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Method

Genome-wide detection of chromosomal rearrangements, indels, and mutations in circular chromosomes by short read sequencing

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Whole-genome sequencing (WGS) with new short-read sequencing technologies has recently been applied for genome-wide identification of mutations. Genomic rearrangements have, however, often remained undetected by WGS, and additional analyses are required for their detection. Here, we have applied a combination of WGS and genome copy number analysis, for the identification of mutations that suppress the growth deficiency imposed by excessive initiations from the *Escherichia coli* origin of replication, *oriC*. The *E. coli* chromosome, like the majority of bacterial chromosomes, is circular, and DNA replication is initiated by assembling two replication complexes at the origin, *oriC*. These complexes then replicate the chromosome bidirectionally toward the terminus, *ter*. In a population of growing cells, this results in a copy number gradient, so that origin-proximal sequences are more frequent than origin-distal sequences. Major rearrangements in the chromosome are, therefore, readily identified by changes in copy number, i.e., certain sequences become over- or under-represented. Of the eight mutations analyzed in detail here, six were found to affect a single gene only, one was a large chromosomal inversion, and one was a large chromosomal duplication. The latter two mutations could not be detected solely by WGS, validating the present approach for identification of genomic rearrangements. We further suggest the use of copy number analysis in combination with WGS for validation of newly assembled bacterial chromosomes.

[Supplemental material is available for this article. The sequence data from this study have been submitted to the NCBI Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi>) under accession no. SRP003789.]

Most bacteria, including *E. coli*, have one circular chromosome. Replication of this chromosome is initiated by assembling a complex of DnaA-ATP at the origin of chromosome replication, *oriC*. This subsequently leads to assembly of two complete replisomes in a highly controlled process (Mott and Berger 2007). Replication proceeds bidirectionally from *oriC* to a termination region, *ter*, at the opposite side of the circular chromosome. Because the replication time may exceed the generation time in fast-growing bacteria, such cells will display overlapping rounds of replication (Cooper and Helmstetter 1968).

The DnaA protein is the master regulator of initiation of chromosomal DNA replication in *Escherichia coli*. DnaA binds ATP and ADP with similar affinities, but only the former is active in the initiation process (Kaguni 2006; Mott and Berger 2007; Katayama et al. 2010). In *E. coli*, several mechanisms regulate the amount and activity of the DnaA protein (Nielsen and Lobner-Olesen 2008). One such mechanism is the RIDA (regulatory inactivation of DnaA) process (Katayama et al. 1998), which reduces the activity of the DnaA protein by hydrolysis of the ATP to ADP. The Hda protein, encoded by the *hda* gene, forms a complex with DNA-bound β -subunits of the DNA polymerase III holoenzyme to stimulate the intrinsic ATPase activity of DnaA. Hda is therefore instrumental for the RIDA process (Kato and Katayama 2001). Inactivation of *hda*

results in an increase in the ATP bound form of the DnaA protein, which, in turn, leads to overinitiation of chromosome replication from *oriC* and compromised cell growth (Kato and Katayama 2001). DnaA-ATP also serves as a repressor of the *nrdAB* genes encoding ribonucleotide reductase (Gon et al. 2006; Olliver et al. 2010). Loss of Hda is, therefore, also expected to result in deprivation of dNTPs in the cell, which could further contribute to the growth defect of *hda* mutant cells.

Once formed, Hda-deficient cells rapidly accumulate suppressor mutations, termed *hsm* (*hda* suppressor mutation) (Riber et al. 2006). Previously, eight *hsm* mutant strains were isolated, but the suppressor mutation was only identified in one of these (a point mutation in the *dnaA* gene) (Riber et al. 2006). Identification and characterization of *hsm* mutations is expected to advance our understanding of how chromosome replication in *E. coli* is controlled and prompted the present study to identify the mutations of the remaining *hsm* strains.

