Evolution of Bacterial Interspecies Hybrids with Enlarged Chromosomes

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Abstract

Conjugation driven by a chromosomally integrated F-plasmid (high frequency of recombination strain) can create bacteria with hybrid chromosomes. Previous studies of interspecies hybrids have focused on hybrids in which a region of donor chromosome replaces an orthologous region of recipient chromosome leaving chromosome size unchanged. Very little is known about hybrids with enlarged chromosomes, the mechanisms of their creation, or their subsequent trajectories of adaptative evolution. We addressed this by selecting 11 interspecies hybrids between *Escherichia coli* and *Salmonella* Typhimurium in which genome size was enlarged. In three cases, this occurred by the creation of an F′-plasmid while in the remaining eight, it was due to recombination of donor DNA into the recipient chromosome. Chromosome length increased by up to 33% and was associated in most cases with reduced growth fitness. Two hybrids, in which chromosome length was increased by the addition of 0.97 and 1.3 Mb, respectively, were evolved to study genetic pathways of fitness cost amelioration. In each case, relative fitness rapidly approached one and this was associated with large deletions involving recombination between repetitive DNA sequences. The locations of these repetitive sequences played a major role in determining the architecture of the evolved genotypes. Notably, in ten out of ten independent evolution experiments, deletions removed DNA of both species, creating high-fitness strains with hybrid chromosomes. In conclusion, we found that enlargement of a bacterial chromosome by acquisition of diverged orthologous DNA is followed by a period of rapid evolutionary adjustment frequently creating irreversibly hybrid chromosomes.

Key words: conjugation, Hfr, experimental evolution, *Escherichia coli*, *Salmonella* Typhimurium, recombination.

Significance

Bacterial conjugation has the potential to create strains with hybrid chromosomes but the genetic architecture, fitness, and subsequent evolution of strains with enlarged genomes have not previously been analyzed. We identified the integration of donor chromosomal DNA circles after interspecies conjugation as a mechanism that can create hybrids with a significantly increased chromosome length without any loss of recipient DNA. Subsequent experimental evolution of such hybrids very rapidly resulted in high-fitness strains, associated with chromosomal deletions that included both donor and recipient DNA, creating strains with irreversibly interspecies hybrid chromosomes.

Introduction

The integration of a conjugative plasmid into a bacterial chromosome can create a strain with the potential to transfer chromosomal DNA to a suitable recipient (Baron et al. 1968; McAshan et al. 1999; Hopwood 2006). In *Escherichia coli*, for example, the conjugative F-factor plasmid carries a number of IS elements and has been shown to integrate into the chromosome by homologous

recombination with similar chromosomal IS elements. The resulting high frequency of recombination (Hfr) strain interacts with a recipient strain via a conjugative pilus, transferring chromosomal DNA into the recipient, where it has the possibility to be recombined into the recipient chromosome (Cavalli-Sforza 1950; Cavalli et al. 1953; Hayes 1953; Davidson et al. 1996; Waksman 2019).

The recent discovery of two globally widespread bacterial pathogens (*Klebsiella pneumoniae* ST258 and *E. coli* ST1193), each with hybrid chromosome architectures (Chen et al. 2014; Tchesnokova et al. 2019), suggests that the processes responsible for creating bacterial hybrids may be important from both a clinical and an evolutionary perspective. In each of these clinical hybrids, at least 20% of the hybrid chromosome originates in a separate strain. Being natural isolates, the mechanisms responsible for creating these hybrids are not certain. However, the fact that in both ST258 and ST1193, a region covering >1 Mb of DNA of the chromosome length has been incorporated from a different strain is consistent with conjugational transfer. This process is known to be able to transfer multimegabase lengths of chromosomal DNA, not only within species but also between different bacterial species and genera (Bartke et al. 2021).

Interspecies hybrids can be created in the laboratory by conjugative mating between *E. coli* Hfr and *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) strains. Cultures of the two strains are mixed, allowed time to conjugate, then transconjugants (hybrids) are obtained by selecting for the growth of bacteria expressing two genetic markers, one present originally on the donor chromosome and the other present on the recipient chromosome. *Escherichia coli* and *S.* Typhimurium, although diverged at the nucleotide level, share a generally co-linear orthologous gene order on their respective chromosomes, a feature that probably contributes to the feasibility of creating viable hybrid strains in these crosses. The process of creating these hybrids depends on RecABCD-mediated homologous recombination activity, with MutSLH mismatch repair activity acting as a barrier (Rayssiguier et al. 1989, 1991). The recombination joint points in such interspecies hybrids occur in regions of greater than average sequence similarity between donor and recipient (Bartke et al. 2021) consistent with hybrid formation depending on homologous recombination. Examination of interspecies hybrid chromosome architecture, either by mapping for the presence or absence of discrete chromosomal markers (Matic et al. 1994) or by whole-genome sequence (WGS) analysis (Bartke et al. 2021) has shown that in the great majority of the hybrid strains studied, a single contiguous segment of DNA replaced an equivalent region of the recipient chromosome.

