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Genetic and Physical Map of Plasmid NR1: Comparison with Other IncFII Antibiotic Resistance Plasmids

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INTRODUCTION

Plasmids are circular extrachromosomal genetic elements that are ubiquitous among the bacterial genera and are also found in some eucaryotic organisms (17, 39, 82, 95). Plasmids encode a number of specialized functions that are generally nonessential for their bacterial hosts. However, these plasmid-encoded functions provide their host cells with versatility and adaptability for growth and survival under a variety of conditions. Plasmids and the transposable elements that they often contain may also have played an integral role in the evolution of bacterial species by promoting the distribution and exchange of genetic information (5). The various plasmid-mediated functions include genetic transfer, bacteriocin production and resistance, production of and resistance to antibiotics, resistance to heavy metals and deoxyribonucleic acid (DNA) damaging agents, metabolism of carbohydrates and hydrocarbons, toxin production, virulence and colonization factors, hemolysin production, and tumorigenicity and nitrogen fixation in plants. In addition, plasmids encode genes and functions that make them capable of autonomous replication. In combination with their dispensable nature, this has made them attractive as model systems for the study of DNA replication and its coordination with cell growth and division. Recently, plasmids have also become indispensable components of the methods of modern molecular biology research.

The three major classes of plasmids that have been characterized most extensively include plasmid F, colicinogenic (Col) plasmids, and drug resistance (R) plasmids (17, 39, 82, 95, 139). The F plasmid is the classic fertility factor that is capable of promoting its own transfer, the transfer of other plasmids, and the transfer of the host bacterial chromosome during bacterial conjugation. Col plasmids mediate the production of colicins, which are toxins that kill certain species of bacteria. Col plasmids also confer resistance to their bacterial hosts for the particular colicin that they specify. R plasmids confer drug resistance to their bacterial host cells, sometimes simultaneously to multiple antibiotics. Some Col plasmids and R plasmids are also sex factors that can promote their own transmission to other bacteria.

When the multiplicity of various types of plasmids was first investigated, it seemed unlikely on the basis of the many varied phenotypes they exhibited that there would be much shared homology among them. Recently, however, it has become apparent that plasmids of seemingly diverse origin share many functions that may be evolutionarily related. Individual studies often have focused on one or only a few genes and functions on selected plasmids and have not considered the entire complement of plasmid genes and their possible interrelationships. This review focuses on the genomes of R plasmids, and in particular on that of the very well-characterized R plasmid NR1, as an illustration of the diversity of interesting plasmid-encoded functions that may be related among many different types of plasmids.

R-plasmid NR1, which is also referred to as R100 or 222, is a 94.5-kilobase (kb), self-transmissible, multiple antibiotic resistance plasmid. NR1 is the original IncFII bacterial resistance factor, the so-called R factor, isolated by Rintaro Nakaya (107) in Japan in the late 1950s and is the archetype of a now large collection of similar R plasmids that have been discovered worldwide (27, 29, 62, 178). Owing to the ability of such plasmids to promote the spread of multiple antibiotic resistance among bacteria, they have created numerous health hazards for humans and other animals. R plasmids are common in bacterial strains whenever the use of antibiotics is common (115).

Bacterial plasmids have been classified by their incompatibility properties, which result in the inability of two closely related plasmids to be maintained together in the descendants of a single cell (26a, 27, 114). NR1 belongs to the FII incompatibility group, which also includes such well-characterized R plasmids as R1 and R6 (27). R plasmids of the IncFII group are composed of two genetically and physically distinguishable components (Fig. 1): a resistance transfer factor (RTF) that harbors the genes for self-transmissibility

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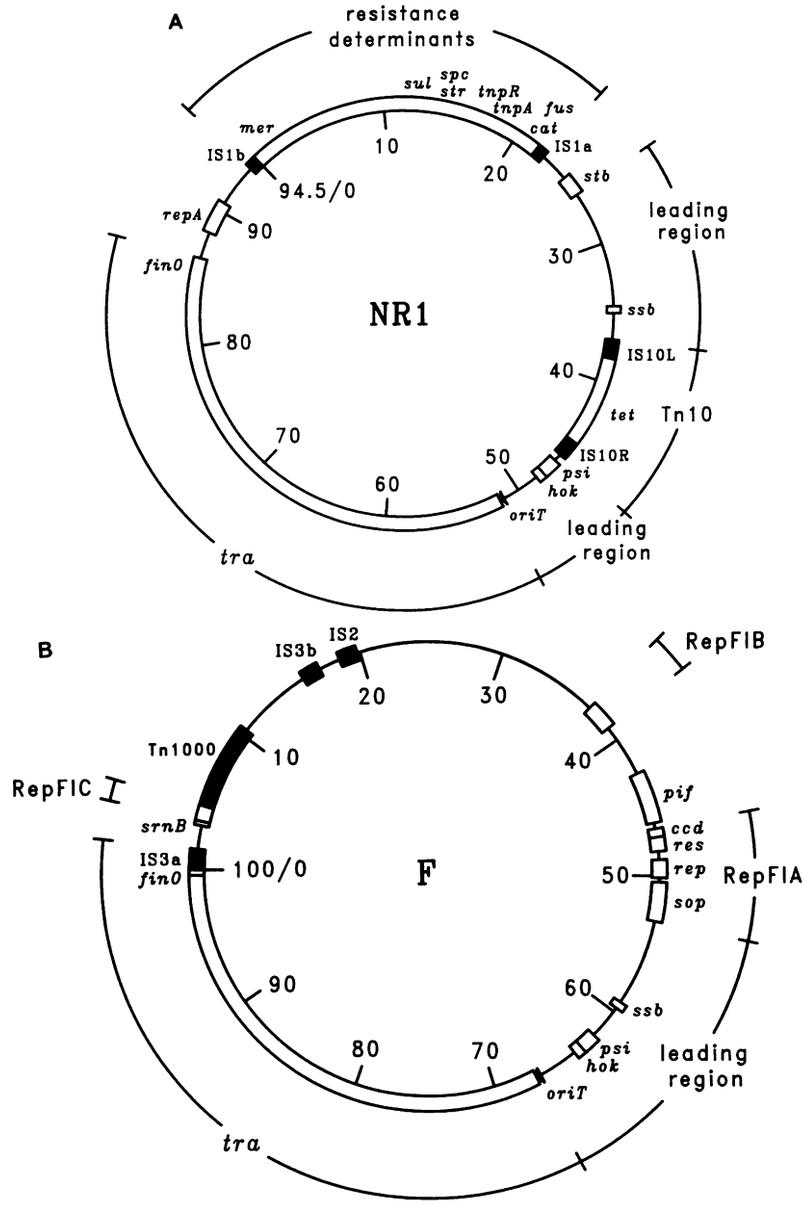

FIG. 1. Genetic maps of IncFII plasmid NR1 (A) and IncFI plasmid F (B). The kilobase coordinate scales begin at the clockwise end of insertion element IS*1*b in NR1 and at the counterclockwise end of IS*3*a in F. The 94.5-kb genome of NR1 can be divided functionally into the antibiotic resistance determinant component, from 93.7 through 22.5 kb, and the RTF component, from 22.5 through 93.7 kb. The r-determinant is bounded by direct repeats IS*1*b and IS*1*a. All symbols for NR1 are described in the text and in Table 2. More detailed maps of the NR1 genes are presented in Fig. 3 and 4. The map of F is derived from the map presented by Willetts and Skurray (183). During conjugal transfer of these plasmids, which begins at *oriT*, the leading region enters the recipient cell first and the *tra* region last. In comparison with the F plasmid, the leading region of NR1 is interrupted by transposon Tn*10*. Therefore, the RTF has sometimes been referred to as RTF-Tc, owing to its ability to confer resistance to tetracycline. The other RTF functions include control of NR1 DNA replication (*repA*) and stable plasmid inheritance (*stb*). For F, the analogous (but apparently not homologous) replication and stability functions are located within the RepFIA region (7, 76, 81, 117, 118, 157, 172).

(*tra*) and autonomous replication (*rep*), and a resistance determinant (r-determinant) component that harbors the majority of the resistance genes (26, 29). The RTF and r-determinant components are separated by direct repeats of the insertion sequence IS*1* (67, 132). NR1 also is the prototypic source of the Tn*10* transposable element, which confers tetracycline resistance (75). Tn*10* is located on the RTF component of NR1 (Fig. 1). IncFII plasmids are compatible with plasmid F, which belongs to group IncFI. However, the *tra* genes on the RTF of IncFII plasmids are closely related to those of plasmid F (Fig. 1), hence the name FII for the incompatibility group of these R plasmids (27).

