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Integration of Heterologous Plasmid DNA into Multiple Sites on the Genome of *Campylobacter coli* following Natural Transformation

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The efficiency of homologous recombination in *Campylobacter coli* following the introduction of DNA by natural transformation was determined by using a series of nonreplicating integrative vectors containing DNA fragments derived from the *C. coli* catalase gene. Homologous recombination occurred with as little as 286 homologous bp present and was not detected when 270 bases of homology was provided. Instead, when plasmids with little or no homology to the chromosome were introduced by natural transformation, the vector DNA became chromosomally integrated at random sites scattered throughout the *C. coli* genome. Southern analysis and nucleotide sequencing revealed that recombination had occurred between nonhomologous sequences and can therefore be described as illegitimate. There were at least five different recombination sites on plasmid pSP105. The ability of *C. coli* to acquire heterologous plasmids by natural transformation, and maintain them by chromosomal integration following illegitimate recombination, has fascinating implications for the genomic diversity and evolution of this species.

Most strains of *Campylobacter coli* and some strains of *Campylobacter jejuni* are naturally competent and, consequently, can be transformed with naked DNA with no special treatment (14, 15). Heterospecific transformation, however, is extremely inefficient because the lack of homology to chromosomal DNA sequences inhibits the successful integration of the heterologous donor DNA into the genome of the recipient (14). The frequency of plasmid transformation in campylobacters, as is the case for other bacterial transformation systems (13), is very low because of the partial degradation of plasmid DNA during uptake (14). If, however, homologous DNA is introduced into the donor molecule, then the homologous region can facilitate the integration of the vector into the host genome by single crossover via "Campbell-like" recombination and transformation efficiencies increase (5, 14). The resulting transformants contain the inserted vector flanked by a duplication of the original chromosomal fragment carried on the plasmid. Integrational plasmids, which are unable to replicate extrachromosomally in their chosen host and which make use of this mechanism, have recently been developed for use in *C. coli* (3). By using this system, we have systematically investigated the dependence of recombination frequency on the amount of homology to chromosomal DNA sequences by using DNA fragments derived from the *C. coli katA* gene (4). Here we show that homologous recombination was not detected when less than 286 bases of homology between plasmid and chromosome was provided. Furthermore, this study reveals that in the absence of functional homology, heterologous plasmid DNA introduced into *C. coli* by natural transformation can integrate at random sites into the genome by illegitimate recombination.

Characterization of requirements for homologous recombination. Derivatives of integrational campylobacter vector pSP105 (Fig. 1) (3) containing various lengths of *C. coli* chromosomal DNA (Table 1) derived from the *katA* gene (4) were constructed. Since pSP105 can replicate only in *Escherichia coli*, the resulting plasmids should confer tetracycline resistance on *C. coli* only after integration into the chromosome (3). The plasmids were introduced into *C. coli* UA585 by natural transformation essentially as described by Wang and Taylor (14), and transformants were recovered by selection for tetracycline resistance (3).

When the lengths of homologous DNA in the integrable vectors decreased from 567 to 125 bases, a concomitant decrease in transformation efficiency from 5×10^{-11} to 3×10^{-13} was apparent (Table 1). Moreover, the location of integration was determined by the size of the homologous insert. Thus, when transformants containing pCK4, which has a 567-base homologous insert, were examined, all contained the plasmid integrated into the *katA* gene (Table 1), as assessed by

TABLE 1. Sequence length requirements for homologous recombination into the *C. coli katA* gene

Donor plasmid[a]	Length (bases) of sequence with homology to chromosome	Transformation efficiency[b]	Frequency of *katA*-specific integration (%)[c]
pSP105	NA[d]	4×10^{-13}	NA
pCK1	125	3×10^{-13}	ND[e]
pCK12	175	3×10^{-13}	ND
pCK2	270	2×10^{-13}	ND
pCK14	286	8×10^{-13}	10
pCK17	370	5×10^{-12}	10
pCK4	567	5×10^{-11}	100

[a] The donor DNA (300 μg in 300 μl of Tris-EDTA buffer) was from *E. coli* JM101.

[b] Number of tetracycline-resistant transformants per microgram of plasmid DNA per cell.

[c] Represents the percentage of transformants arising from site-specific integration. Specificity of integration was determined by Southern hybridization with the relevant homologous probes contained in the derivatives of pSP105 (data not shown), and at least 10 transformants were tested for each case.

