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High-Frequency Secondary Mutations after Suicide-Driven Allelic Exchange Mutagenesis in Extraintestinal Pathogenic *Escherichia coli*

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Frequent unintended secondary mutations occurred in extraintestinal pathogenic *Escherichia coli* strains CP9, CFT073, and RS218 during suicide plasmid-mediated, putatively specific deletions of *hlyA*, *papG* allele III, and *iha*. Pulsed-field gel electrophoresis and PCR analyses demonstrated genomic alterations and/or unintended loss of defined virulence genes (*papG*, the F7-2 *papA* allele, *iutA*, *sat*, *hlyD*, and *cnf*). Caution is warranted when attributing the observed phenotypic changes to the intended mutation.

Background. Bacteria differing only for a particular gene are often used to assess the phenotypic significance of specific genes (5–7, 10, 16, 21, 22). One technique for producing isogenic *Escherichia coli* mutants involves introducing a replication-defective suicide plasmid containing a modified version of the target gene into a wild-type strain. Derivatives with a mutant allele exchanged for a wild-type allele are isolated through serial selection and screening steps (8, 23). Mutant validation is usually limited to confirming the intended allelic exchange. Here we describe inapparent secondary mutations in allelic exchange mutants created in different laboratories and wild-type strains by contemporary suicide plasmid methods.

Strains. CP9 (O4:K10/54/96:H5), CFT073 (O6:K2:H1), and RS218 (O18:K1:H7), wild-type pathogenic *E. coli* strains (1, 11, 13, 14, 23, 26–28), contain established or putative virulence genes, such as *pap* (P fimbriae), *hly* (hemolysin), *cnf1* (cytotoxic necrotizing factor 1), *iha* (iron-regulated gene homologue adhesin), *sat* (secreted autotransporter toxin), *iutA* (aerobactin system), and *malX* (pathogenicity-associated island [PAI] marker) (20). UPEC76 is a double *pap* mutant of CFT073 (23).

Mutagenesis. The two CP9 *hlyA* genes, encoded on separate PAIs (18), were targeted for mutation (in the laboratory of H.A.L., Ohio State University, Columbus, Ohio) by using pWAM1746, a derivative of the sucrose-counterselectable suicide vector pCVD442 (24). The *hlyA*::Kan^r mutation from pWAM1746 was introduced into one copy of *hlyA* in CP9. In a second round of allelic exchange targeting the second CP9 *hlyA* gene, selection for mutants with two copies of *hlyA*::Kan^r yielded derivatives such as WEX139 (Table 1). Additionally, the Kan^r gene in pWAM1746 was replaced with a chloramphenicol resistance (Chl^r) gene; this construct was used to generate *hlyA*::Kan^r *hlyA*:Chl^r derivatives (e.g., WEX404 in Table 1). Putative double *hlyA* mutants were screened for antibiotic resistance, hemolysin, and CNF1 production (25) and were examined in Southern blots (30) for *hlyA*, *cnf1*, and pCVD442 sequences (8).

The *cnf1* gene of CP9 was targeted for allelic exchange (by H.A.L.) by using a pSC101-based suicide plasmid and was extensively confirmed (25).

To delete papG alleles I and III in strain CP9, in-frame deleted versions of these genes were ligated separately into suicide vector pCVD442 and the resulting plasmids were introduced into a nalidixic acid-resistant CP9 derivative (CP9Nal^r) in the laboratory of J.R.J. (University of Minnesota, Minneapolis). Dual antibiotic selection yielded the desired integrants. After nonselective growth, derivatives having resolved the plasmid were selected on 5% sucrose agar without sodium chloride. PCR using flanking and internal *papG* primers confirmed allele exchange. A double *papG* mutant was created in a *papG* allele I single mutant by using the *papG* allele III suicide plasmid, described above.

papG allele III of a Nal^r RS218 derivative (RS218Nal^r) was deleted (by J.R.J) as described for CP9Nal^r.

iha was deleted (in the laboratory of P.I.T., University of Washington, Seattle) from CFT073Nal^r, a Nal^r CFT073 derivative. An in-frame-deleted version of *iha* was ligated into pCVD442, yielding the construct used to derive allelic exchange *iha* mutants of CFT074Nal^r, as described above for *papG* in CP9Nal^r. Exchange was confirmed by PCR with internal and flanking *iha* primers. *iha* was similarly deleted from UPEC76.