In this review we bring the physical and genetic map of NR1 (Fig. 1) up to date and compare it with those of some other IncFII plasmids and also with plasmid F, whose map was recently updated by Willetts and Skurray (183). Bacte-

rial strains that harbored NR1 were provided by R. Nakaya to other researchers (see below), who have sometimes referred to the plasmid by other names such as R100 or 222. This has sometimes resulted in confusion in the literature concerning the relationship of NR1 to the other members of the IncFII group of R plasmids. Occasionally, tables or lists of IncFII plasmids have included NR1 and R100 as separate entries. To help allay such confusion, we also present a brief historical perspective that describes the evolution of R-plasmid nomenclature during the early studies of NR1 and its relatives.

DISCOVERY AND CHARACTERIZATION OF RESISTANCE PLASMIDS

During the late 1950s in Japan, there was an alarming increase in the prevalence of antibiotic resistance among clinical isolates of bacterial pathogens that paralleled the clinical use of antibiotics (reviewed in references 102 and 178). R. Nakaya and his associates (107) investigated *Shigella flexneri* 2b strain 222/CTS, which was isolated from a clinical sample of human feces, and found it to be simultaneously resistant to chloramphenicol, tetracycline, and streptomycin (hence, CTS). Watanabe and Fukasawa also found the strain to be resistant to sulfonamides (179). In their initial studies, this strain was found capable of transferring the multiple resistances to other strains of *Shigella* spp. as well as to numerous other members of the family *Enterobacteriaceae* through cell-to-cell contact, i.e., by conjugation (107, 179). Usually all four resistances were transferred together, but there were occasional rare examples of the transfer of only chloramphenicol and streptomycin or tetracycline resistance. Unlike previous studies of conjugation promoted by the F factor, the transfer of chromosomal markers from donor to recipient cells was not observed during resistance transfer. Transduction of resistance by bacteriophage P1 *kc* indicated that all of the resistances and their transferability were linked closely to each other, but not to chromosomal genes. Occasional single-step reversion to sensitivity for all four antibiotics was also observed, which suggested that there might be a single, autonomous cytoplasmic element responsible for the antibiotic resistances and their transfer, which Nakaya called *Rta* for resistance transfer agent (107). The term R-factor, subsequently proposed by S. Mitsuhashi, was eventually adopted to describe such elements (102), and the R-factor of *S. flexneri* 2b strain 222/CTS was given the name NR1, for National Institutes of Health (Tokyo) R-factor number 1 (147). More recently, such elements have generally been referred to as R plasmids. Numerous other R plasmids were soon isolated and characterized, but NR1 was and has remained the most thoroughly studied for many years. Like many other R plasmids (161), NR1 was eventually found also to confer resistance to mercuric ions (77, 78).

In their initial characterization of NR1, Nakaya et al. (107) reported that there was no effect on R-plasmid transfer if either donor or recipient strains were Hfr or F^-. However, they noted that the presence of NR1 in an Hfr donor strain significantly inhibited the transfer of chromosomal markers to F^- recipients. Nakaya provided T. Watanabe with *S. flexneri* 2b strain 222/CTS, and Watanabe and Fukasawa (180) demonstrated that the R plasmid, which they called RTF for resistance transfer factor, inhibited transfer of the F plasmid from donor strains that contained both RTF and F. Later, Watanabe and his associates would refer to this R-factor as 222, in reference to the original strain of *S. flexneri* from which it was isolated (40).

Y. Hirota obtained the resistant *S. flexneri* strain 222 from T. Watanabe and referred to the R plasmid it harbored as R_{100} (162). This designation was later modified to R100, i.e., without a subscript, by S. Mitsuhashi (61). Sugino and Hirota (162) reported the isolation of R_{100} mutants that were defective for transferrability but not for autonomous replication. They also reported the isolation by R. Egawa of mutant $R_{100\text{-}1}$, which had a fertility 10^3 to 10^4 more efficient than that of R_{100}, similar to that of the F plasmid. Like F, $R_{100\text{-}1}$ also could promote the Hfr-like transfer of chromosomal markers (162). Very recently, it was found by Ohtsubo and his colleagues (193) that mutant $R_{100\text{-}1}$ contained a single-base-pair insertion mutation in the gene *finO*, which is responsible for repression of conjugal transfer (31, 72, 182). At the same time they demonstrated that the F plasmid was also *finO*, by virtue of insertion of IS*3* into the *finO* gene of some presumed ancestor of F (193). This explained the original observations of Sugino and Hirota (162) concerning the relative transfer efficiencies of R100 (NR1) and F and the inhibitory effects of NR1 on transfer from Hfr (107) and F^+ strains (180). In a series of complementation experiments, Hirota et al. (65) found that some mutants of F that were defective for transfer (*tra*) could be restored to transmissibility by the presence of R100 (NR1). Likewise, *tra* mutants of the R plasmid could be complemented by F. These results suggested that the transfer genes of NR1 and F might be quite similar. Later, it was shown by Guerry and Falkow (56) by DNA-DNA hybridization experiments that F was approximately 40% homologous with IncFII group R plasmids. This homology is now known to result from their nearly identical *tra* operons (158, 182).

By transferring an F′*lac* plasmid from *Escherichia coli* to *Serratia marcescens*, whose chromosomal DNA has a high (58%) guanine-plus-cytosine (G+C) content, it was demonstrated that the F′ plasmid DNA could be visualized as a satellite band in an analytical CsCl density gradient (91). F′ DNA also could be purified from *S. marcescens* chromosomal DNA in a preparative CsCl density gradient formed during ultracentrifugation (91). Likewise, transfer of NR1 to *Proteus mirabilis*, which has a low (40%) G+C content, and to *S. marcescens* allowed visualization of R-factor DNA as satellite bands in CsCl density gradients that contained DNA isolated from these strains (40, 147). The R-plasmid DNA was reported to have a high molecular weight and was lost from strains "cured" of antibiotic resistance. The number of copies of NR1 DNA molecules present in *E. coli* and *S. marcescens* hosts was estimated to be approximately one for each bacterial chromosome (147), a conclusion later confirmed by more direct methods of analysis (103, 149, 190).

In their characterization of a series of deletion mutants of NR1 that had lost the ability to confer tetracycline resistance, Hashimoto et al. (61) observed that several of the mutants also had become defective in transfer. In addition, some of these R-plasmid mutants were lost at a relatively high frequency from the cells during subculture; i.e., they were unstable. Hashimoto et al. (61) proposed that a stability locus, *stb*, was deleted from the mutants. Further, they noted that the mutations did not inactivate the autonomous replication (*rep*) functions of the R plasmid, nor were *stb* deletion mutants complemented by introduction of stb^+ R plasmids into the same host cells (61). It was suggested that both *rep* and *stb* functions were required for stable maintenance of R plasmids (61, 62).

In the original characterization of NR1, it was observed that the behavior of this plasmid in a *P. mirabilis* host was quite different from that in other host strains. The number of

copies of the r-determinants was observed to be much greater in *P. mirabilis* than in *E. coli* (138, 147), and it was speculated that the r-determinants and RTF components of NR1 could dissociate in *P. mirabilis* (26, 144). Eventually, this behavior was shown to result from the amplification of the r-determinant component to form very large poly-r-determinant R plasmids with multiple, tandem copies of the r-determinants (124, 140, 141, 143, 144). Originally referred to as a "transition," this amplification of the r-determinant component conferred very high-level antibiotic resistance on the *P. mirabilis* host cells for chloramphenicol, streptomycin, and sulfonamide (63, 68). The high-level resistance to these antibiotics resulted from the high gene dosage of the antibiotic resistance genes on the poly-r-determinant R plasmids (63, 70). Interestingly, it was found that the level of mercuric ion resistance, genes for which also reside on the r-determinants, was independent of gene dosage (105). The level of tetracycline resistance, encoded by the RTF component of NR1, did not change in a transition. Eventually, the level of tetracycline resistance was found to be inversely related to gene dosage (70, 96, 170). The amplification of the resistance determinants of NR1 was the first well-characterized system of gene amplification, a phenomenon now commonly observed in many organisms (155, 156).

GENETIC AND PHYSICAL MAPPING OF IncFII PLASMID GENES AND FUNCTIONS

Using P1 *kc* transduction of NR1 to *E. coli* and P-22 transduction to *Salmonella typhimurium*, Watanabe and Fukasawa (179) proposed that the order of genetic markers on NR1 was: *sul-str-cat-tet*-RTF (for, respectively, resistance to sulfonamide, streptomycin, chloramphenicol, and tetracycline and for resistance transfer factor). Hashimoto and Mitsuhashi (61, 62) examined deletion mutants of NR1 and recombination between them and proposed the linkage order: (*stb*, *tet*, *tra*)-*rep*-(*sul*, *str*)-*cat*. The new markers represented stability (*stb*), transmissibility (*tra*), and autonomous replication (*rep*). In genetic complementation experiments between transfer-defective (*tra*) mutants of NR1 and F, Ohtsubo et al. (121) found that the *tra* mutants fell into multiple complementation groups. Deletion mapping by Ohtsubo (119) indicated that the various *tra* cistrons were linked together on NR1, and their relative order was determined.