Traditional genetic approaches to map unmarked mutations in *E. coli* require a readily identified phenotype of the mutation in question. These approaches include Hfr mating and co-transduction frequencies with known markers that identify a candidate region for sequencing. The main disadvantages of these classical mapping techniques are that they are time-consuming and inefficient (not all mutations can be mapped this way), and the genetic tools needed are only available for a few organisms. Large chromosomal rearrangements are particularly difficult to map with genetic tools and have only been identified in a few cases, including the *rrnD-rrnE* inversion in *E. coli* strain W3110, which was mapped with DNA-RNA

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hybridization (Hill and Harnish 1981). The emergence of complete genomic sequences allows comparison of genomes of related bacteria. Such comparisons indicate that fixed inversions have a preference to pivot around *oriC* (Eisen et al. 2000). Optical mapping has also been applied to identify genomic rearrangements and linking these to sequence information (Zhou et al. 2004).

An alternative to the classical genetic mapping of mutations is based on whole-genome sequencing (WGS) by “next-generation DNA sequencing technologies” (NGS) (Shendure and Ji 2008), in which massive parallel sequencing of millions of short reads of DNA sequence can provide many-fold coverage of the entire genome. The many short reads can be aligned to a reference sequence similar to the experimental sequence in resequencing experiments and the differences enumerated.

There are several examples of the use of short read WGS for mutation detection. Srivatsan et al. (2008) sequenced a selection of *Bacillus subtilis* laboratory strains, using Illumina WGS, and identified a variety of mutations including two synthetic *relA*-suppressing mutations, each residing in a separate *relA* homolog and each having only a partial suppressing effect. Davis and Waldor (2009) sequenced *maE* mutants of *Vibrio cholera*, using Illumina WGS, in a search for *maE* suppressors and report single-nucleotide substitutions and single-nucleotide indels compared to the reference sequence.

Traditional WGS may not solve the difficulty with detection of large chromosomal rearrangements. This was demonstrated by Herring and Palsson (2007), who resequenced *E. coli* W3110 and used *E. coli* MG1655 as a reference sequence with the Comparative Genome Sequencing (CGS) service provided by Nimblegen Systems Inc. They reported that CGS was efficient in detection of SNPs (single-nucleotide polymorphisms), small indels, IS element insertions, and deletions compared to a reference sequence, but they failed to detect the known *rmD-rmE* inversion in W3110 (Hill and Harnish 1981). In a long-term *E. coli* adaptation experiment, SNPs, small indels, three larger deletions, and IS element insertions were readily detected. However, a major inversion between *citC* and *gatZ* (Schneider et al. 2000) was not detected (Barrick et al. 2009). These publications collectively indicate that point mutations and small insertions or deletions (indels) can be identified by short-read-based WGS, whereas larger chromosomal rearrangements are difficult to identify due to the limited ability of these methods to span the repeated sequences surrounding the chromosomal rearrangements.

Here we report the Illumina WGS sequencing of eight *hsm* strains. Apart from detecting point mutations and small insertions or deletions, the additional use of copy number analysis of template DNA isolated from fast-growing bacterial cultures allowed for easy detection of large chromosomal rearrangements. These rearrangements would have been very difficult to identify with classical genetic methods. Our results suggest that chromosomes are frequently rearranged, but only a few rearrangements are fixed.

Results and Discussion

Detection of point mutations and small indels

In previous work (Riber et al. 2006), we isolated eight independent *E. coli* strains with *hda*-suppressing mutations and named the mutations *hsm-1* to *hsm-8* (*hda* suppressor mutation). We sequenced the origin of replication, *oriC*, from all *hsm* strains, and we further sequenced the *dnaA* gene and the *ygfZ* gene in four of the *hsm* strains (Riber et al. 2006), since mutations in *ygfZ* were reported to suppress *hda* deficiency (Ote et al. 2006). We found that

the *hsm-2* strain had a point mutation in the *dnaA* gene that substituted phenylalanine with valine at position 349 in the protein and that this mutation was likely to cause *hda* suppression (Riber et al. 2006). The seven other *hsm* mutations remained unidentified.

Since *hsm* mutations arise spontaneously with a high frequency, it would be very difficult to map and eventually sequence the *hsm* mutations using any genetic approach. We have therefore investigated the use of the Illumina sequencing platform to detect mutations genome-wide without any prior mapping.