Extended virulence genotypes. Isolates were tested in duplicate for 35 virulence-associated markers by multiplex PCR (19, 20).

PFGE. *Xba*I- with or without *Avr*II-generated macrorestriction profiles were examined by pulsed-field gel electrophoresis

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TABLE 1. Characteristics of selected *E. coli* parent strains and their putatively isogenic derivatives

Vector-strain combination	Parent (laboratory)	Strain(s)[a]	Immediate parent	Intended knockout	Secondary mutation(s)[b]	PFGE profile[c]	
						*Xba*I	*Avr*II
NA[d]	CP9 (H.A.L.)	CP9	NA	NA	NA	1	ND
1		WEX135	CP9	*cnf1*	None	1	ND
2[e]		WEX139	CP9	*hlyA* (2nd copy)	*cnf1*	1	ND
3[e]		WEX404	CP9	*hlyA* (2nd copy)	*cnf1*, *papG* allele III, PFGE shift	2	ND
NA	CP9 (J.R.J.)	CP9Nal^r	NA	NA	NA	1	ND
4		JJ969-JJ971	CP9Nal^r	*papG* allele III	None	1	ND
5		JJ972-JJ975	CP9Nal^r	*papG* allele I	None	1	ND
6		JJ985-JJ987	JJ972	*papG* alleles I + III	None	1	ND
6		JJ988, JJ989	JJ972	*papG* alleles I + III	PFGE shift	2	ND
NA	CFT073 (P.I.T.)	CP9Nal^r	NA	NA	NA	1	1
7		JJ1079, JJ1091, JJ1094	CP9Nal^r	*iha*	None	1	1
7		JJ1074–5, JJ1078, JJ1080	CP9Nal^r	*iha*	F7-2 *papA* allele, *iha* flanking regions, *sat*, *iutA*, PFGE shift	3	3
7		JJ1073, JJ1076–7	CP9Nal^r	*iha*	*hlyD*, F7-2 *papA* allele, *iha* flanking regions, *sat*, *iutA*, PFGE shift	2	2

[a] Mutants analogous to JJ988 and JJ989 were obtained with attempted deletion of *papG* allele III in RS218Nal (vector -strain combination 8), and mutants analogous to JJ1074–5, JJ1078, and JJ1080 were obtained with attempted deletion of *iha* in UPEC76 (vector-strain combination 9).
[b] As detected by PCR in extended virulence marker screening (with or without Southern blot confirmation) and/or PFGE.
[c] PFGE profile designations are specific to the particular parent strain (always designated as pattern 1) and its derivatives from a particular laboratory and do not apply across parent strains or laboratories. ND, not done.
[d] NA, not applicable.
[e] Different *hlyA* suicide plasmid constructs were used to generate WEX139 and WEX404.

(PFGE) (performed according to the 1998 Centers for Disease Control and Prevention training course "Standardized molecular subtyping of foodborne bacterial pathogens by pulsed-field gel electrophoresis"). Altered profiles were confirmed in duplicate.

Findings. In six of the nine unique (host strain-target gene-suicide vector) combinations, unintended nonparental genotype alterations were observed (Table 1 and Fig. 1 to 3).

cnf1 was deleted from strain CP9 without detectable secondary mutations (WEX135 in Table 1 and Fig. 1), but WEX139 (intentionally deleted of *hlyA*) neither produced CNF1 nor contained *cnf1* by Southern hybridization (data not shown) or PCR analysis (Table 1). Also, its PFGE profiles were nonparental (Fig. 1).

Forty-six (6%) of 800 second-round derivatives (e.g., WEX404), created with a Chl^r derivative of pWAM1746, were phenotypically Kan^r Chl^r Amp^s Hly$^-$ as expected. However, like WEX139, each was CNF1$^-$ and contained no *cnf1* by Southern hybridization. These isolates also lost *papG* allele III (Table 1) and exhibited altered PFGE profiles (Fig. 1).

papG alleles I and III were deleted individually from CP9Nal^r without detectable secondary mutations (Table 1). However, deletion of *papG* allele III from an allele I single mutant (JJ972), although producing several apparently isogenic double *papG* mutants (e.g., JJ985 to -987), also yielded derivatives (e.g., JJ988 to -989) with altered *Xba*I PFGE profiles (Fig. 2) despite retaining their virulence genotype (Table 1). PFGE alterations with retained virulence genotyping also occurred with attempted deletion of *papG* allele III from RS218Nal^r (data not shown).