Yoshikawa (192) examined the ability of NR1 to integratively suppress a *dnaA*(Ts) mutant of *E. coli*. After mapping of the NR1 genes by conjugation and transduction, he proposed a circular map with the order: *repA-sul-str-cat-repB-tet-tra-repA* (192). The *repA* locus was required for integrative suppression of the *dnaA*(Ts) host. In comparison with the map of Hashimoto and Mitsuhashi, one could surmise that *repA* might correspond to *rep*, for autonomous replication. The *repB* locus was required for stable autonomous replication of NR1, but not for replication itself (192). Again, one could surmise that *repB* might correspond to the stability locus, *stb*, described by Hashimoto and Mitsuhashi (61, 62).

Electron microscopic analysis of DNA heteroduplexes among plasmids F, R100 (NR1), R1, R6, and ColV-K94 by Sharp et al. (158) indicated that NR1 and R6 were nearly identical, whereas R1 was more distantly related. All five plasmids were found to be closely related in their *tra* regions. The physical mapping of the r-determinants, RTF, *tet*, and *tra* regions in the heteroduplexes was shown to be consistent with the genetic maps described above. Additional physical mapping of NR1 and several deletion mutants by the method of partial denaturation and electron microscopy confirmed the relative locations of the r-determinants, *tet*, and RTF (126). Analysis of replicating NR1 molecules by the same method revealed the location of an origin of replication that approximately corresponded to the *repA* locus (125, 126, 148). Further analysis of DNA heteroduplexes indicated that insertion sequence IS*1* was present at each of the two boundaries of RTF and r-determinants of NR1 and that the two IS*1* elements were oriented in the same direction (67, 132). The direct repetition of IS*1* at the RTF/r-determinants borders suggested models for recombinational events that might be involved in the process of dissociation and amplification of r-determinant DNA in *P. mirabilis* host cells (67, 128, 129, 132, 140, 145, 146, 167).

Owing to gene dosage effects, it was possible to isolate mutants of IncFII plasmids that conferred high-level antibiotic resistance as a result of an increased copy number of the plasmids in the host cells (70, 103, 104, 110, 190). These copy number mutants suggested that plasmids encoded functions involved in the control of their own replication. However, such copy number mutants were often genetically unstable in *E. coli*. This was found to result from the formation of miniplasmids by IS*1*-mediated deletion of various amounts of R-plasmid DNA (51, 59, 60, 96, 97, 170). The miniplasmids from different isolates were of various sizes. However, all were found to contain DNA corresponding to the *repA* region, which suggested that this region was both necessary and sufficient for autonomous replication. Timmis et al. (172) demonstrated that the second largest *Eco*RI restriction endonuclease fragment from plasmid R6, isolated in vitro and referred to as *Eco*RI-2, was capable of autonomous replication in vivo after recircularization and introduction into an *E. coli* host by transformation. This *Eco*RI-2 fragment was shown to have a map position that corresponded to the *repA* locus and was the only *Eco*RI fragment of R6 that was capable of autonomous replication (172). Electron microscopic analysis of replicating plasmid DNA molecules isolated from *E. coli* cells subsequently located the origin of replication (*ori*) of NR1 (159) and of miniplasmids derived from NR1 (120). The NR1 *ori* was located within the second largest *Eco*RI fragment and corresponded approximately to *repA*. Replication proceeded unidirectionally toward the r-determinants and on around the plasmid (120, 159). Although the replication properties of miniplasmids containing the second largest *Eco*RI fragment of NR1 were indistinguishable from those of NR1 itself, such miniplasmids were found to be unstable in the absence of selection, indicating that they lacked the *stb* function (99, 142). The *stb* locus of NR1 was found to reside on the largest *Eco*RI fragment (99, 142).

Dempsey and Willetts (32) examined deletion mutants induced in a series of cointegrates of NR1 and bacteriophage λ. They were able to map the locations of 11 *tra* cistrons, confirmed the order of the resistance genes, and indicated that mercuric ion resistance (*mer*) was located between *repA* in the RTF and *sul* in the r-determinants (32). A physical map of the 13 largest *Eco*RI restriction endonuclease fragments of NR1 was constructed (167), followed by similar maps for the *Eco*RI and *Sal*I fragments of plasmids NR1, NR84, and R6 (10). By analysis of the properties of clones of each of the 13 largest *Eco*RI fragments from NR1 and the knowledge of their locations on the NR1 map, the physical position of each of the antibiotic resistance genes of NR1 was determined (80, 98).

Timmis et al. (175) analyzed the *Eco*RI and *Hin*dIII restriction fragments of R6 and found their order on the R6

map to be the same as the equivalent fragments on the map of NR1. By analyzing clones of the largest R6 fragments, they were able to map three functions referred to as *repA*, *repB*, and *repC*. In agreement with their earlier work, *repA* was found to be located on *Eco*RI-2, which corresponded to *Eco*RI-B of NR1 and was sufficient for autonomous replication. *repB* and *repC* were mapped to R6 fragments *Eco*RI-1 and *Eco*RI-4, respectively, which corresponded to fragments *Eco*RI-A and *Eco*RI-D of NR1, respectively. The *Eco*RI fragments with *repB* and *repC* were found to be incapable of autonomous replication (99, 175). However, when cloned into a ColE1 vector plasmid, they were each found capable of partial stabilization of ColE1 in a *polA*(Ts) host (175). From these results, it now seems likely that the *repB* locus of R6 corresponds to the stability (*stb*) locus of NR1 (99, 142) and to the equivalent locus on plasmid R1 referred to as *parA* (48, 49). In addition, it seems likely that the *repC* locus of R6 corresponds to the locus on plasmid R1 referred to as *parB* or *hok* (48, 50). That region of plasmid DNA is homologous for NR1, R1, R6, F, and ColV-K94 (158) and is contained within the so-called leading region of these plasmids (Fig. 1). A corresponding locus on F, referred to as *parL* (183), is capable of stabilizing a pACYC184 vector plasmid but not of autonomous replication (133).

Earlier estimates of the sizes of F and NR1 suggested they were composed of 94.5 and 89.3 kb, respectively (158). The measurements by electron microscopy (158) and agarose gel electrophoresis (167) were made in comparison with bacteriophage λ DNA standards. Because the exact size of λ DNA is now known (154), the map of F was recently revised to reflect a corrected size of 100 kb (183). A similar revision of the NR1 map to reflect a size of 94.5 kb and to take into account a substantial amount of new information available since the last map of NR1 was prepared (142) is presented below.

RESTRICTION ENDONUCLEASE CLEAVAGE MAP OF NR1

The positions of cleavage of NR1 DNA by nine different restriction endonucleases are listed in Table 1 and shown in Fig. 2. The data in Fig. 2 are based on earlier maps of NR1 for cleavage by *Eco*RI (98, 167), *Sal*I (10), and other enzymes (142) and on numerous more detailed analyses of specific regions of NR1 cited in Table 1. Restriction endonuclease fragments are labeled alphabetically in order of decreasing size; i.e., A is largest (167). The reference point for the kilobase coordinate system is the clockwise (Fig. 1) or right-hand (Fig. 2) end of insertion sequence IS*1*b (97, 120, 122). The sizes of the *Eco*RI restriction fragments are based on recent measurements by Fee and Dempsey (41) and others (Table 1), including nucleotide sequences of several regions of NR1. Not all positions of restriction endonuclease cleavage sites (Table 1) or gene locations (Table 2) relative to IS*1*b are known to the base-pair precision presented in the tables. However, many individual regions are known to that accuracy (Table 3), and therefore, within local areas of the map the precision is justified. As more nucleotide sequencing information becomes available, it will be simple to adjust all of the coordinates as needed to define the precise positions of all of the genes and sites on NR1.

The percent G+C plotted in Fig. 2 is based on the buoyant density analysis of individual restriction endonuclease fragments (142) and on the available nucleotide sequence information (Table 3). The variation in base composition from region to region is sometimes abrupt and most likely reflects the original acquisition of certain segments of DNA during the evolution of NR1. For example, the Tn*10* transposon is of much lower G+C content than that of the leading region into which it is inserted (Fig. 1). The *mer* region of the r-determinants is, by amusing coincidence, the densest region of all. It is also interesting to note that the *repA* and *stb* regions, both necessary for stable maintenance of NR1, are of similar base composition. It seems possible that they were at one time more closely linked together in a progenitor plasmid that was composed of about 46% G+C. Subsequent insertion of the various other functional regions of the plasmid may then have led to the development of the present day structure of NR1, in which the *repA* and *stb* regions are separated by about 28 kb.