[d] NA, not applicable.

[e] ND, not detected (less than 1% of transformants). Similar results were reproducibly obtained in several experiments.

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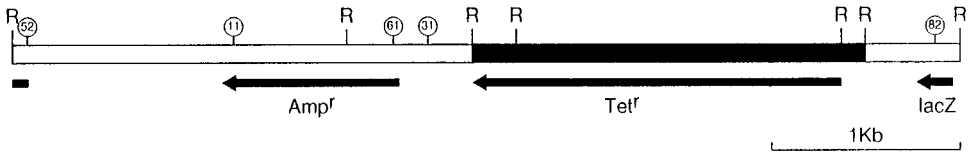

FIG. 1. Partial restriction enzyme map of the integrative plasmid pSP105. The three open reading frames are indicated as *lacZ*, Amp^r, and Tet^r. The filled region in pSP105 represents the extent of the sequence which did not appear to mediate chromosomal integration. The sites which mediated the integration of pR11, pR31, pR52, pR61, and pR82 are denoted by circled numbers. R, *Rsa*I.

phenotypic analysis and Southern hybridization (data not shown). In contrast, only 10% of the transformants derived from pCK14 and pCK17, containing *katA* fragments of 370 and 286 bases, respectively, harbored plasmids integrated specifically into *katA*. Furthermore, no site-specific integration into the *katA* gene was detected when homologies of 125 and 175 bases were provided. When pSP105, which has no extensive homology to the *C. coli* genome, was used as a donor molecule, transformation still occurred and with efficiencies similar to that seen for pCK1 and pCK12 (Table 1). To assess the nature of these integrative events, the chromosomal DNA from 16 transformants containing integrated copies of pSP105 was isolated (11) and subjected to analysis by Southern hybridization as described previously (12), with linearized pSP105 as a DNA probe. The hybridization results (Fig. 2A) demonstrate that the site of plasmid integration was different for at least 9 of the 16 independently isolated clones, as deduced from the size variability of the hybridizing fragments. Similar results were obtained when transformants containing pCK1, pCK2, and pCK12 were analyzed in the same way (data not shown), suggesting that although the 125-, 175-, and 270-base inserts in these vectors had homology to the chromosome, the length of each homologous sequence was not sufficient to permit detectable homologous recombination in *C. coli.*

Integration of DNA occurs at multiple sites within the heterologous plasmid. There are six sites for *Rsa*I in pSP105 (Fig. 1). The integration of this plasmid into the chromosome must occur at regions bordered by at least two *Rsa*I sites. The particular restriction fragment delineated by any two sites will not, therefore, be present in the pSP105 replicon contained in a particular integrant if the recombination has occurred at a region between these restriction targets. To determine the location of the site(s), within the heterologous plasmid DNA that mediated the integrative events, genomic DNA was isolated from transformants containing integrated copies of the plasmid, digested with *Rsa*I, and analyzed by Southern hybridization with pSP105 as a DNA probe. From the hybridization results, it is apparent that integration had occurred via sequences contained in the 1,799-, 676-, and 474-base fragments since these bands were absent from the DNA of at least one integrant (Fig. 2B). In most cases, two additional bands of homology were apparent and these represent fragments generated by restriction at *Rsa*I sites within pSP105 and in the chromosomal DNA flanking the recombination junction. In three cases, only one additional band was apparent and here the additional *Rsa*I fragments may have been too small to be resolved by the agarose gel. These results suggest that the recombination is mediated by multiple sites within pSP105. No transformants in which sites present in the 1,743-, 225-, or 110-base *Rsa*I fragment of pSP105 had mediated the recombination were apparent (Fig. 2B). This may reflect not a lack of suitable sites in this DNA but merely the facts that each of