In the early literature on interspecies conjugation, it was observed that Hfr-mediated crosses between *E. coli* and *Salmonella* (with either species as donor or recipient) could also result in the creation of merodiploid strains, that is, strains apparently carrying orthologous genes from both species (Baron et al. 1959, 1968; Johnson et al. 1972, 1973, 1975; Lehner and Hill 1985). Genetic analysis showed that in some cases, the merodiploid state was due to the inheritance of an F′-plasmid carrying donor chromosomal DNA (Baron et al. 1968; Leavitt et al. 1971) but in other cases, it appeared to have involved recombination of the inherited DNA into the recipient chromosome. In some but not all cases, genetic mapping showed that the recombination occurred between non-identical *rrn* operons, resulting in tandem or non-tandem chromosomal regions carrying many orthologous genes from each parent strain (Baron et al. 1968; Johnson et al. 1970; Lehner and Hill 1985). Many of these early studies also observed genetic instability in the hybrids with enlarged genomes. The techniques available at the time did not allow for detailed investigations into either the organization of the hybrid genomes or how subsequent evolution could be affected by the interplay between the relative fitness and the genetic architecture of the hybrid strains.

In nature, the chromosomal region in which recombination can occur to generate a viable transconjugant, either inter- or intraspecies, might be limited in length, as a consequence of sequence or organizational divergence between the conjugating strains, and the demands of the specific selection regime. We reasoned that under such conditions, where a simple genetic replacement would not generate a viable hybrid with the selected phenotype, that transconjugant hybrids might instead be generated by recombination events resulting in acquisition of additional genetic material, enlarging the size of the genome. Accordingly, we selected transconjugants from crosses between an *E. coli* Hfr and *S.* Typhimurium recipients in which the selected donor and recipient genetic markers were located within a short (~35 kb) region of genetic co-linearity. This resulted in the selection of hybrid strains with enlarged genomes. Here, we used genome sequence analysis to determine the different mechanisms of genome enlargement, the relative fitness of the hybrid strains, and applied experimental evolution to study the genetic changes that occurred under selection for amelioration of the growth fitness costs.

Results and Discussion

Selection of Hybrids with Enlarged Genome Size

Initial conjugations were made as previously described (Bartke et al. 2021) between *E. coli* Hfr CH6941 and *S.* Typhimurium TH10684 (supplementary table S1, Supplementary Material online), selecting for widely separated donor and recipient markers. Diagnostic polymerase chain reaction (PCR) was used to assess the presence of

additional *E. coli* DNA in transconjugants by species-specific amplification of orthologues of a gene mapping in close proximity to the selected *E. coli* and *S.* Typhimurium resistance genes. Two transconjugants carrying additional donor DNA were identified in these selections and included in this study (CH7199 and CH7205). However, because >90% of transconjugants selected in this way carried simple replacements of orthologous regions, rather than additional DNA, it was decided to employ a different approach to selecting transconjugants. This involved conjugational crosses between *E. coli* Hfr and *S.* Typhimurium in which the selected donor and recipient genetic markers were located within a ~35-kb region of genetic co-linearity (fig. 1a). This approach was applied to five independent selections, each directed at three different regions of the chromosome, at different distances from the origin of transfer in the Hfr: 1) *yiiF—yiiQ*, ≥1 Mb; 2) *ymdA—yceD*, ≥3 Mb; and 3) *queE—ygdI*, ≥4.5 Mb (supplementary table S2, Supplementary Material online). The gene names are those in which a selective antibiotic-resistance cassette is located in the donor and recipient strain, respectively, and the size in megabase is the approximate distance of the selected genes from the Hfr origin of transfer. The orthologues amplified to test potential hybrids for the presence of genetic material from both species were, *yiiD* (close to the locations of *yiiF* and *yiiQ*), *csgA* (close to the locations of *ymdA* and *yceD*), and *pyrG* (close to the locations of *queE* and *ygdI*). Transconjugants were obtained from 9 of the 15 independent crosses (frequency ~10^{-10} per recipient cfu) and in every case, the diagnostic PCR assay confirmed the presence of orthologues from both species, indicative of additional DNA. In total, 11 transconjugants (two from the old selection and nine from the new selection) were stored for WGS analysis to identify the extent and locations of the additional DNA.

Genetic Architecture and Mechanism of Hybrid Formation

We determined the WGS of each of the 11 hybrid strains and confirmed that each had an enlarged genome that included orthologous chromosomal regions from both *E. coli* and *S.* Typhimurium. Based on our analysis of the sequence data, we could identify three different causative mechanisms to explain the additions of *E. coli* genetic material to the *S.* Typhimurium genome (fig. 1b and supplementary table S3, Supplementary Material online).

Three of the 11 transconjugants, CH7204, CH7209, and CH8725, obtained by selection for *queE<>gen*, the marker most distant from the Hfr origin of transfer, occurred by the creation of an F′-plasmid (fig. 1b). These carried the complete F-plasmid sequence (~100 kb), together with *E. coli* chromosomal DNA ranging in length from ~300 to 400 kb that originated from each side of the site of

F-plasmid integration site in the Hfr donor strain (supplementary table S3 and fig. S1, Supplementary Material online). Because this is a mechanism that does not enlarge the size of the chromosome these hybrids are not considered further. In the remaining eight hybrid strains, *E. coli* DNA had recombined into the *Salmonella* chromosome resulting in a net increase in chromosome length (fig. 1b). In five cases, this involved replacement of *Salmonella* DNA with a larger length of *E. coli* DNA (fig. 2a). In the remaining three cases, hybrid formation occurred by the integration of a circle of *E. coli* DNA into the *Salmonella* chromosome, increasing the length of the chromosome without replacing any *Salmonella* DNA (fig. 2b).