We obtained 5 to 13 million reads per mutant, each with a length of 35 nt, to cover the 4.62-Mb genome for each sample (Supplemental Table S1). The short sequences were aligned to the published sequence of *E. coli* MG1655 using a set of Perl scripts that we tentatively call “From Reads to Results, R2R” (Supplemental Table S1).

We found seven mutations to be common to some or all strains, and we found one unique mutation in each of *hsm-1-hsm-6* (Supplemental Table S2).

In the two strains (*hsm-7* and *hsm-8*) in which we failed to detect any point mutations or small indels, we identified larger rearrangements by copy number analysis of exponentially growing cultures (see below). We subsequently analyzed read frequencies from exponentially growing cultures of the *hsm-1* to *hsm-4* mutants and verified that they did not carry any large chromosomal rearrangement (data not shown).

Copy number analysis can detect chromosomal rearrangements

The replication time in fast-growing *E. coli* exceeds the doubling time, and the *oriC* proximal regions will, therefore, have more than a twofold higher copy number than *ter* proximal regions. The frequency of a sequence on the circular chromosome in a balanced culture with doubling time τ and replication time C will depend on the fractional distance m from the origin to the terminus ($m_{oriC}=0$, $m_{ter}=1$) of this sequence. Assuming that the replication fork velocity is constant from initiation at *oriC* to termination at *ter* and is the same for each of the two replication forks, the frequency N_x of a sequence X at position m_x is given by (Bremer and Churchward 1977):

$$\frac{N_x}{N_{oriC}} = 2^{-\frac{m_x C}{\tau}}$$

which can be rewritten as:

$$\log_2 N_x = \log_2 \left(N_{oriC} \times 2^{-\frac{m_x C}{\tau}} \right) = \log_2 (N_{oriC}) - \frac{m_x C}{\tau}.$$

The slope of $\log_2 N_x$ plotted as a function of m thus becomes:

$$\frac{d(\log_2 N_x)}{dm_x} = -\frac{C}{\tau}.$$

A plot of $\log_2 N_x$ as a function of m gives straight lines with maximum at *oriC* and minimum at *ter*. Any deviation from the straight line indicates either that the replication speed C varies with the position on the chromosome or that the position m is wrong, as it will be if part of the chromosome is missing, relocated, inverted, or duplicated. Variations in replication speed will show as bends on a continuous curve, whereas chromosomal rearrangements will show as a discontinuous curve.

We prepared chromosomal DNA from an exponentially growing culture and a stationary-phase culture of the wild-type strain MG1655 and used the frequency of reads per base pair to analyze copy number variations. The read frequency of the stationary-phase culture where replication does not take place (Fig. 1A) was uniform for all parts of the chromosome, whereas the read frequency of the exponentially grown culture (Fig. 1B) showed a different distribution with the origin proximal regions being 2.7-fold more frequent compared to the *ter* proximal regions. The “tent-shape” of the curve reflects the bidirectionality of the DNA replication.

We also grew *hsm-7* and *hsm-8* strains exponentially before preparing the chromosomal DNA. The read frequency distribution generated from these two strains indicated a ratio of *oriC* to *ter* that was increased in *hsm-7* (6.4) and *hsm-8* (9.8) compared to wild type (2.7) (Fig. 1). Previous publications have noted similar patterns in strains in which the activity of DnaA protein was increased by DnaA overproduction (Atlung et al. 1987; Simmons et al. 2004), the *dnaAcos* mutation (Simmons and Kaguni 2003) or the *seqA* mutation (von Freiesleben et al. 1994; Riber et al. 2006). The increased *oriC*-to-*ter* ratio in the *hsm-7* and *hsm-8* strains suggests that the activity of the DnaA protein is still elevated and that the rearrangements only partially relieve the defects of Hda deficiency. The shape of the read frequency distribution of *hsm-7* and *hsm-8* deviated clearly from the “tent-shape.” A region of *hsm-7* covering 521,000 bp showed an inverted distribution compared to wild type (Fig. 1C). This region is flanked by two IS5 insertion sequences in