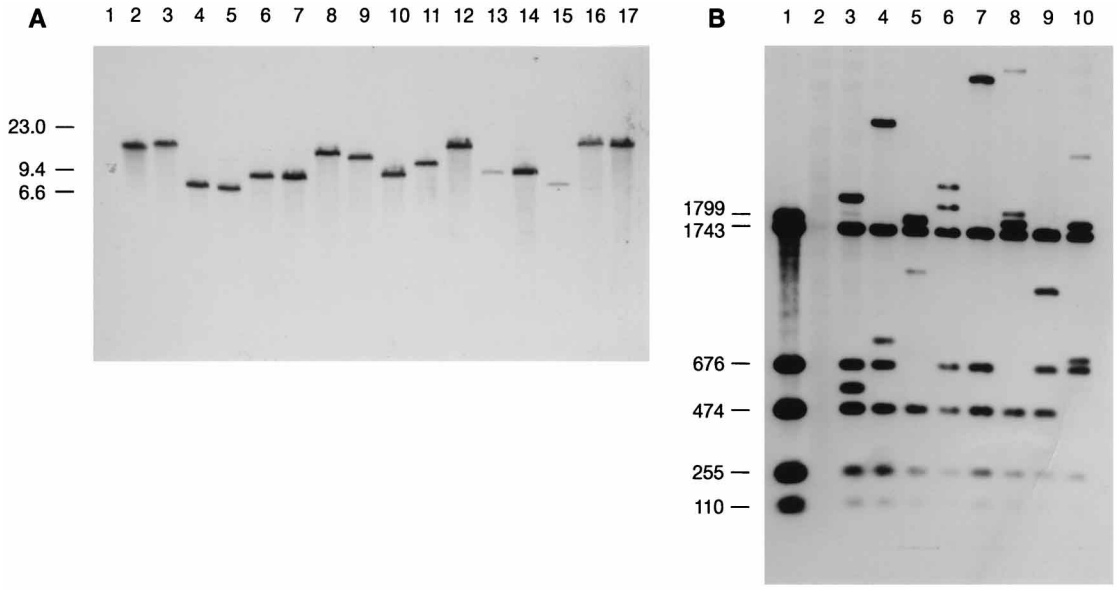

FIG. 2. Demonstration of nonhomologous recombination in *C. coli* by Southern analysis. (A) Chromosomal DNA from *C. coli* UA585 (lane 1) and from 16 tetracycline-resistant integrant transformants (lanes 2 to 17) was digested with *Eco*RV and probed with linearized pSP105. The positions of molecular standards are indicated by their sizes (in kilobases). (B) pSP105 DNA (lane 1) and chromosomal DNA from *C. coli* UA585 (lane 2) and from eight tetracycline-resistant integrant transformants (lanes 3 to 10) were digested with *Rsa*I and probed with linearized pSP105. The sizes of the *Rsa*I restriction fragments of pSP105 are given in bases.

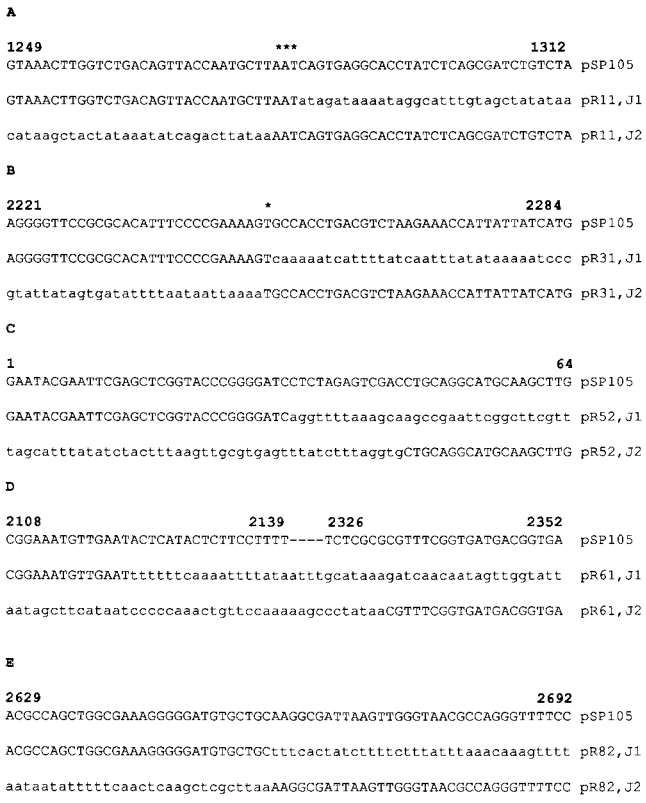

FIG. 3. Nucleotide sequences of recombination junctions. The nucleotide sequences on both sides (J1 and J2) of the recombination junctions in resolved integrons pR11 (A), pR31 (B), pR52 (C), pR61 (D), and pR82 (E) are given. Chromosomal and plasmid sequences are in lower- and uppercase type, respectively. The nucleotide sequence of pSP105 prior to the recombination was derived from that of pGEM-4Z (Promega). The additional bases added in pR11 and pR31 during the recombination event are denoted by asterisks.

these fragments is present in the *tetO* gene (Fig. 1) and that integration at sites within these would have disrupted the integrity of this gene, leading to the loss of tetracycline resistance by which the transformants were selected.