Enlargement of Chromosome Length by Integration of Linear DNA

Five transconjugants obtained by selecting for donor and recipient markers within the co-linear regions defined by the *yiiF—yiiQ* or *ymdA—yceD* intervals can be explained by recombination of a linear fragment of *E. coli* DNA, involving recombination between orthologous donor and recipient sites at one end and non-orthologous sites at the other end of the linear fragment (fig. 2a). The result is the replacement of a region of *Salmonella* chromosome by a longer region of *E. coli* chromosome resulting in a net increase in genome size. In three of the five hybrids in this category (CH8723, CH9323, and CH9324), all selected for *yiiF<>kan*, the sites of non-orthologous recombination were different *rrn* operons. In the remaining two hybrids selected for *ymdA<>rif* (CH8728 and CH9325), the sites of non-orthologous recombination were different IS elements. One of these strains (CH8728) acquired the complete sequence of the F-plasmid, turning it into a new Hfr strain. CH8728 could be explained by the formation of a circular F′-factor in the donor strain that after transfer to the recipient is involved in recombination of a linear fragment into the recipient chromosome, or by the rolling circle transfer of greater than one *E. coli* chromosome length (thus including all of the integrated F-factor), followed by recombination of a linear fragment into the recipient chromosome. The genomic architecture of CH8728 is complex and best explained if in addition to acquiring a linear segment of *E. coli* DNA, it also underwent two inversion events (supplementary fig. S2, Supplementary Material online). The length of chromosomal DNA replaced in these hybrids ranged from 530 kb to 2.4 Mb (CH9324 and CH8728, respectively), while the net increase in chromosome length ranged from 151 to 521 kb (CH8723 and CH8728, respectively). The growth rates of the five strains in this class, range from 0.70 to 0.89, with no correlation between growth rate and the net increase in genome size (fig. 2a).
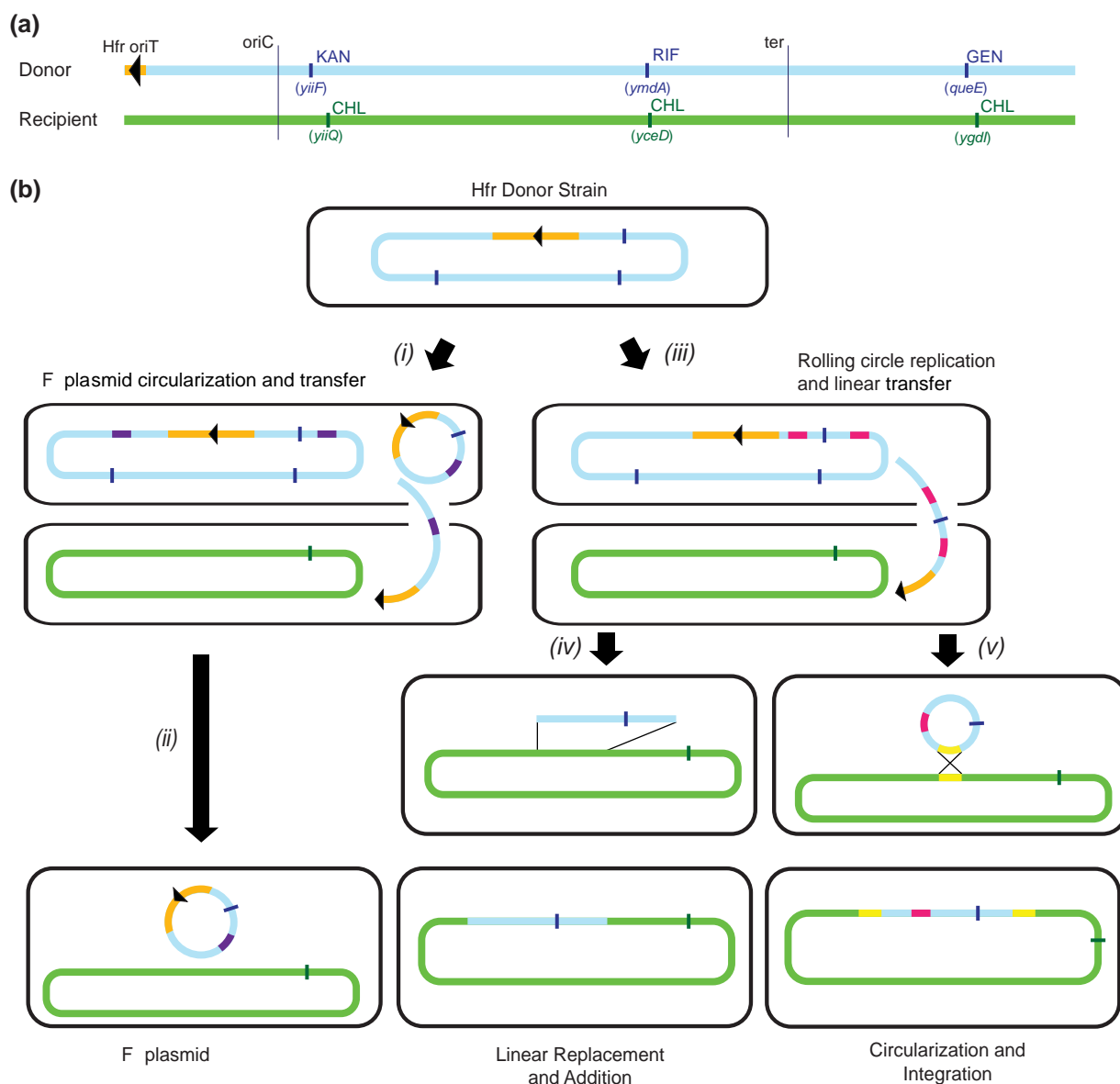
FIG. 1.—Selection and formation of transconjugants with enlarged genomes. (a) Linear representation of donor Hfr and recipient chromosomes showing the relative locations of the Hfr origin of transfer, and donor and recipient selection markers. (b) Different mechanisms by which genome size was enlarged in transconjugant hybrids: (i) formation of an F'-plasmid carrying part of the donor chromosome; (ii) its transfer into a recipient strain; (iii) rolling circle replication from the Hfr oriT and transfer of linear chromosomal DNA into a recipient; (iv) where it recombines as a linear fragment into the recipient chromosome replacing a section of recipient with a larger section of donor chromosomal DNA; or (v) where the linear donor DNA recombines into a circular molecule which then recombines into the recipient chromosome using a different region of sequence identity, resulting in chromosome enlargement.