TABLE 1. Locations of selected restriction endonuclease cleavage sites on the map of IncFII plasmid NR1[a]

Site	Locations (kb)
*Eco*RI	2.779, 7.319, 8.979, 9.224, 11.034, 16.434, 16.732, 16.978, 18.258, 21.258, 42.858, 54.158, 65.727, 73.317, 79.687, 92.293
*Sal*I	12.034, 61.858, 80.067, 87.112, 89.203, 89.227
*Bgl*II	12.721, 24.309, 25.374, 41.298, 40.088, 53.100, 88.376
*Bam*HI	7.878, 9.058, 12.014, 12.274, 15.798, 35.608, 40.098, 50.439, 52.109, 76.317
*Hin*dIII	9.514, 10.714, 13.574, 13.638, 16.617, 16.966, 38.488, 39.048, 43.748, 92.803
*Sac*I	12.294, 68.227, 81.687
*Kpn*I	18.809, 20.409
*Xba*I	42.168
*Xho*I	8.498, 92.340
*Pst*I[b]	13.116, 21.875, 24.075, 24.181, 25.257, 30.258, 34.458, 35.258, —, 79.937, 80.737, 81.137, 81.637, 83.317, 83.817, 88.162, 89.254, 90.849, 93.907
*Sma*I[c]	79.917, 80.917, 82.592, 82.767, 83.818, 84.632, 85.302, 85.912, 89.379, 89.667

[a] Site locations were obtained from references 2, 4, 10, 23, 25, 30, 41, 46, 52, 53, 58, 66, 68, 69, 74, 80, 98, 129, 142, 164, 166, 167, 169, 175, and 194.

[b] *Pst*I sites within *Eco*RI fragments D, C, E, and F (coordinates 42.858 through 79.687) are not listed.

[c] *Sma*I sites are listed for *Eco*RI fragment B only (coordinates 79.687 to 92.293).

DETAILED MAP OF IncFII PLASMID GENES AND FUNCTIONS

The known genes and functions of NR1 are listed in Table 2 in order of their positions on the NR1 map (Fig. 3). The information used to compile the details of the map was obtained from the references cited in Table 2. Each of the regions of NR1 shown in Fig. 3 is discussed below.

Antibiotic Resistance Determinants

The r-determinant component of NR1 has a size of 23.23 kb and is bounded by direct repeats of IS*1*b and IS*1*a between coordinates 93.73 and 22.46 kb. The r-determinant DNA is homologous to several transposable elements and is itself transposable as Tn*2670* (58, 69). A second complete transposon, Tn*21*, is located within the r-determinants between coordinates 0.06 and 20.04 kb and is bounded by inverted repeats IR_I and IR_r (30, 54, 194). The mercuric ion resistance (*mer*) genes and transposition functions of Tn*21*, including the resolution site (*res*), resolvase/repressor gene (*tnpR*), and transposase gene (*tnpA*), are closely related to

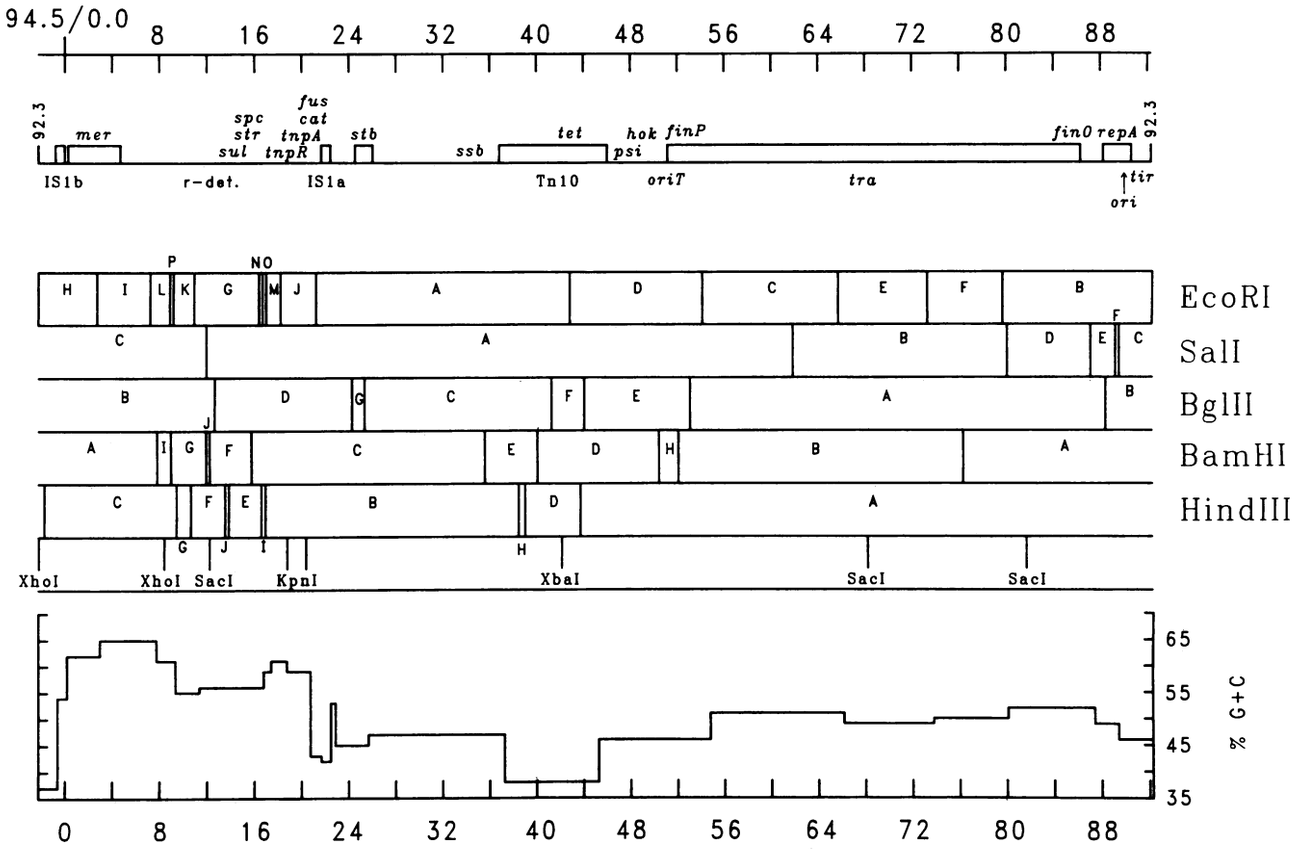

FIG. 2. Restriction endonuclease cleavage map of plasmid NR1. The kilobase coordinates begin and end at the right end of insertion element IS*1*b. Plasmid NR1 is circularly continuous, but the map has been linearized for simplicity of presentation. The locations of all known cleavage sites for *Eco*RI, *Sal*I, *Bgl*II, *Bam*HI, *Hin*dIII, *Sac*I, *Xho*I, *Kpn*I, and *Xba*I restriction endonucleases (Table 1) are shown in the midsection of the map. The various fragments created by cleavage with each enzyme are labeled alphabetically in order of decreasing size (i.e., A is largest). The locations of the known genes and functions of NR1 (Table 2) are indicated at the top of the map. The average base composition of various regions of NR1, determined from buoyant density analysis (142), is indicated at the bottom of the map.

those of Tn*501* (33, 55, 100, 101). The evolution of Tn*21* apparently has included the insertion of approximately 3.4 kb of DNA between coordinates 12.6 and 16.0 kb that includes genes for resistance to streptomycin and spectinomycin (*str/spc*) and sulfonamides (*sul*). The insertion of another segment of approximately 7.9 kb between *mer* and *sul* is also apparent (58, 166). Interestingly, no genes or phenotypic traits have as yet been assigned to this last region, between coordinates 4.7 and 12.6 kb, or to the region between *str* and *res*.

Tn*9* is composed of the *cat* gene for chloramphenicol resistance bounded by direct repeats of IS*1* (3) and is therefore homologous to most of the remainder of the r-determinant component of NR1 (142), from coordinates 20.59 to 22.46 kb. The *cat* gene also confers resistance to fusidic acid (*fus*) (130, 177) and crystal violet (130, 131). The chloramphenicol acetyltransferase encoded by the *cat* gene of NR1 binds to and sequesters these hydrophobic molecules. This has enabled the use of such dyes as crystal violet and basic fuchsin as indicators for chloramphenicol acetyltransferase in colonies formed on agar media (131). The DNA sequence between the right end of IS*1*b and the left end of IR_l (9, 100) of NR1 is not related to Tn*9* (3), and there are additional DNA sequences between the right end of IR_r and the *cat* gene of NR1 that are not included in Tn*9*. Therefore, it is possible that Tn*9* arose from an IS*1*-mediated deletion between IS*1*b and *cat* in the r-determinants of NR1 or in some common ancestor of both NR1 and Tn*9*. Introduction of NR1 into a streptomycin-dependent host results in the selection of mutants that have inactivated or deleted the *str* gene from the r-determinants (146). If selection is maintained for chloramphenicol resistance, many of the resulting deletion mutants retain an r-determinant component that has a structure similar to that of Tn*9*, composed of only the *cat* gene between the two IS*1* elements (129, 146).