opposite orientations that provide a 1203-bp inverted repeat sequence ideal for generating an inversion by recombination (Fig. 2A). Indeed, we verified the inversion by PCR analysis (Fig. 2B). For the *hsm-8* strain, a region from *rmaA* to *rmaE* ribosomal operons had twice the expected read depth compared to wild type (Fig. 1D), indicating that this region is duplicated. The *rmaA* and *rmaE* operons are oriented in the same direction, and they provide long, near perfect repeat sequences ideal for recombination (Fig. 3A). A detailed analysis of the read frequencies of the few unique sequences within each operon indicated that recombination had taken place between the two genes for 16S RNA *rrsA* and *rrsE* (Fig. 3B). We verified this chromosomal rearrangement with PCR analysis across the *rmaE*-*rmaA* junction (Fig. 3C). The absence of other detectable mutations in *hsm-7* and *hsm-8* suggests that the observed chromosomal rearrangements cause the *hsm* phenotype. We propose that the *hsm* phenotype in *hsm-7* and *hsm-8* is caused by altered expression of one or more gene(s) within the inverted and duplicated regions, respectively, resulting from their altered copy number(s).

Large chromosomal rearrangements may be frequent, but few are fixed

A number of clinical *E. coli* isolates have now been completely sequenced. Rasko et al. (2008) compared 10 completely sequenced *E. coli* genomes from four different pathovars. They observed only one large inversion in the EHEC strain EDL933 compared to the other *E. coli* strains. Similarly, Touchon et al. (2009) compared 12 clinical *E. coli* isolates and six clinical *Shigella* isolates representing four different species and found only a limited number of rearrangements in the *E. coli* isolates, whereas the *Shigella* isolates had 16 to 64 rearrangements compared to *E. coli*. The *Shigella* isolates also had a much higher number of IS-like elements in their genome (from 549 to 1165) compared to most *E. coli* isolates (from 42 to 150). One exceptional *E. coli* isolate, IAI39, had 224 IS-like elements and 10 rearrangements compared to other *E. coli*.

The low frequency of large chromosomal rearrangements found in these studies contrasts with our observation of both a large inversion and a large segmental duplication in only eight samples. Tandem duplications of regions between rRNA operons have been known for long to occur in a small percentage of cells in a population of *Salmonella enterica* cells (Anderson and Roth 1981), and this is likely true for *E. coli* as well. Tandem duplications may form in a RecA-independent way, but duplication loss is RecA-dependent and the steady state of duplication frequency is a balance between formation and loss due to a fitness cost of duplication (Reams et al. 2010). This fitness cost may explain why duplications are rarely fixed in populations. The duplications may meanwhile provide the substrate for more stable mutations before they are lost, leading to, for instance, short junction mutations