Enlargement by Chromosomal Integration of a Donor DNA Circle

Three hybrids (CH7199, CH7205, and CH8726) are explained by a mechanism that involves the acquisition of a linear fragment of donor *E. coli* DNA that circularized, then recombined into the *S.* Typhimurium chromosome in a single crossover recombination event (fig. 2b). In each case, the initial circle formation appears to have involved recombination between genes with a high degree of sequence similarity present at each end of the acquired donor fragment: *rhsD* and *rhsE* in CH7199; *rrfA* and *rrfE* in CH7205; *insC* and *insD* in CH8726. Each circle then integrated into the *S.* Typhimurium chromosome by homologous recombination between a different pair of genes
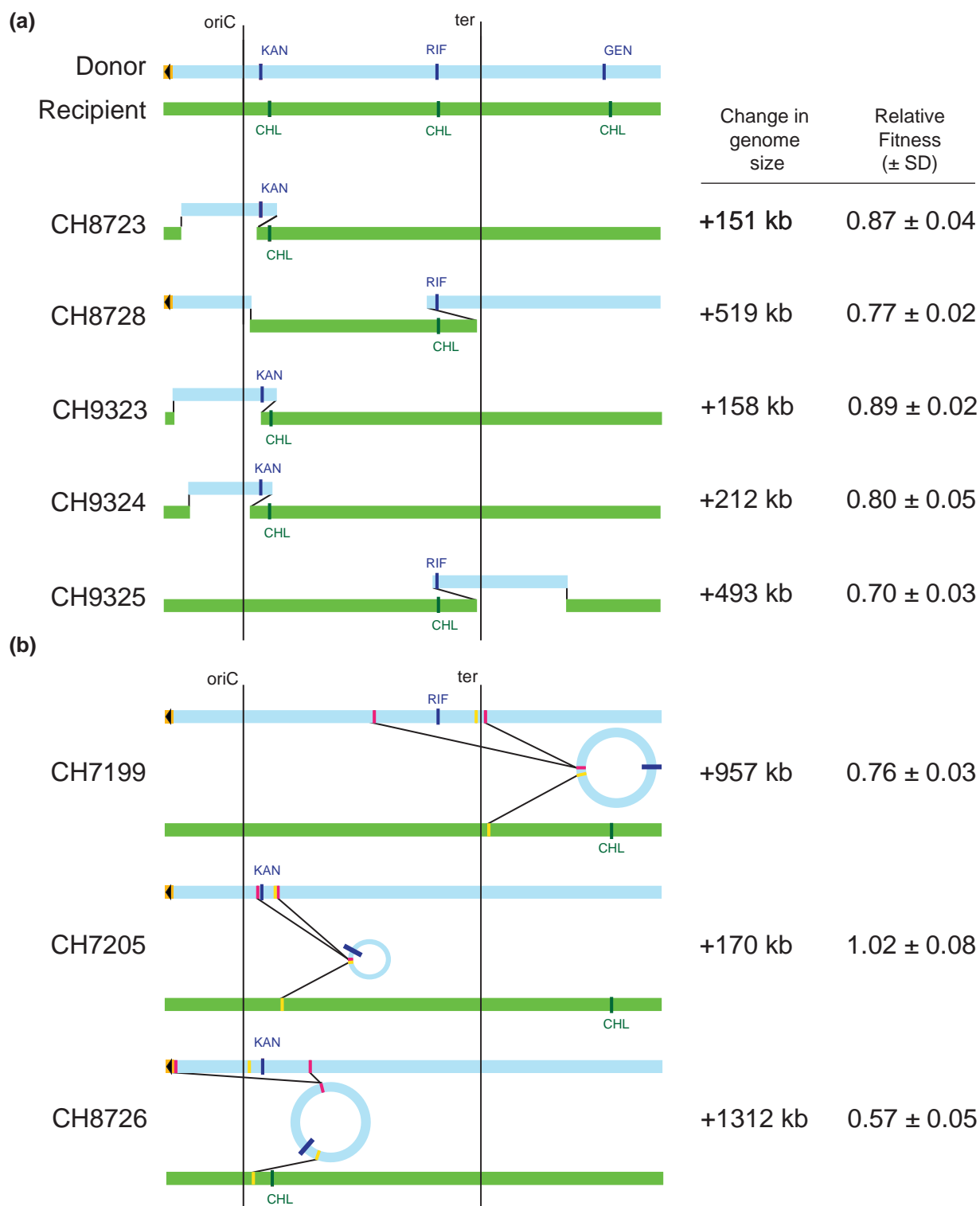
FIG. 2.—Formation of transconjugants with hybrid chromosomes. (a) Recombination resulting in replacement of a region of *Salmonella* chromosomal DNA with a longer segment of *Escherichia coli* DNA, resulting in a net increase in chromosome length. (b) Recombination of a circle on *E. coli* DNA into the *Salmonella* chromosome resulting in an increase in chromosome length without any replacement of *Salmonella* DNA. Sequences involved in circle formation or circle recombination into the chromosome are indicated.
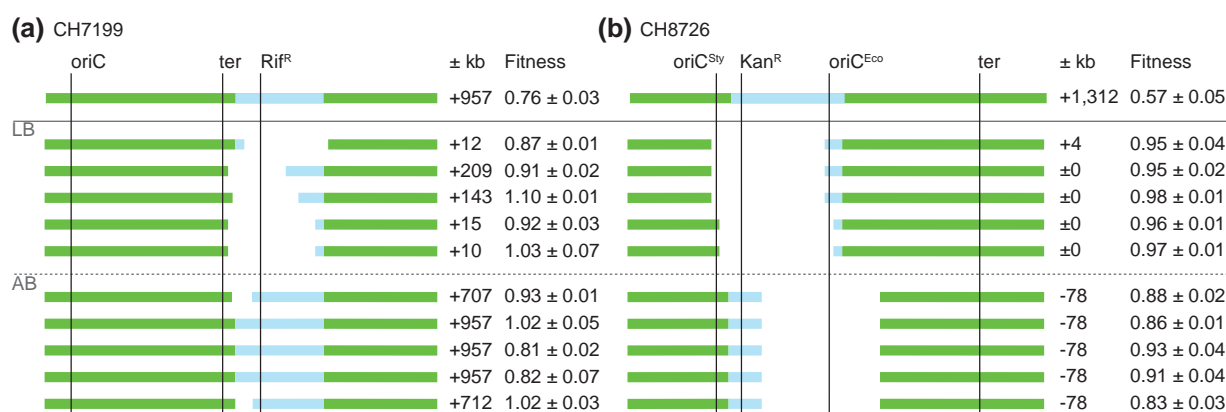
Fig. 3.—Changes occurring during experimental evolution of hybrid strains with enlarged chromosomes: (*a*) CH7199, (*b*) CH8726, evolved 100 generation in LB or LB with the addition-selective antibiotic (AB: RIF or KAN). *Salmonella* Typhimurium DNA is shown in green, *Escherichia coli* DNA in blue. Deletions are shown as a gap in the linear sequence. Chromosome length relative to the parental *Salmonella* is shown in the column headed ± kb. Fitness is calculated from the exponential growth rate of each hybrid and evolved strain relative to that of the parental *Salmonella*.