Attempted deletion of *iha* in strain CFT073Nal^r yielded *iha* mutants with the parental PFGE profile (e.g., JJ079, etc.); these accounted for approximately 25% of colonies from plates containing no salt or sucrose. Except for *iha*, such derivatives exhibited preserved virulence profiles, including *iha* flanking sequences. However, an approximately equal number of derivatives (e.g., JJ1074, etc.) also lost *iha* flanking sequences, *iutA*, *sat*, and the F7-2 *papA* (structural subunit) allele and exhibited altered PFGE profiles (Table 1 and Fig. 2 and 3). Additional isolates (e.g., JJ1073) were nonhemolytic, having lost *hlyD* in

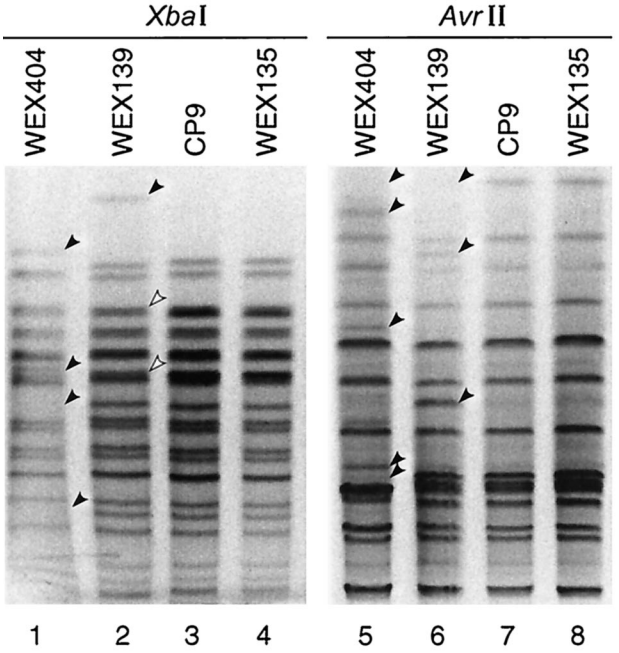

FIG. 1. PFGE profiles of strain CP9 and selected derivatives. Lanes 1 to 4 contain *Xba*I digests, and lanes 5 to 8 contain *Avr*II digests. Lanes: 1 and 5, WEX404 (*hlyA cnf1 papG* allele III); 2 and 6, WEX139 (*hlyA cnf1*); 3 and 7, CP9 (parent); and 4 and 8, WEX135. Arrowheads indicate positions of profile differences between parent and derivatives. Solid arrowheads indicate complete loss or gain of band, and open arrowheads indicate diminished intensity of band.