Nucleotide sequence at recombinant DNA junctions. To clone the chromosomal-plasmid junctions from transformants, we took advantage of the ability of pSP105 to replicate in *E. coli* (3). Chromosomal DNA from integrants represented in lanes 3, 5, 7, 8, and 10 of Fig. 2B was digested with *Eco*RV, which has no target site within pSP105, and ligated at DNA concentrations of less than 10 μg ml^{-1} to direct the preferential formation of circular monomers. The ligation mixture was used to transform *E. coli* JM101. Recircularization of the DNA fragments composed of the complete pSP105 replicon and adjacent chromosomal DNA resulted in the formation of hybrid plasmids from each transformant which were designated pR11 (lane 3), pR31 (lane 5), pR52 (lane 7), pR61 (lane 8), and pR82 (lane 10). Based on the data in Fig. 2B, the points of insertion were estimated and oligonucleotides were designed to sequence across the recombinant DNA junctions. Illegitimate recombination between plasmids and chromosome can occur in *Bacillus subtilis* (1, 6) and *Streptomyces* spp. (9, 10) via short stretches of homology. If integration of plasmids in *C. coli* had occurred via this mechanism, the region of homology would have been expected to be duplicated during integration by the "Campbell-like" recombination mechanism. However, the results of the nucleotide sequence determination (Fig. 3) indicated that no significant target site duplication had occurred, although an additional T base and AAT bases were found in pR31 and pR11, respectively. Furthermore, plasmids pR52 and pR61 contained deletions of 13 and 211 bases, respectively, in the pSP105-derived sequence at one of the recombination junctions.

Concluding remarks. Following natural transformation, integration of suicide vectors into the *C. coli* genome was shown to occur by two independent mechanisms. When 567 bases of homology was provided, homologous recombination appeared to be the dominant process. However, as the size of the homologous insert was reduced, this process became less efficient and transformants generated by this mechanism were not detected when 270 bases of homology between plasmid and chromosome was provided. Instead, transformants appeared to have arisen following random integration of plasmids into the chromosome. Furthermore, pSP105, which has no extensive homology to the *C. coli* genome, still gave rise to tetracycline-resistant transformants. The recombination which generated these transformants occurred between sequences with little or no homology and can therefore be described as illegitimate. There were at least five different recombination sites on plasmid pSP105, and the integration appeared to occur at random sites scattered throughout the *C. coli* genome. The homology requirement for homologous recombination in *C. coli* appears to be higher than that measured for other bacteria. In *B. subtilis*, for example, approximately 70 bases is required for detectable homologous recombination (8). The reason for this variation is unclear, but it may reflect differences between the efficiencies of the mechanisms of illegitimate and homologous recombination in these species.

Illegitimate recombination is rare in prokaryotes, although it has been shown to occur at low frequencies in *B. subtilis* (1, 6), *Mycobacterium* spp. (7), and *Rhodococcus fascians* (2). Although the molecular basis has not been fully elucidated in any system, it may be associated with efficient DNA repair mechanisms and with ligation of linear DNA fragments into the chromosome (2, 7). Such a mechanism may account for the illegitimate recombination observed in *C. coli* since the integration occurred at sites without significant homology and lacking obvious structural features. Moreover, two of the integrants contained deletions in the plasmid-derived DNA sequence, suggesting that the mechanism of integration involved the action of nucleases. The ability of *C. coli* to acquire a heterologous plasmid by natural transformation and maintain it by chromosomal integration following illegitimate recombination has fascinating implications for the genomic diversity and evolution of this species. Finally, the ability to select for insertions of a tetracycline resistance element at multiple sites on the chromosome may provide a useful tool for the genetic analysis of *C. coli.*

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