with sequence similarity, where one is present on the circle and the other is in the recipient chromosome: in CH7199, the ~957 kb circle recombined into *ydaO*, close to the terminus of replication; in CH7205, the ~170 kb circle recombined into *rrsE*; and in CH8726, the ~1.3 Mb circle recombined into *rrlC*. Interestingly, after circle integration CH8726 contains two chromosomal origins of replication, one from each species. Each of the acquired fragments contained, as expected, the selected donor drug-resistance marker: *yiiF<>kan* in CH7205 and CH8726 and *ymdA<>rif* in CH7199. The absence of a growth cost for the circle integration in CH7205 (fig. 2*b*) may be a consequence of the integration duplicating a region with many highly expressed gene encoding important components of the translation and transcription machinery, a region where spontaneous duplications are frequently selected during fast growth in rich medium (Anderson and Roth 1981; Huseby et al. 2020).

In summary, we have shown that Hfr-mediated chromosomal conjugation can, by at least three different mechanisms (F′-plasmid formation, linear fragment integration, circle integration), create hybrid bacterial strains in which the genome of the recipient has been enlarged by the addition of donor chromosomal DNA. The net increases in genome size observed in these 11 hybrids ranged from ~158 kb to 1.3 Mb and in 10 out of 11 cases, hybrid genomes were associated with a reduction in growth rate.

Experimental Evolution of Transconjugants with Enlarged Chromosomes

Escherichia coli and *S. enterica*, although evolving as separate species for >100 Myr, have a largely co-linear arrangement of functional orthologues on their respective chromosomes (Ochman and Groisman 1994). Consequently, each hybrid

strain with an enlarged chromosome is a partial merodiploid carrying orthologous sequences from both species. This raises the question, whether genetic adjustments selected to improve growth fitness would occur primarily by alterations to donor or recipient DNA, or to DNA from both species. In particular, strains in which the chromosome was enlarged without replacing any recipient DNA (circle integration) would have the possibility to completely reverse any associated fitness costs by precisely removing the acquired DNA. To address this question, we performed experimental evolution of two hybrid strains (CH7199 and CH8726) and asked whether and how the chromosomal architecture would change to accommodate the acquired DNA and increase growth fitness (fig. 3). Five independent lineages per strain were serially passaged for 100 generations in rich medium under each of two different conditions: without antibiotic selection [Luria broth (LB)], and in the presence of the appropriate addition-selective antibiotic (AB).