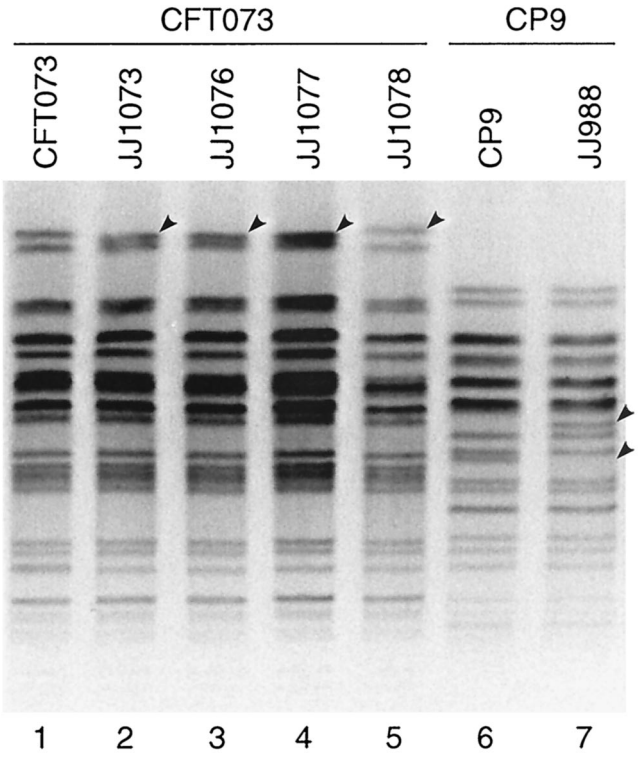

FIG. 2. *Xba*I PFGE profiles of strains CFT073Nal^r, CP9Nal^r, and selected derivatives. Lanes: 1, CFT073Nal^r; 2 to 4, JJ1073, JJ1076, and JJ1077, respectively (all *iha iutA hlyD* mutants of CFT073Nal^r); 5, JJ1078 (*iha iutA* mutant of CFT073Nal^r); 6, CP9Nal^r; 7, JJ988 (double *papG* mutant of CP9Nal^r). Arrowheads indicate positions of profile differences between parents and respective derivatives.

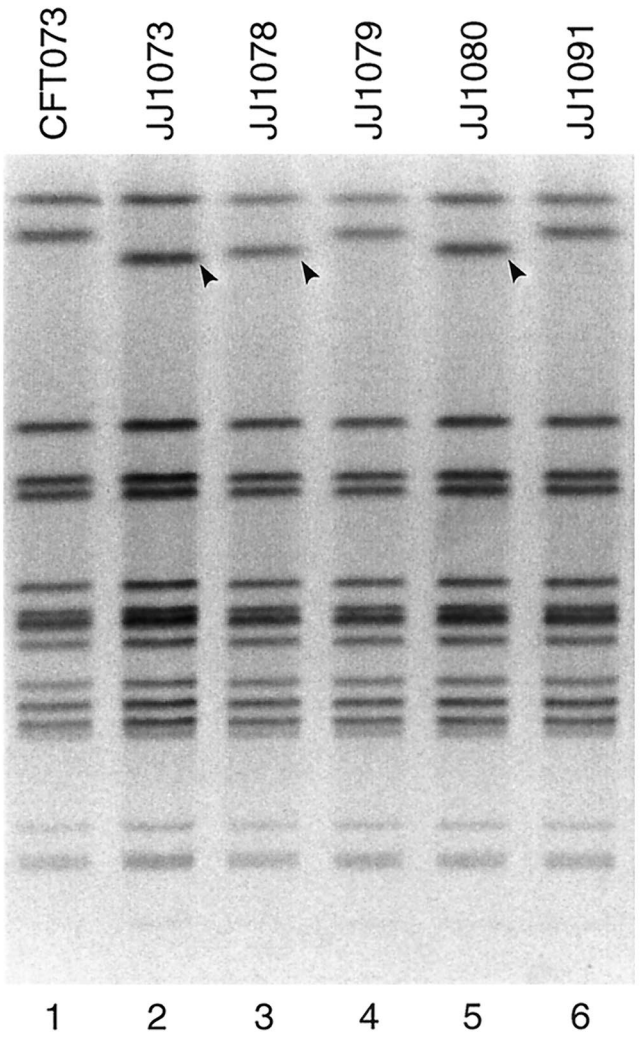

FIG. 3. *Avr*II PFGE profiles of strains CFT073Nal^r and selected derivatives. Lanes: 1, CFT073Nal^r; 2, JJ1073 (*iha iutA hlyD* mutant of CFT073Nal^r); 3 and 5, JJ1078 and JJ1080, respectively (both, *iha iutA* mutants of CFT073Nal^r); 4 and 6, JJ1079 and JJ1091, respectively (both, *iha* mutants of CFT073Nal^r). Arrowheads indicate positions of profile differences between parent and derivatives.

addition to the inadvertent deletions described above, and exhibited yet different PFGE profiles (Table 1 and Fig. 2 and 3). Downward shifts in the second-highest band in the *Avr*II profiles were more pronounced for the latter than for the former group of mutants, consistent with larger deletions (Fig. 3). The orientation of the corresponding genes within the *pheV* PAI of CFT073 suggested possible large deletions flanking *iha*, with minimum estimated losses of 26.6 and 35.6 kb, respectively, for deletions II and III (Fig. 4).

Attempted excision of *iha* from UPEC76 also yielded *iutA iha* double mutants (not shown).

Implications of findings. The high frequency of unanticipated, inadvertent, secondary mutations involving shifts in PFGE patterns and loss of other known virulence loci when often-employed suicide vectors are used is of concern, because the central tenet of allelic exchange is that the resulting phenotypic differences are specifically attributable to the target mutation. However, if additional mutations result, they, and not the intended mutation, may cause the differences. This is particularly problematic when secondary mutations involve known or putative virulence genes and the phenotype of interest is virulence (10).

