTGCT (reverse).

Soft-agar swimming assays. Semisolid motility plates were prepared using tryptone broth (13 g of Bacto tryptone and 7 g of NaCl per liter of medium) and 0.25% Difco agar. Plates were prewarmed to 37°C prior to inoculation. , Plates were poked with a pipette tip and inoculated with 3 μl of cell culture grown to an OD_{600} of 0.1 in 10 ml of tryptone broth in flasks. Plates were incubated in 37°C for 8 h and imaged on a Bio-Rad Gel Doc XR+ gel documentation system (no. 1708195). Culture diameters were measured using ImageJ software and normalized to the diameter of the petri dishes.

Swimming assays for overnight cultures. Two sets of liquid cultures were started for all strains in 10 ml of tryptone broth. One set was shaken at 250 rpm continuously while the other set was kept on the shelf without shaking at 37°C for 16 to 20 h. At the end of the 16- to 20-h duration, 1 ml was taken from each culture and diluted to an OD_{600} of 0.1 and plated for soft-agar swimming assays as explained above. Prior to plating, the resting cultures were shaken at 250 rpm for a minute.

After each day of overnight incubation, liquid cultures were diluted 1:200 to continue with a new day of overnight cultures.

P1 transduction. To generate fresh deletion strains, genomic DNA from the Keio strains was transduced into wild-type BW25113 strains using bacteriophage P1 and selected on LB agar plates with 50 ng/μl kanamycin. For transduction, the protocol outlined by Thomason et al. was used (35). The lysates for each fresh deletion were prepared from the strain directly obtained from CGSC. For example, the Δ*pykA*^{transduced} strain was made from Δ*pykA*^{Keio} strain lysate. Knockouts were validated by amplifying the regions surrounding the deletion of interest and confirming the gene replacement by a kanamycin cassette.

Whole-genome sequencing. DNA from overnight shaking liquid cultures of the Δ*pykA*^{Keio}, Δ*sodA*^{Keio}, Δ*cspA*^{Keio}, Δ*sodB*^{Keio}, and BW25113 strains was extracted with a Promega Wizard genomic DNA purification kit (A1120) according to the manufacturer's protocol. Samples were prepared using a Nextera XT DNA library prep kit (Illumina) and paired-end sequenced (150 bp plus 150 bp) on an Illumina MiSeq system (version 2 chemistry). Sequencing data were analyzed for mutations using the breseq package using BW25113 as the reference strain (36).

Sanger sequencing. Regions of interest in the *lrhA* and *flhD* neighborhood for all six Keio strains studied were amplified using NEB Q5 high-fidelity polymerase and PCR. PCR conditions were as follows: 30 s of initial denaturation at 98°C, 35 cycles of amplification for 20 s at 65°C, and 2 min of final extension at 72°C. Primers used for PCR were as follows: *flhD* region, GTTGATGTCATAAATGTGTTTCAGCAAC (forward) and CATTAACATTAAGTTGATTGTTGCCTTTCTTTG (reverse); *lrhA* region, CGCGATTTAACAGGAA AGGTAAGATC (forward) and CAAACACGCGCATGTCATAAATAG (reverse).

Data availability. RNA sequencing data are available at Gene Expression Omnibus under accession number GSE129161. Whole-genome sequencing data are available in the NCBI Sequence Read Archive (SRA) under accession number PRJNA530228.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00259-19.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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D.J.P., P.D., and G.-W.L. conceived the study and wrote the manuscript. D.J.P. designed and completed the DNA and RNA sequencing. P.D. performed the qPCR, overnight incubation, and swimming assays. G.-W.L. supervised the project.

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