After the tenth cycle of growth (100 generations), individual clones were isolated from each culture as follows: a clone evolved in the absence of antibiotic selection that had lost the addition-associated resistance marker (LB) and a clone evolved in the presence of the addition-selective antibiotic (AB). Note that 100 generations of evolution in LB was associated with the loss of the addition-associated antibiotic-resistance marker from the great majority of the population, showing that loss of at least some part of the acquired *E. coli* DNA was a very common and rapid response to growth selection (supplementary fig. S3, Supplementary Material online). These 20 clones were stored at −80 °C and were used for measurements of the relative growth fitness of the evolved strains, and for WGS to analyze the genetic alterations that occurred during the experimental evolution. All of the evolved clones increased in relative growth rate from their

initial low values (CH7199 ~0.76 and CH8726 ~0.57), in some cases reaching a value of 1 (fig. 3). The For CH8726, the difference in relative growth rate improvement is significantly better for the strains evolved in LB versus the strains evolved with selection to maintain the antibiotic-resistance marker ($P = 0.006$ for evolved in LB vs. AB, Mann–Whitney test).

Genetic Changes that Occurred During Experimental Evolution of Hybrid Strains

Evolution of CH7199 (957 kb Circle Inserted Close the Terminus of Replication)

All five clones evolved in LB (without selection to maintain the Rif[R] marker) deleted large parts of the acquired *E. coli* DNA together with shorter adjoining sections of the *S.* Typhimurium chromosome (fig. 3a). As a result of these deletions, all five evolved strains are interspecies hybrids in which the chromosome length is 10–209 kb longer than that of the parental *Salmonella* strain. In four out of five strains, the deletions can be explained by recombination between homologous sequences (supplementary text S1, Supplementary Material online). All five clones evolved in the presence of the addition-selective antibiotic had acquired mutations associated with the movement of IS elements into coding sequences distributed around the genome, with two of them also acquiring deletions that were smaller than those acquired by the clones evolved without antibiotic selection (fig. 3 and supplementary text S1, Supplementary Material online). One clone, CH9855, that acquired an IS10 insertion into *mutS*, a key enzyme of the DNA mismatch repair system, accumulated 34 SNPs distributed across the chromosome (31 SNPs in *Salmonella* DNA and 3 in *E. coli* DNA), probably associated with having an increased mutation rate due to the *mutS* mutation (supplementary table S4, Supplementary Material online). The main conclusions from the experimental evolution of CH7199 are that, in the absence of antibiotic selection to maintain the acquired *E. coli* Rif[R] marker, the evolved hybrid strains rapidly acquired large deletions in the area of the *E. coli* DNA insertion that extended out on either side into *Salmonella* DNA. On average, these changes resulted in close to a full restoration of growth fitness. The deletions are not identical and have a variety of different endpoints (supplementary text S1, Supplementary Material online) but in every case, the resulting strain has a hybrid chromosome, with DNA from both species, and which is larger, by up to 209 kb, than the chromosome of the parental *Salmonella* strain (fig. 3a). The trajectory of evolution in these strains is largely determined by the interplay between selection (for growth fitness and/or maintenance of resistance) and the locations of repetitive sequences that can act as substrates for recombination leading to deletion formation (supplementary text S1, Supplementary Material online).

Evolution of CH8726 (~1.3 Mb Circle Inserted Close to the Origin of Replication)

CH8726 is an interesting hybrid because its chromosome is not only increased in length by the addition of more than 1,000 *E. coli* genes, but it has also acquired a second origin of chromosome replication. All ten of the clones isolated after evolution of this strain, regardless of the selection conditions (LB or AB), had acquired large deletions that ranged in size from 1,259 to 1,390 kb, reducing net chromosome length in many cases to below that of the *Salmonella* parent strain (fig. 3b). The deletions always included one of the two origins of replication. All ten evolved clones had deleted some *Salmonella* chromosomal DNA but also retained some of the acquired *E. coli* DNA. Accordingly, all evolved clones had hybrid chromosomes, with DNA originating from both species. Among the five clones evolved in LB, two retained the *Salmonella* origin of replication while three retained the *E. coli* origin. The only genetic event that occurred in these five clones was a single deletion (no point mutations), that in four out of five cases resulted in a chromosome with the original *Salmonella* length, and in one case, a chromosome that was slightly longer by 4 kb. Each of these deletions was unique and occurred by recombination between different short regions of perfect homology, ranging in length from 8 to 67 bp (supplementary text S2, Supplementary Material online).

All clones of CH8726 evolved in the presence of the addition-selective antibiotic retained the *Salmonella* origin, probably because the proximity of the drug-resistance marker to *oriC* restricted the possibility for deletions extending in that direction (fig. 3b). These five clones had acquired non-identical deletions of 1,390 kb, that removed both *E. coli* and *Salmonella* DNA, creating hybrid chromosomes that were in all cases shorter by 78 kb than the original length of the *Salmonella* recipient chromosome. These non-identical deletions occurred by recombination between short regions of sequence homology ranging in length from 14 to 26 bp (supplementary text S2, Supplementary Material online). Three of the kanamycin (KAN)-resistant strains had also acquired one or two IS insertions in coding sequences, in addition to the deletions (supplementary text S2, Supplementary Material online).