The high frequency of secondary mutations using unrelated strains and a diversity of target alleles suggests that inadvertent mutations may be common with pCVD442 and, perhaps, other allelic exchange systems. It should be noted that we targeted genes within PAIs, which are structures with a propensity for spontaneous deletions and rearrangements (3, 4, 12, 29, 32), and the unintentionally deleted genes occurred within the same PAIs. We speculate that aberrant integration and/or excision of plasmids caused changes in neighboring DNA, analogous to perturbations in genomic architecture in *E. coli* K-12 when lysogenized by a Shiga toxin 2-encoding bacteriophage (17). The patterns of virulence gene loss support this hypothesis with CFT073, for which the structure of the relevant PAI is defined (Fig. 4).

Exclusion of inadvertent secondary mutations, short of whole-genome sequencing (2, 32) or other methods that detect small interchromosomal differences (31), poses challenges. That PFGE detected all secondary mutants recommends this modality. Alternatively, multiple independent deletants for the same target gene could be prepared, possibly even in different

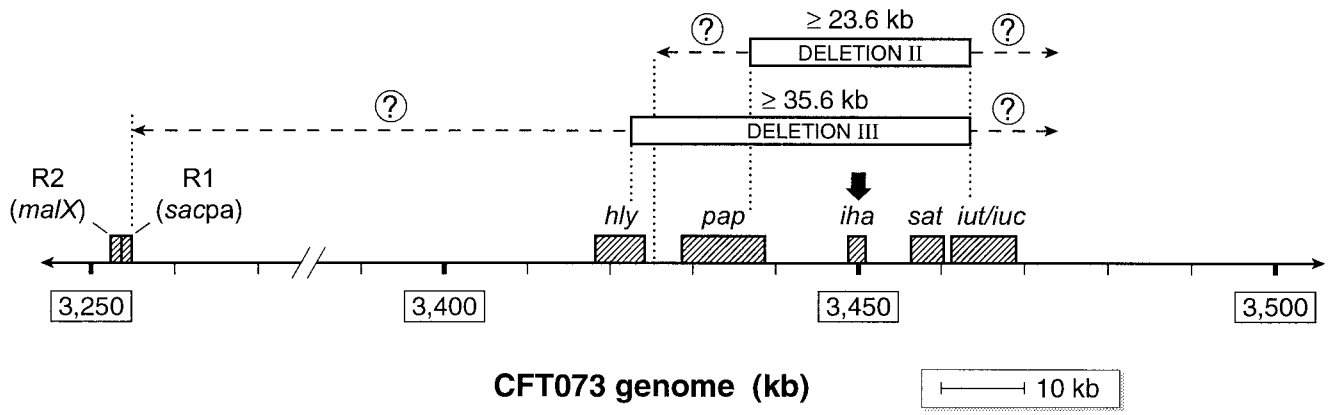

FIG. 4. Map of the regions of the CFT073 genome adjacent to *iha.* Positions of relevant genes (hatched boxes) are shown according to their coordinates in the CFT073 genome. Open boxes represent putative deletions involving *iha* (downward arrow) and flanking regions. Deletion I *iha* mutants (not shown) exhibit only an internal deletion within *iha*. Deletion II *iha* mutants also have lost the F7-2 *papA* allele, *iha* flanking sequences, *sat*, and *iutA*. Deletion III mutants have lost all these markers plus *hlyD*. All mutants retain R2 (*malX*) and R1 (*sac*pa). Dashed arrows indicate possible extension of deletions, and dotted vertical lines indicate known minimal or maximal boundaries of deletions.

wild-type backgrounds (25), subjected to confirmation of retention of a panel of non-target loci, and then tested in various systems to confirm uniformity of effect.

Complementation of presumed isogenic in-frame mutants would also help corroborate specific attribution of effect (10), but this approach too is imperfect, because gene regulation and copy number may significantly influence phenotype (9, 15, 25), although low-copy-number vectors may help (15). Alternatively, the wild-type allele could be restored to the genome at the native locus by reverse allelic exchange. These considerations illustrate the challenges inherent in attempting to define causal relationships in complex biological systems, the use of contemporary molecular methods notwithstanding.

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