The general structures of the r-determinant components of other IncFII plasmids are similar to that of NR1 (2, 10, 25, 158, 167, 175). R6 contains a kanamycin resistance determinant inserted into *Eco*RI-J, and NR84 contains an ampicillin resistance determinant inserted into *Eco*RI-L (10). Plasmid R1 contains an ampicillin resistance determinant inserted into *Eco*RI-H. In plasmid R1, there is also a kanamycin resistance determinant unrelated to that of R6 located between IS*1*b and a third direct repeat of the IS*1* element, IS*1*c (24, 25). This explains the different stability patterns of the various resistance genes of R1, some segregants of which had lost only kanamycin resistance or retained only kanamycin resistance (25, 79). A detailed comparison of the r-determinant components of these IncFII plasmids is presented below.

TABLE 2. Locations of genes and functions on the map of IncFII plasmid NR1

Gene(s)	Function	Location[a] (kb)	Reference(s)
IS*1*b	Insertion sequence	93.727–94.495/0.000	97, 122
insA	IS*1* transposition	93.727–94.054	6, 89, 122
insB	IS*1* transposition	94.102–94.476	6, 89, 122
Tn*21*	Transposon	0.059–20.035	30, 55, 79, 142, 163, 194
IR_i	Left inverted repeat of Tn*21*	0.059–0.096	54, 194
mer	Mercuric ion resistance	0.092–4.723	9, 11, 18, 45, 80, 98, 100, 101, 106, 163
merR	Repressor of *mer*	0.092–0.526	
merT	Mercuric ion resistance	0.598–0.948	
merP	Mercuric ion resistance	0.962–1.237	
merC	Mercuric ion resistance	1.273–1.695	
merA	Mercuric reductase	1.747–3.441	
merD	Mercuric ion resistance	3.459–3.821	
urf1	Uncertain	3.818–4.054	
urf2	Uncertain	4.051–4.723	
sul	Sulfonamide resistance	12.65–13.48	66, 80, 98, 164
str/spc	Streptomycin/spectinomycin resistance	13.80–14.60	66, 80, 98
res	Tn*21* resolution site	16.344–16.470	33
tnpR	Tn*21* resolvase	16.471–17.032	33
tnpA	Tn*21* transposase	17.035–20.002	19
IR_r	Right inverted repeat of Tn*21*	19.998–20.035	54, 194
Tn*9*	Homology with Tn*9*	20.593–22.463	3, 142
cat/fus	Chloramphenicol/fusidic acid resistance	20.816–21.476	3, 80, 98
IS*1*a	Insertion sequence	21.695–22.463	3, 97
r-det.	Resistance determinants (Tn*2670*)	93.727–22.463	25, 58, 68, 69, 79, 80, 158, 167
stb	Stable inheritance (also *repB parA*)	24.559–26.053	48, 49, 99, 111, 142, 165, 175, 192
stbB	Stable inheritance	24.559–24.909	165
stbA	Stable inheritance	24.910–25.871	49, 165
ssb	Single-stranded DNA binding	34.50–35.00	52
Tn*10*	Transposon	36.85–46.05	57, 74, 75, 158
IS*10*L	Insertion sequence	36.85–38.18	57
tetR	*tet* repressor	41.59–42.20	13, 14, 108
tetA	Tetracycline resistance	42.23–43.46	13, 14, 64, 108
IS*10*R	Insertion sequence	44.72–46.05	57
psi	Plasmid-mediated SOS interference	46.60–47.60	8
hok	Host killing (also *repC parB parL*)	47.84–48.42	48, 50, 175, 183
tra	Conjugal transfer	51.20–86.33	23, 31, 41–44, 46, 72, 73, 90, 94, 116, 158, 160, 171, 173, 182–184, 193
oriT	Origin of conjugal transfer	51.200	
traM	Transfer	51.521–51.904	
finP	*tra* regulation	52.00	
traJ	*tra* regulation	52.100–52.706	
traY	Transfer	53.883–53.110	
traA	Transfer	53.143–53.509	
traL	Transfer	53.524–53.90	
traE	Transfer	53.9–54.4	
traK	Transfer	54.4–55.0	
traB	Transfer	55.0–56.5	
traP	Transfer	56.5–57.2	
traV	Transfer	57.6–58.3	
traC	Transfer	59.0–61.6	
traW	Transfer	61.8–62.35	
traU	Transfer	62.35–62.9	
traN	Transfer	62.9–65.1	
traF	Transfer	66.7–67.4	
traQ	Transfer	68.1–68.6	
traH	Transfer	69.5–70.6	
traG	Transfer	70.8–73.5	
traS	Transfer	73.8–74.3	
traT	Transfer	74.45–75.19	
traD	Transfer	75.36–76.83	
traI	Transfer	78.46–83.59	
32K	32-kDa protein[b]	84.552–85.402	23
finO	*tra* regulation	85.767–86.328	23, 31, 94, 193
orf4	23-kDa protein	87.635–88.222	6, 123

Continued on following page

TABLE 2—*Continued*

Gene(s)	Function	Location[a] (kb)	Reference(s)
repA	Replication	88.250–90.628	4, 6, 12, 99, 123, 136, 169, 172, 175, 192
repA2	Regulation of replication (also *copB*)	88.466–88.717	6, 34, 123, 136, 185
repA3	Uncertain	88.827–88.996	6, 123, 134
*inc*RNA	Regulation of replication (also *copA*)	88.850–88.940	37, 38, 137, 142, 169, 174, 185, 187
repA1	Replication initiation	89.025–89.879	6, 123, 136, 185
*repA1**	Uncertain	89.517–89.879	189
ori	Origin of replication	90.070–90.218	93, 120, 123, 135, 136, 159
repA4	Uncertain	90.245–90.628	6, 123, 136, 151
tir	Inhibition of RP4 transfer (*orf1*)	90.821–91.471	6, 123, 168
orf2	9.3-kDa protein (*pemI parD*)	91.567–91.821	6, 16, 123, 176
orf3	12-kDa protein (*pemK parD*)	91.826–92.115	6, 16, 123, 176

[a] The average size of the peptide-coding regions of these genes is approximately 600 ± 280 base pairs. The NR1 genome therefore could contain between 100 and 300 genes of this size, of which approximately 70 have been identified.
[b] kDa, Kilodalton.

Although the resistance determinants of NR1 could be amplified by tandem duplication in a *P. mirabilis* host, in repeated attempts no amplification was observed in *E. coli* (68, 146). However, amplification of the r-determinants in *E. coli* was obtained for NR1 mutants that had deleted an r-determinant segment containing the Tn*21* resolution functions (68, 146). It seems likely that these functions might inhibit amplification in an *E. coli* host by "resolving" the tandem duplications of r-determinant DNA (141). The absence of interference with amplification in *P. mirabilis* might indicate that the *tnpR* gene is poorly expressed in that host or that some host factors necessary for Tn*21* resolution are missing from or are of insufficient activity in *P. mirabilis*.

Although the directly repeated IS*1* elements participate in the amplification of the r-determinant DNA of NR1, the process was found to be *recA* dependent and therefore not a result of common transposition-type mechanisms (68, 140, 146). Moreover, it was shown that the IS*1* elements could be replaced by other directly repeated DNA sequences of similar size without interference in the amplification process (127, 129). It has been suggested that any selectable genetic marker that responds to gene dosage could be amplified by this process if there were a sufficient amount of directly repeated DNA homology flanking that marker (127, 140, 141). In the case of amplification of the r-determinants of NR1 in *P. mirabilis*, there exists a minor subpopulation of cells that contain spontaneously amplified plasmid DNA (63, 128, 140, 145). When selection for high-level drug resistance is applied to such a culture, it seems likely that the subpopulation of more highly resistant cells has a significant growth advantage and is able to take over the culture.