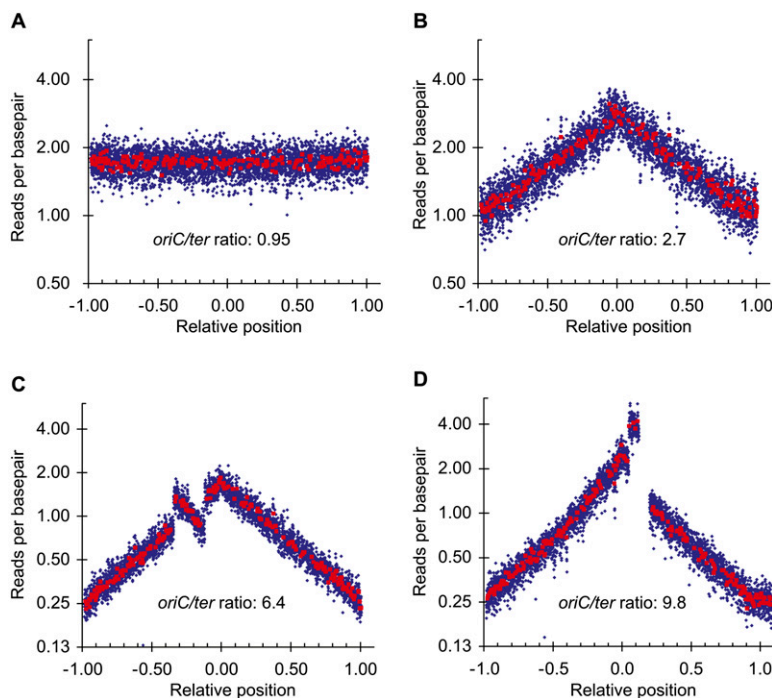


Figure 1. Chromosomal rearrangements detected by read frequency analysis. The number of reads starting at each base is plotted as a function of the relative position on the reference chromosome; *oriC* is set to 0, and *ter* is set to ± 1 to reflect the bidirectional DNA replication. (Blue rhombus) The average of 1000-bp windows; (red squares) the average of 10,000-bp windows. Any window containing repeat sequences is omitted. The *oriC/ter* ratio is the ratio of reads per base pair at *oriC* to reads per base pair at *ter*. Sequencing template was isolated from: (A) an overnight culture of MG1655; (B) an exponentially growing culture of MG1655; (C) an exponentially growing culture of *hsm-7*; and (D) an exponentially growing culture of *hsm-8*. (C,D) Clear deviations from the pattern of the host strain MG1655 (B) indicating an inversion and a duplication, respectively.

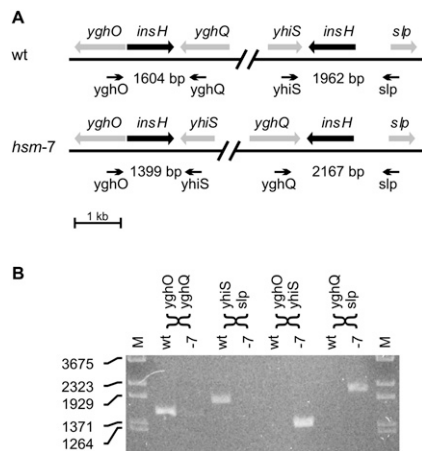


Figure 2. The chromosome of *hsm-7* is inverted between two ISS elements. The read frequency analysis of *hsm-7* (Fig. 1C) indicates an inversion between two inverted copies of the *insH* gene encoding the ISS transposase and *trans*-activator. (A) Genetic map of the two copies of *insH* and their neighboring genes in the wild-type (wt) and in the suggested *hsm-7* configuration. The heads of the arrows indicate the approximate locations of PCR primers designed to differentiate between the wt and the *hsm-7* configuration by amplifying the *insH* genes with neighboring sequences. The expected size of each PCR product is indicated. (B) PCR analysis of MG1655 DNA (wt) and *hsm-7* DNA (-7) with the primers shown in A. MG1655 shows the expected wild-type fragments of 1604 bp and 1962 bp. *hsm-7* shows the 1399-bp and the 2167-bp fragment expected from the inversion.

(Kugelberg et al. 2006) or point mutations (Pranting and Andersson 2011). We suggest that also inversions may be formed frequently and then lost again because of a fitness cost. In the case of our *hsm* mutants, the chromosomal rearrangements provide the host an immediate advantage, i.e., the ability to stay alive, and hence there is a selection for keeping the suppressing rearrangement. The ease of formation and removal of chromosomal rearrangements may explain why they are rare in wild isolates compared to laboratory strains.

In general, large chromosomal rearrangements in closely related species/strains may easily be overlooked in resequencing experiments with short read sequencing technologies. In this case, the inversion was between 1203 bp perfectly inverted repeats, and the duplication was between ~5 kb nearly perfectly repeats. The paired-end technology and the recent mate pair technology from Illumina may span 200–500 bp and 2–5 kb repeats, respectively. Paired-end sequencing will not span any of these repeat sequences. Mate pair sequencing is most efficient in combination with single-end or paired-end sequencing and will thus require additional sequencing.