Summary and Conclusions

We identified three routes to genome enlargement following bacterial conjugation. One of these, circle formation and recombination into the chromosome, is in principle universal and could occur between closely or distantly related species. The circle integration mechanism enlarges the chromosome without replacing existing genetic

material, and does not require donor and recipient genomes to have close similarity, either in overall nucleotide identity or in gene order. We found that the reductions in exponential growth rate suffered by hybrids with integrated circles of foreign DNA could be rapidly and almost completely ameliorated in <100 generations of experimental evolution. This rapid adaptive evolution occurred primarily by a process of deletion, where the substrates for recombination were short regions of sequence identity, in several cases <10 bp (supplementary text S1 and S2, Supplementary Material online). Interestingly, in all ten independent evolution experiments made on strains with hybrid chromosomes (without selection to maintain the addition-associated antibiotic-resistance marker), the adaptive deletions included parts of both the *E. coli* and *Salmonella* chromosomal DNA. Consequently, all of the resulting high-fitness strains had chromosomes that were hybrids, with DNA sequences from both species. Because deletions dominated the rapid adaptive evolutionary trajectory, the evolved chromosome architectures are strongly influenced by the interplay between the selection pressure for increased growth fitness and the chromosomal distribution of sequences that can act as substrates for deletion by homologous recombination. Because it occurs at a high frequency relative to point mutations, recombination between repetitive sequences is likely to be the dominant initial mode of evolution of hybrid adaptation to increase relative fitness. A similar mode of chromosome evolution has recently been observed in strains in which niche adaptation selects bacteria with a large tandem duplication in the chromosome, and where subsequent evolution is initially dominated by mutations deleting parts of the duplicated region (Brandis and Hughes 2020; Cao et al. 2022). In conclusion, our data show that conjugational HGT can enlarge a bacterial genome by the recombination of megabase lengths of DNA into the chromosome, and that subsequent evolution to ameliorate growth fitness costs can occur rapidly and very frequently results in the creation of strains with irreversibly hybrid chromosomes.

Materials and Methods

Bacterial Strains and Genetic Constructions

All bacterial strains are derivatives of either *S. enterica* serovar Typhimurium LT2 or *E. coli* K-12 MG1655. Exact strain genotypes are shown in supplementary table S1, Supplementary Material online. For the placement of antibiotic-resistance gene cassettes into the *S.* Typhimurium genome, Lambda Red recombineering with the pSIM6 plasmid (Sharan et al. 2009) was used. Universal chloramphenicol (CHL) resistance cassettes were placed into the *S.* Typhimurium genes *yiiQ* (TH11925) and *STM1190* (TH11926) for selection of tranconjugants, and a universal rifampicin (RIF) resistance

cassette into *STM1190* (TH11954) for Bioscreen measurements with RIF. All oligonucleotides are listed in supplementary table S5, Supplementary Material online.

Media, Growth Conditions, and Antibiotic Concentrations

Liquid cultures of bacteria were grown in LB at 37 °C with 180–200 rpm shaking, culturing on solid medium was done on Luria Agar (LB with 1.5% agarose, LA) at 37 °C overnight. As appropriate, LA was supplemented with KAN at 50 mg/l; RIF at 100 mg/l; gentamicin (GEN) at 50 mg/l or CHL at 60 mg/l.

Hfr Conjugation Protocol

All conjugations were done using an *E. coli* Hfr donor (CH6941) and an *S.* Typhimurium recipient (TH10684, TH11925, and TH11926). Liquid cultures of the *E. coli* donor and an *S.* Typhimurium recipient were mixed in equal volumes. A nitrocellulose filter (pore size 0.22 μm, diameter 25 mm; Sigma Aldrich) was placed on an antibiotic-free LA plate. One hundred microliters of the donor–recipient mixture was evenly spread evenly on the filter and the plate then incubated at 37 °C undisturbed for 12 h. Using sterile tweezers, the filters were then picked up from the LA plate and transferred to a 50-ml microfuge tube containing 1 ml of 1× phosphate-buffered saline. To resuspend the bacteria, tubes were vortexed for 45 s. Cells were plated in 250 μl aliquots on LA plates containing CHL together with KAN, RIF, or GEN as appropriate. For an overview, over which strain pairs and selections were used to obtain each transconjugant, see supplementary table S2, Supplementary Material online. Selective plates were incubated at 37 °C for 24–48 h and colonies were picked and pure streaked on the selective medium. PCR was used as an initial test of the presence of genetic material from both donor and recipient strains in the one genome, and also to test whether any hybrid genomes carried orthologues of particular genes from each species (putative hybrids with additional DNA enlarging the genome). Hybrid strains with PCR-confirmed additions were stored in LB containing 10% glycerol at −80 °C for further analysis.

Polymerase Chain Reaction

Drug-resistance cassettes for use in Lambda red recombineering were PCR amplified using VeraSeq 2.0 High-Fidelity DNA Polymerase (Enzymatics, Massachusetts, USA), according to the manufacturer's protocol. Prior to recombineering PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Germany). To identify transconjugants with additions, a gene in close proximity of the transferred *E. coli* resistance cassette (conjugation selecting KAN—*yiiD*; conjugation selecting RIF—*csgA*; conjugation selecting GEN—*pyrG*); as well as the *S.* Typhimurium

orthologue were PCR amplified using Taq PCR Master Mix (Thermo Scientific, Waltham, USA). Oligonucleotides used for PCR are listed in supplementary table S5, Supplementary Material online.