TABLE 3. Nucleotide sequence information available for IncFII plasmid NR1

Coordinates (kb)	Gene(s)/function(s)	Reference(s)
93.625–4.786	IS*1*b, *mer*	6, 9, 18, 100, 101, 122, 123, 194
12.420–13.574	*sul* (R538-1)[a]	S. Hollingshead
13.638–15.081	*str/spc* (R538-1)[a]	66
15.960–17.076	*res*, *tnpR*	33
17.077–19.996	*tnpA* (Tn*501*)[a]	19
19.955–20.035	IR_r	54, 194
20.593–22.463	*cat/fus* (Tn9),[a] IS*1*a	3, 122
23.894–26.053	*stb*	165
36.85–38.18	IS*10*L	57
42.11–43.75	*tetR*, *tetA*	64, 108
44.72–46.05	IS*10*R	57
51.06–53.55	*oriT*, *traM*, *finP*, *traJ*, *traY*	41–44, 46, 71
74.271–75.317	*traT*	116
85.581–86.586	*finO*	94, 193
87.491–92.527	*repA*	6, 123, 135, 136, 151, 152

[a] The nucleotide sequences were obtained from the related plasmid R538-1 (2) or the related transposons Tn*501* and Tn9, rather than from NR1 itself.

Stability and Leading Regions

The DNA to the right of (or clockwise from) IS*1*a between coordinates 22.46 and 26.05 kb is homologous in all IncFII plasmids examined and includes an essential *cis*-acting site as well as two genes, *stbA* and *stbB*, that encode *trans*-acting proteins essential for the stability function (165). The *stb* locus is thought to participate in the partition of plasmid DNA molecules to daughter cells at the time of cell division (99, 111, 142).

The DNA between the *stb* locus and the origin of transfer, *oriT*, from coordinates 26.05 to 51.20 kb, is referred to as the leading region, because it is the region of the plasmid that is first introduced into a recipient cell during conjugal transfer (72, 182–184). The leading region is homologous in IncFII plasmids, F, and ColV-K94 (158). Although not essential for conjugal transfer, the leading region may impart some advantages to the process that might explain its evolutionary conservation among these plasmids (183). The leading region is known to include a gene for a single-stranded DNA-binding protein (*ssb*) that is homologous to that of *E. coli* (21, 52), and genes for plasmid-mediated SOS interference (*psi*) (8). A second plasmid-stabilizing function, *hok*, was identified on plasmid R1 (48, 50) and is among the very first genes introduced into a recipient during conjugation. The equivalent locus on various plasmids has been referred to as *repC* (175), *parB* (48, 50), or *parL* (183). However, Molin and his associates (47, 50) have shown that the locus encodes neither replication nor partitioning functions, but instead encodes a host killing function (*hok*) that results in the death of plasmid-free segregants. Therefore, it is possible that the *hok* locus functions after conjugal transfer by killing any cells unlucky enough to divide and segregate a plasmid-free daughter before the plasmid has had a chance to replicate in its new host cell. The *hok* locus of plasmid R1 is homologous to the *relF* locus of *E. coli* (47). The homologous locus on plasmid F is now referred to as *flm*, for F leading-region maintenance (87).

In plasmids NR1 and R6, but not R1, F, or ColV-K94 (158), the leading region is interrupted by insertion of the 9.2-kb transposon Tn*10* (75). Tn*10* is located between coordinates 36.85 and 46.05 kb and is composed of the *tetA* gene

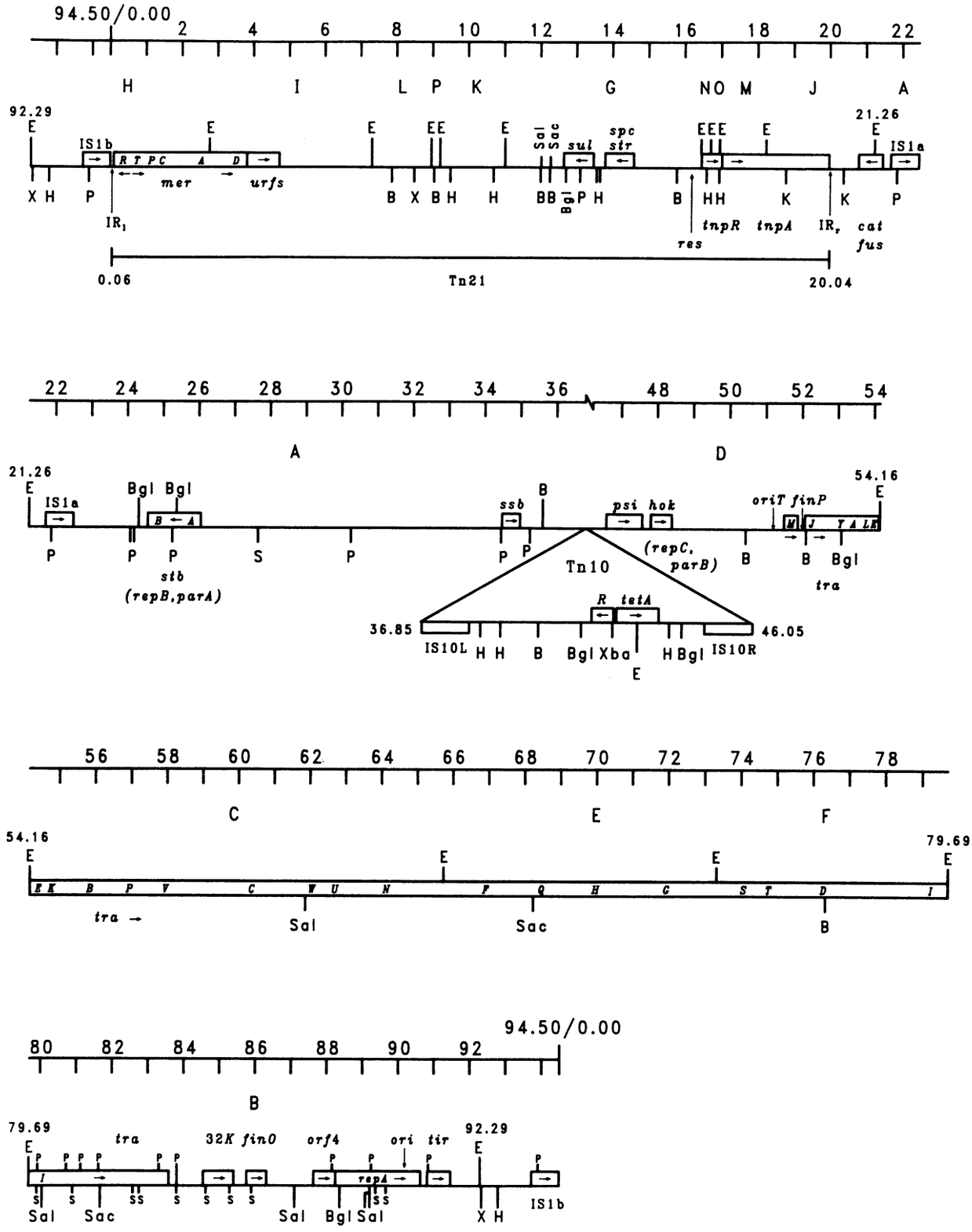

FIG. 3. Expanded map of NR1 genes and functions. The positions of the genes and functions are from Table 2 and are indicated by the boxes. Letters within the boxes indicate individual protein-coding regions, and the horizontal arrows indicate their directions of coding. Transcripts and transcription promoters are not indicated. The kilobase scale is interrupted at coordinate 36.85, the site of insertion by Tn*10*. The letters above the map indicate the *Eco*RI restriction fragments from Fig. 2. Restriction endonuclease cleavage sites from Table 1 are abbreviated: E, *Eco*RI; P, *Pst*I; Bgl, *Bgl*II; S, *Sma*I; B, *Bam*HI; H, *Hin*dIII; X, *Xho*I; Xba, *Xba*I; Sal, *Sal*I; Sac, *Sac*I; K, *Kpn*I.