Application of copy number analysis for assembly of de novo sequencing

We suggest that an additional copy-number analysis derived from using sequencing templates from fast-growing cultures is a cost- and labor-effective strategy for detection of assembly errors in de novo sequencing projects. To test this hypothesis, we mapped the sequence reads obtained from MG1655 with the sequence of W3110 as a reference sequence (Fig. 4). W3110 is almost identical to MG1665 except for the inversion between *rmD*–*rmE* (Fig. 4A; Hayashi et al. 2006). Mapping reads obtained from a stationary-phase culture, where all sequences are present in the same copy

number, did not reveal the inversion in W3110 compared to MG1655 (Fig. 4B). In contrast, a clear discontinuity in the curve was observed when the reads obtained from the fast-growing culture were mapped to W3110 compared to MG1655 (Fig. 4C, cf. right and left part of panel) revealing the inversion (Fig. 4C). This demonstrates that genomic copy number analysis can greatly contribute to detection of assembly errors in de novo sequencing of bacterial genomes and to the detection of large chromosomal rearrangements in resequencing bacterial genomes.

A specific variation in the copy number of different positions in a genome is required to benefit from copy number analysis. The extent of such variations depends on the ratio of the time required to replicate the genome, C , to the doubling time of the organism, τ . This analysis can therefore be applied directly to all fast-growing organisms with one or few origins for DNA replication. Also, slow-growing organisms may be susceptible to copy number analysis.

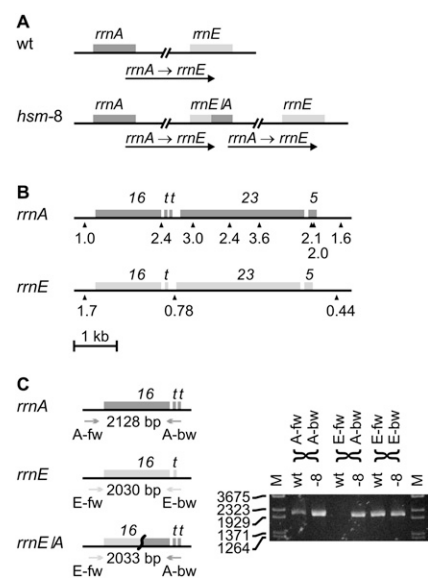


Figure 3. The *rrmA*–*rrmE* region of the chromosome of *hsm-8* is duplicated. The read frequency analysis of *hsm-8* (Fig. 1D) indicates a duplication of the *rrmA*–*rrmE* region. This duplication can be caused by homologous recombination between sequences repeated in the *rrmA* and *rrmE* ribosomal RNA operons creating a *rrmE/A* chimeric operon and duplicating the entire sequence between *rrmA* and *rrmE*. (A) The duplication in *hsm-8* compared to wild type (wt). (B) A detailed read frequency analysis was made to narrow down the recombination point. (16) *rrs* genes for 16S RNA; (t) genes for tRNA; (23) *rrl* genes for 23S RNA; (5) *rrf* genes for 5S RNA. The read frequency of a 500-bp window on each side of the *rrmA* and *rrmE* operons and at positions with unique sequences (shown by triangles) within the *rrm* operons in *hsm-8* was normalized to the read frequencies of the same positions in wt. The relative read frequency of the 500-bp window to the left of *rrmA* was set to 1. The read frequency shifts from 1.0 to higher than 2 on the opposite side of the 16S RNA gene. This indicates that the entire part of the *rrmA* operon downstream from the 16S RNA gene is duplicated. In the *rrmE* operon the read frequency drops fourfold from the left side to the right side of the operon. This fourfold drop is in agreement with Figure 1D and indicates a long DNA replication time for the *rrmE* operon. The read frequency at the only measurable point in the *rrmE* operon is lower than half the read frequency of the left side and higher than the read frequency of the right side and is less conclusive. The combined analysis of *rrmA* and *rrmE* indicates that recombination took place within the 16S RNA genes. (C, left) PCR primers were designed to amplify the *rrsA*, *rrsE*, and the *rrsE/A* chimeric genes. (Heads of the arrows) The approximate locations of the primers and the expected PCR product sizes are indicated. (C, right) PCR products obtained from MG1655 (wt) and from *hsm-8* (-8). *hsm-8* shows the native *rrmA* and *rrmE* operons as well as the *rrmE/A* chimeric operon, whereas MG1655 only shows the native *rrmA* and *rrmE* operons.