Whole-Genome Sequence and Analysis

To determine the genetic architecture of the genetic hybrids, both short-read (Illumina) and long-read (Nanopore) sequencing technologies were utilized. For all genomic DNA (gDNA) preparations, bacteria were scraped directly from an LA plate containing the antibiotic selecting for the additional *E. coli* DNA (KAN, RIF, or GEN, as appropriate) and resuspended in 0.9% NaCl at a density of $\sim 10^9$ cfu/ml. For Illumina sequencing, gDNA was prepared using MasterPure DNA and RNA Purification Kit (Epicenter, Chicago, USA). For Nanopore sequencing gDNA was prepared using Genomic DNA Buffer Set and Genomic-tip 100/G (Qiagen, Germany). Extracted gDNA was resuspended in Elution Buffer (Qiagen, Germany; 10 mM Tris-Cl, pH 8.5) and incubated at 55 °C for 2 h to allow for resuspension. gDNA concentrations were measured in a Qubit 3.0 Fluorometer (Invitrogen, California, USA) and diluted in milliQ water (Merck KGaA, Germany) to a final concentration of 0.2 ng/ml for Illumina sequencing and 52 ng/ml for Nanopore sequencing. For Illumina sequencing, samples were prepared according to Nextera XT DNA Library Preparation Guide (Illumina Inc., Wisconsin, USA). Prior to the sequencing run, the sample's DNA fragment size distribution was verified using the Agilent High Sensitivity D1000 ScreenTape System (Agilent Technologies, California, USA). Sequencing was performed in a MiSeq™ desktop sequencer, according to the manufacturer's instructions (Illumina Inc.). Samples were prepared for Nanopore sequencing following the Rapid Barcoding Sequencing Protocol (SQK-RBK004, Oxford Nanopore, UK). Sequencing was performed in a MinION Mk1B flow cell (Oxford Nanopore, UK). Nanopore sequencing data were combined with each strain's corresponding Illumina sequencing data using the hybrid assembler function of the Unicycler (Wick et al. 2017). All sequence analyses were done in version 11.0.1 of the CLC Genomics Workbench (CLCbio, Qiagen, Denmark).

Fitness Measurements

Bacterial growth fitness was measured by changes in optical density (OD) over time. For fitness measurements of un-evolved transconjugants, bacterial colonies were scraped off an LA plate containing the appropriate antibiotic (to maintain selection for the genomic addition), resuspended in 0.9% NaCl to a density of $\sim 2 \times 10^9$ cfu/ml then diluted 1:1,000 in LB plus addition-selective antibiotic. For fitness measurements of experimentally evolved transconjugants, liquid overnight cultures were initially grown in antibiotic-

free LB (for cultures evolved in LB), or LB plus addition-selective antibiotic (for cultures evolved in the presence of antibiotic), then diluted 1:1,000 in the same medium. Three hundred microliters of each dilution was then pipetted into a honeycomb well plate and incubated at 37 °C with continuous shaking in a Bioscreen C machine (Oy Growth Curves Ab Ltd, Finland). Measurements of OD_{600} were taken every 5 min for 24 h. All Bioscreen growth measurements were performed on four independent biological replicates per strain. Minimal doubling time was calculated by determining the linear slope of these measurements at maximum exponential growth. Relative fitness was calculated by dividing minimal doubling times of the transconjugants with the minimal doubling time of the *S.* Typhimurium recipient. For fitness measurements performed in LB plus RIF, the parental comparator strain was TH11954 that carried the same RIF resistance cassette as the RIF-resistant transconjugants (supplementary table S1, Supplementary Material online).

Experimental Evolution and Stability Measurements

Two hybrid strains (CH7199 and CH8726) were serially passaged in LB for 100 generations by transferring 2 µl of an overnight culture into 2 ml of fresh LB every day for 10 days. Five independent lineages per strain were passaged in antibiotic-free LB, and another five were passaged in LB plus the antibiotic selective for the additional *E. coli* DNA (CH7199—RIF, CH8726—KAN). Cultures were grown at 37 °C at 180 rpm on a rotary shaker. At generations 10, 30, 50, 70, and 90 serial dilutions of all lineages grown in antibiotic-free LB were plated on LA and LA plus the addition-selective antibiotic. Colonies were counted after incubation at 37 °C overnight to determine the proportion of bacteria that still retained the resistance marker. After evolution, one clone from each lineage grown in LB and one clone from each lineage grown in LB plus the addition-selective antibiotic were picked and WGS.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

Data Availability

The whole-genome sequence data underlying this article have been deposited at the NCBI Bioproject database. Illumina sequences of parental *Salmonella* Typhimurium and *E. coli* strains (named TH6767 and CH6459, respectively) can be found at PRJNA667834, http://www.ncbi.nlm.nih.gov/bioproject/667834. Illumina and Oxford Nanopore sequences of interspecies hybrids and Illumina sequences of evolved hybrids can be found at PRJNA692818, http://www.ncbi.nlm.nih.gov/bioproject/692818.

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