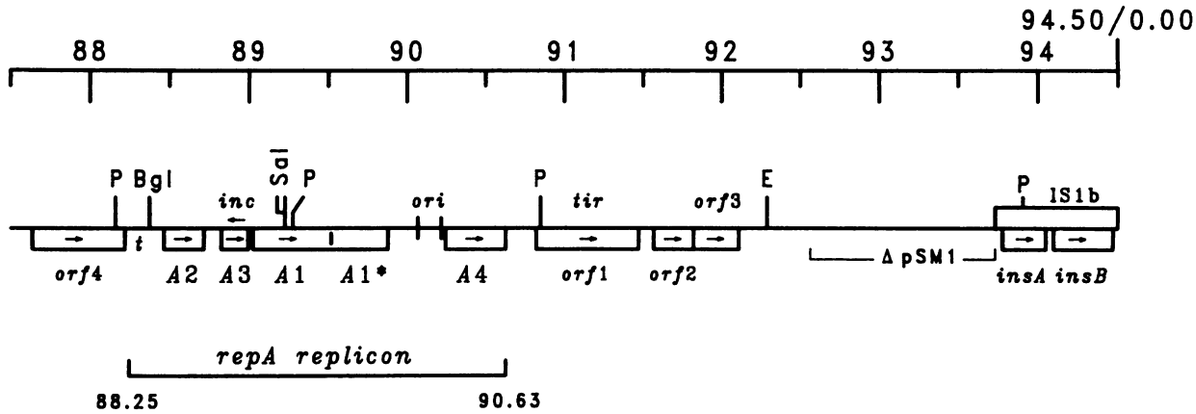

FIG. 4. Detailed map of the NR1 *repA* region. Ohtsubo and his colleagues have determined the nucleotide sequence of all but 1.2 kb from this region (Table 3), using miniplasmids pSM1 and pSM2. These miniplasmids were generated from plasmid pRR12, a copy number mutant of NR1, by deletion from the ends of IS*1*b (97). The locations of the genes listed in Table 2 are indicated by the boxes, and the horizontal arrows indicate the directions of coding. Transcription promoters are not indicated. The origin of replication (*ori*) is located between genes *repA1* and *repA4*. Plasmid pSM1 has deleted DNA between coordinates 92.527 and 93.727, in addition to the deletion of DNA between coordinates 0.000 and 87.491. Abbreviations for restriction endonuclease sites are as given in the legend to Fig. 3. The letter *t* near the left end of the *repA* region represents a transcription terminator that insulates the *repA* region from transcription initiated upstream of the replicon (189).

for tetracycline resistance, the *tetR* repressor gene, and inverted repeats of the insertion sequence IS*10*. The insertion of Tn*10* at this position has no obvious consequences on the conjugal transfer of NR1 and R6. However, it is interesting to note that the one resistance determinant, *tet*, that responds inversely to gene dosage is also the one that is located outside of the amplifiable region between the two IS*1* elements.

Transfer Operon

The *tra* region of NR1 between coordinates 51.20 and 86.33 kb is largely homologous to that of F, which recently has been described in detail by others (72, 183). The gene products of the *traY-I* operon serve various roles in the synthesis of pili and in the process of conjugation. The *traY-I* operon is regulated in a two-step process (72, 183). The *traJ* gene product is required as a positive regulator for expression of *traY-I*. In turn, the expression of *traJ* is negatively regulated by the products of genes *finO* and *finP*. Between coordinates 84.4 and 85.7 kb, a gene of unknown function that encodes a 32-kilodalton protein is located between *traI* and *finO* of NR1 and R6 (23) but not of F (see below). The *finO* gene of F contains an insertion of IS*3* (22, 193). F is therefore phenotypically FinO$^-$, which results in the derepression of the F *tra* operon. Consequently, the frequency of conjugal transfer of F is about 1,000-fold higher than that of *finO*$^+$ IncFII plasmids. A comparison of the *tra* operons of NR1, R1, and F in the region of *traJ* and *finP* has revealed multiple nonhomologies and differences in nucleotide sequence (41, 42, 44). These differences apparently impart plasmid specificity to the *traJ* and *finP* regulatory components of these plasmids (182, 184). However, the *finO* gene is apparently not specific among these plasmids, which accounts for the inhibition of F plasmid transfer by *finO*$^+$ IncFII plasmids.

Replication Control Region

A detailed map of the *repA* replication control region of NR1 from coordinates 88.25 to 90.63 kb that is based on the nucleotide sequence (6, 122, 123, 135, 136, 152) is shown in Fig. 4. The genes and gene products that serve in the regulation of IncFII plasmid replication are well characterized (6, 35, 36–38, 83, 84, 92, 99, 112, 136, 137, 139, 142, 151, 157, 169, 185, 187–189). The essential components include the *repA1* gene, which encodes a 33-kilodalton *cis*-acting initiation protein, and the replication origin, *ori*. The copy number of NR1 is maintained at a low level of one to two copies per chromosome by regulating the synthesis of the RepA1 initiation protein. Transcription of the *repA1* gene is initiated at two promoter sites, a weak promoter located just upstream of *repA2* and a moderate-strength promoter just downstream from *repA2* (34, 35, 38, 137, 189). Transcription from the upstream promoter is constitutive, whereas transcription from the downstream promoter is regulated by a tetramer of the 9.4-kilodalton repressor protein product of *repA2* (34, 85, 86, 185). Translation of *repA1* messenger ribonucleic acid (mRNA) is regulated by the *trans*-acting 91-base *inc*RNA, which is transcribed from the opposite DNA strand and is complementary to the leader region of the *repA1* mRNA (15, 35, 37, 38, 84, 137, 185–187). The *inc*RNA is not translated, is unstable, and is transcribed constitutively from a strong promoter located just upstream from *repA1*. The activities of both the RepA2 transcription repressor and the *inc*RNA translation inhibitor, which are synthesized constitutively, are related to gene dosage and therefore to plasmid copy number (34, 189). This results in a regulation of synthesis of the RepA1 initiation protein that is inversely related to plasmid copy number, which can therefore compensate for fluctuations and maintain a steady balance of plasmid copies during cell growth and division (188).

An excess of RepA2 repressor protein provided in *trans* from a cloned copy of *repA2* does not express incompatibility against NR1 and results in only a small decrease in copy number (34, 85, 86, 99, 113, 150, 185). This indicates that the level of *repA1* mRNA synthesis provided by transcription from the constitutive upstream promoter is sufficient to maintain a near-normal replication frequency for NR1. In contrast, *inc*RNA provided in *trans* from a cloned copy of the *inc* gene expresses strong incompatibility against NR1 by

severely inhibiting the translation of the *repA1* mRNA (15, 37, 38, 84, 99, 150, 185, 187). Therefore, the IncFII plasmid incompatibility specificity results from the regulation of translation of *repA1* mRNA by the *inc*RNA (150, 186, 187). A comparison of the nucleotide sequences of IncFII plasmids NR1 and R1 revealed numerous substitutions in their *repA2* genes and respective operator sites (134, 136, 151, 152). These differences result in different plasmid specificities for control of the transcription of *repA1* in these two plasmids (113, 134). However, the *inc*RNA genes of NR1, R1, and R6 are identical (15, 136, 137, 151, 152), which accounts for their mutual incompatibility. The incompatibility specificity and control of replication of some plasmids from various other IncF groups (12, 153), including ColV2-K94 (181), and also from groups IncB and IncI (109), may be determined by similar interactions of *inc*RNA with a complementary mRNA transcript. However, the nucleotide sequences of the *inc*RNA genes of these plasmids apparently have diverged sufficiently to allow mutual compatibility between the different groups of plasmids.

Genes *repA3* and *repA4* are of unknown function but could theoretically encode proteins of 7.2 and 14 kilodaltons, respectively (6, 136, 152). Deletion or insertion mutants of *repA4* are replication proficient but are not inherited stably (191; Y. Min, A. Tabuchi, Y. Fan, D. D. Womble, and R. H. Rownd, J. Mol. Biol., in press) and may form plasmid multimers (123). The function of another potential gene, *repA1**, also is not known. The *repA1** gene could encode a protein of 14 kilodaltons composed of the carboxyl-terminal portion of RepA1 (189). There are two LexA boxes in the nucleotide sequence preceding *repA1** (189). Therefore, transcription of *repA1** may be regulated by the host SOS system.

There are four other known protein-coding genes of unknown function around the *repA* region, referred to as *orf1*, *orf2*, *orf3*, and *orf4* (6). The 24-kilodalton product of *orf1* inhibits conjugal transfer of the unrelated broad-host-range plasmid RP4, and the gene has been referred to as *tir* (168). The 23-kilodalton product of *orf4* may cause a modest inhibition of F-mediated conjugation when present in high gene dosage (6), and therefore *orf4* was once mistaken for gene *finO* (173). Both the *orf1* and *orf4* products may be membrane proteins, which might contribute to their respective phenotypes (6). A transcription terminator located downstream from *orf4* ensures that transcription of the *repA* region is regulated only by the endogenous *repA* promoters (189).

The functions of the wild-type *orf2* and *orf3* gene products, with sizes of 9.3 and 12 kilodaltons, respectively (6), have not yet been discovered. However, mutations in *orf2* cause inhibition of host cell growth and lead to increased stability of *repA*-containing miniplasmids derived from R1 (16). Phenotypic reversion to normal cell growth and normal plasmid stability can occur by subsequent mutation in *orf3* (16). Therefore, these genes have been referred to as *parD*, suggesting that they participate in plasmid partitioning (16). When cloned together into a pBR322 vector plasmid, the *orf2* and *orf3* genes appear to cause plasmid-free segregants to die when a *polA*(Ts) host is incubated at the nonpermissive temperature, conditions which inhibit the replication of pBR322 (176). As a result of this postsegregational killing phenotype, the *orf2* and *orf3* genes have been referred to as *pemI* and *pemK*, respectively, for plasmid emergency maintenance (176). However, the presence of *orf2* and *orf3*, which are contained within *Eco*RI-B of NR1, on otherwise wild-type IncFII plasmids is neither necessary nor sufficient for stable plasmid inheritance (48, 99, 142, 165). The mechanisms responsible for these observations are not understood. Owing to a deletion in miniplasmid pSM1 that was studied by Ohtsubo and his colleagues (120, 122), there is a 1.2-kb gap in the nucleotide sequence between *orf3* and IS*1*b from coordinates 92.53 to 93.73 kb. There are two protein-coding genes within IS*1*, *insA* and *insB*, that are important for IS*1* transposition (6, 20, 89). These genes encode proteins of 10 and 15 kilodaltons, respectively.