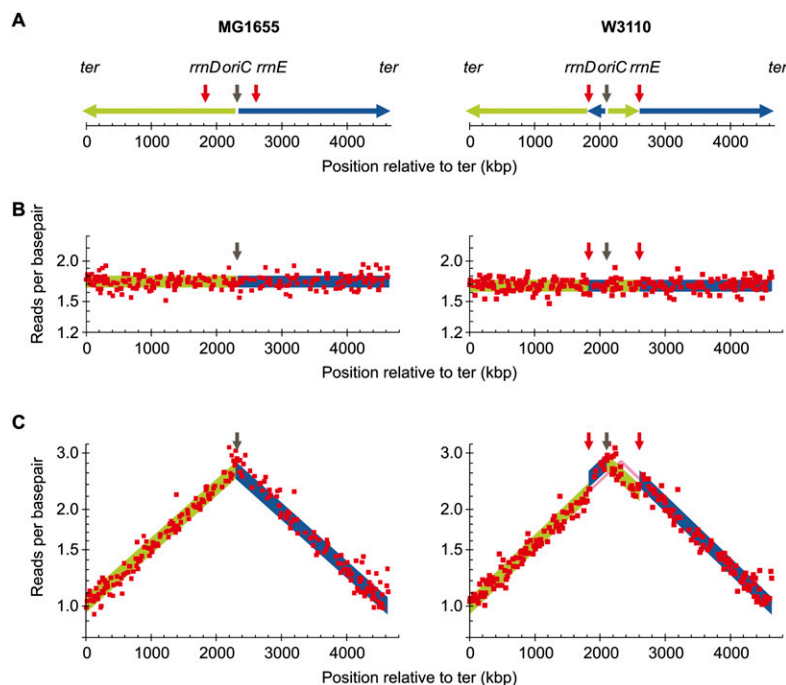


Figure 4. Simulation of detection of errors in de novo assembled bacterial sequences. (A) Chromosomal DNA is replicated bidirectionally from *oriC* (black arrow) indicated by the green line (left arm) and blue line (right arm). Strain W3110 (right) carries an inversion between the ribosomal operons *rrnD* and *rrnE* (red arrows) but is otherwise very similar to MG1655 (left). This inversion includes the origin of replication, *oriC*, and displaces *oriC* 215 kb to the left compared to MG1655. (Green and blue) The inverted chromosomal arms. Reads generated from DNA template of a stationary phase (B) and an exponentially growing culture of MG1655 (C) were mapped to the genomic sequence of MG1655 (left) and W3110 (right). The number of reads starting at each base pair was calculated for a 10-kb-wide sliding window (red squares), and windows covering repeat sequence were removed. The ideal read frequencies were calculated using an *oriC/ter* ratio of 2.7 and shown with the same color code as in A.

For instance, Lundgren et al. (2004) demonstrated that the hyperthermophilic archaeons *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* show significant variations in copy number along the chromosome even with doubling times of 3–4 h. This led us to suggest that most cultivable prokaryotes can be subjected to copy number analysis.

Conclusion

We have detected one specific mutation in each of eight isolated *hsm* mutant strains, as well as seven other mutations common to most or all the strains solely by analyzing the short read sequences. This yield is superior to classical genetic mapping techniques, and our analysis required only a fraction of the workload of classical techniques. The detected mutations comprise point mutations, single and dinucleotide indels, insertion and deletion of transposable IS elements, insertions and deletions between short tandem repeats, and major genomic rearrangements. The chromosomal rearrangements identified in this study were only detected because we used DNA template isolated from a fast-growing bacterial culture and combined sequence determination and copy number variation analysis. These rearrangements would not have been detectable using the standard paired-end sequencing technique since the paired sequences usually span <500 bp, and in these cases, the repeated sequences enclosing the rearrangements exceed 1000 bp. The frequency with which we observed large chromosomal rearrangements led us to speculate that they may

normally constitute a significant fraction of mutations.

Methods

Strains and growth conditions

Our isolate of the *E. coli* K-12 strain MG1655 was originally obtained as CGSC6300 from the Coli Genetic Stock Center and have traveled through the laboratories of Donald Biek and Martin G. Marinus; the isolate was stored as a frozen glycerol stock in these laboratories and in our laboratory (MG Marinus, pers. comm.). Several variants of MG1655 are currently in circulation in various laboratories, some of which contain a major chromosomal deletion in the *fir* region (Soupene et al. 2003). Overall, the MG1655 sequenced here is quite similar to the strain for which the sequence is published as U00096 (Blattner et al. 1997).

Construction of the *hda::cat* insertion and isolation of the MG1655 derived strains ALO1917 (*hsm-1*), ALO2515 (*hsm-2*), ALO2516 (*hsm-3*), ALO2517 (*hsm-4*), ALO2518 (*hsm-5*), ALO2519 (*hsm-6*), ALO2520 (*hsm-7*), and ALO2521 (*hsm-8*) were described previously (Riber et al. 2006).

Strains were grown in AB minimal medium (Clark and Maaløe 1967) supplemented with 10 μ g/mL thiamine, 0.2% glucose, and 0.5% acid-hydrolyzed caseamino acids (BD Difco Bacto Casamino Acids, technical grade). Chromosomal DNA was extracted (Grimberg et al. 1989) for sequencing either from stationary-phase cultures or sampled at $OD_{450} \sim 0.2$ from exponentially growing cultures as indicated.

Sequencing, sequence analysis R2R, and read frequency analysis

DNA was prepared for sequencing following the Illumina recommended procedure. Briefly, genomic DNA was randomly sheared to 200–800 bp using a Nebulizer. DNA fragments were blunt ended, and A-overhangs were added to the 3' end of the fragments. Sequencing adapters were ligated to the fragments, and the library was subjected to 18 cycles of PCR amplification. The final library was sequenced using the GA II (Illumina) instrument following the protocol of the manufacturer. Base calling of the 35-bp short read sequences was done using Illumina's analysis GOAT Pipeline.

We mapped the short reads to the published sequence of strain MG1655 (or W3110, AC_000091) as a resequencing analysis using our own set of Perl scripts, that we call R2R. The key steps of R2R are (1) indexing of the reference sequence and call of any sequence with length equal to or longer than the read length and present two or more times in the reference sequence as a repeat sequence; (2) mapping and quality monitoring of the short reads with the reference sequence; and (3) presentation, call of mutations, and statistics of read frequencies and sequence coverage, etc. The mapping of reads was done in the following order: First, all matching reads were mapped and subtracted; the remaining reads were quality-filtered; all reads with one misfit were mapped and subtracted; all reads with two misfits were mapped and subtracted;

and the remaining reads were analyzed for having at least 15 exactly matching nucleotides from either end. We call this latter class of reads “indel-reads” since they potentially mark sites for insertions or deletions. The filter for quality was set so the first 30 nt should be called with an average quality of at least 35 using the Illumina raw quality, which is a phred-like value with a maximum value of 40 for one nucleotide.

Mapped reads are presented together with reference sequence and annotations, and in the same run tables of called mutations are generated. A nucleotide is set “confirmed” if a matching read overlaps this nucleotide, and the read extends for at least n nucleotides before and after. For SNPs, simple indels, and read statistics, n was set to 3; for calling duplications between short tandem repeats, n was set to 7. SNPs and simple indels were called with the criteria that at least two reads should indicate a mutation, and the number of matching reads should be <50% of the nonmatching reads. Duplications between short tandem repeats were called with the criteria that at least 10 indel reads should mark the position, and the number of matching reads should be less than twice the indel reads.

Read frequency analysis was visualized with Microsoft Excel, using the R2R-generated frequency data as input.

R2R is available at <http://milne.ruc.dk/R2R/>; details of the R2R scripts will be reported elsewhere.

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