COMPARISON OF THE NR1 MAP WITH OTHER PLASMIDS

Comparison with Other IncFII Plasmids

IncFII plasmid R6 has a size of approximately 101.8 kb (175). All of the nucleotide sequences of NR1 are contained in plasmid R6, but R6 has several insertions of additional DNA not included in NR1 (158). R6 contains an insert of approximately 4.0 kb approximately at position 19.8 kb of NR1 (158). The insertion is near the 3′ end of the *tnpA* gene of Tn*21* (Fig. 3). The insertion includes transposon Tn*903* and confers resistance to kanamycin (10, 53, 79, 88). Tn*903* contains one *Hin*dIII site (53), and the additional DNA sequences in the insertion that are clockwise from Tn*903* contain one *Sal*I site (10), which result in different restriction maps for plasmids NR1 and R6 (10, 142, 175).

R6 also contains a 1.8-kb insertion at position 30.2 kb of NR1. Compared with the leading region of F, plasmids NR1 and R1 have an additional 3.1 kb of DNA inserted at this position, whereas R6 has an insertion of 4.9 kb (158). This region is of unknown function. R6 and NR1 have Tn*10* inserted between *ssb* and *psi* (Fig. 3). A variant of R6 that has lost tetracycline resistance, R6-5, has an insertion of approximately 1.5 kb at position 43.4 kb of NR1 (158). The insertion apparently includes a copy of IS*10* (158) and is located within the *tetA* gene of Tn*10*.

The R6-5 and R100 (NR1) derivatives used by Sharp et al. (158) in their heteroduplex studies had an insertion of 1.6 kb at position 61.1 kb of NR1. This is within the *traC* gene near its 3′ end (Fig. 3). A variant of NR1 having a similar insertion was called NR1-C$^+$, because its *Eco*RI-C fragment (Fig. 2 and 3) was 1.6 kb larger than what was considered to be the normal *Eco*RI-C fragment (167). All of these derivatives apparently are transfer proficient. The final known difference between R6 and NR1 is that R6 lacks the *Sal*I site present at position 87.1 kb of NR1 (10).

IncFII plasmid R1 has multiple differences from NR1 and R6 (25, 158). The main differences concern the antibiotic resistance genes and the specificities of the *tra* and *repA* regulatory functions (25, 72, 158, 182, 184). Of the major differences, R1 contains an insert of 10.3 kb at position 0.0 kb of NR1 (Fig. 3) (25, 158). The insert includes a direct repeat of IS*1*, referred to as IS*1*c, at its clockwise (right-hand) end. The gene *kan*, which confers resistance to kanamycin, is located between IS*1*b and IS*1*c of R1. The *kan* gene bounded by IS*1*b and IS*1*c of R1 is transposable as Tn*2350* (24). R1 also contains an insertion of Tn*3* at position 1.8 kb of NR1, which confers ampicillin resistance (25). This 5.0-kb insertion is within the *merA* gene near its 5′ end (Fig. 3). In R1, the equivalent of Tn*21* is called Tn*4* (79), which confers ampicillin resistance but not mercuric ion resistance. R1 is lacking 1.5 kb of DNA between positions 8.8 and 10.3 kb in the silent region of the NR1 r-determinants (Fig. 3) and therefore is lacking the *Eco*RI sites that bound the *Eco*RI P fragment of NR1 (25). R1 also does not contain transposon

Tn*10* (158). Therefore, R1 lacks the *Eco*RI site within Tn*10* that separates *Eco*RI-A and *Eco*RI-D of NR1, which results in the *stb*, *ssb*, *psi*, and *hok* loci of R1 all being located on a single *Eco*RI fragment (48, 50).

There are multiple substitutions and insertions in the *tra* regions between NR1 and R1 (158). These include (i) a substitution of 0.42 kb in a region of unknown function between positions 49.16 and 49.58 kb of NR1; (ii) a substitution of 0.42 kb between positions 51.25 and 51.66 kb of NR1, including part of *traM*; (iii) a substitution of 1.04 kb between positions 52.12 and 53.16 kb of NR1, including *traJ* and *traY*; (iv) an insertion of 1.8 kb in R1 at position 63.2 kb of NR1, within *traN*; (v) a substitution of 0.4 kb between positions 64.7 and 65.1 kb of NR1, at the 3′ end of *traN*; and (vi) an insertion of 0.89 kb in R1 at position 75.36 kb of NR1, between *traT* and *traD*.

In the *repA* region of R1, there is a substitution of 0.34 kb immediately downstream from *finO*, between positions 86.33 and 86.67 kb of NR1. A final substitution of 0.258 kb is between positions 88.498 and 88.755 kb of NR1, within the *repA2* gene (136, 137, 151, 152). There are multiple-base-pair substitutions in the *repA* regions that result in several alterations of the detailed restriction map of R1 compared with NR1 (151, 152). Finally, in comparison with NR1, the size of R1 is estimated to be 101.8 kb.

Comparison with F

A detailed map of plasmid F has been prepared by Willetts and Skurray (183). F and NR1 (Fig. 1) are largely homologous between positions 53.8 and 4.1 kb of F and 26.0 through 89.0 kb of NR1 (158). This includes the leading region, the *tra* operon, and part of *repA* (referred to as RepFIC in F and other related plasmids [12, 153, 183]). F and NR1 are completely nonhomologous between positions 4.1 and 53.8 kb of F and 89.0 through 26.0 kb of NR1 (158). This includes the r-determinants and *stb* regions of NR1 and the Tn*1000* (γδ), IS*3*, IS*2*, RepFIB, *pif*, *ccd*, RepFIA, and *sop* (*par*) regions of F (183).

Within the homologous regions of F and NR1, there are multiple substitutions, insertions, and deletions (72, 90, 158). These include (i) a deletion in F of 3.1 kb in a region of unknown function from positions 30.2 through 33.3 kb of NR1 (58.0 kb of F); (ii) insertion in NR1 of Tn*10* (9.2 kb) at position 36.85 kb of NR1 (61.5 kb of F); (iii) a substitution of 0.42 kb from positions 49.16 through 49.58 kb of NR1 (64.66 through 65.08 kb of F); (iv) a substitution of 1.04 kb from positions 52.14 through 53.16 kb of NR1 (67.62 through 68.66 kb of F), including *traJ* and *traY*, that affects the specificity of *tra* operon regulation; (v) a substitution of 0.2 kb in F for 0.5 kb in NR1 at position 63.4 kb of NR1 (78.9 kb of F), within *traN*; (vi) a substitution of 0.4 kb from positions 64.7 through 65.1 kb of NR1 (79.9 through 80.3 kb of F), at the 3′ end of *traN*; (vii) a substitution of 0.4 kb from positions 70.3 through 70.7 kb of NR1 (85.5 through 85.9 kb of F), at the 3′ end of *traH*; (viii) a substitution of 0.76 kb from positions 73.6 through 74.4 kb of NR1 (88.8 through 89.6 kb of F), including *traS*; (ix) an insertion in F of 0.89 kb at position 75.36 kb of NR1 (90.54 kb of F), at the 5′ end of *traD*; (x) a deletion in F of 0.86 kb at position 77.6 kb of NR1 (93.7 kb of F), between *traD* and *traI*; (xi) a deletion in F of 1.38 kb at position 84.39 kb of NR1 (99.6 kb of F), which includes the *32K* gene of NR1; (xii) an insertion in F of IS*3* (1.26 kb) at position 86.16 kb of NR1 (100/0.00 through 1.26 kb of F), within *finO* (193); (xiii) a substitution of 0.34 kb at position 86.33 kb of NR1 (1.42 kb of F); (xiv) a substitution of 1.30 kb at 87.02 kb of NR1 (2.11 kb of F), which includes the nonhomologous *srnB* gene of F (1,153) and the *orf4* gene of NR1 (6); (xv) a substitution of 0.26 kb at position 88.50 kb of NR1 (3.60 kb of F), which includes the *repA2* genes of RepFIC and *repA* of F and NR1, respectively (153); (xvi) a substitution of 0.27 kb at position 89.03 kb of NR1 (4.13 kb of F), which includes the 5′ ends of the *repA1* genes of

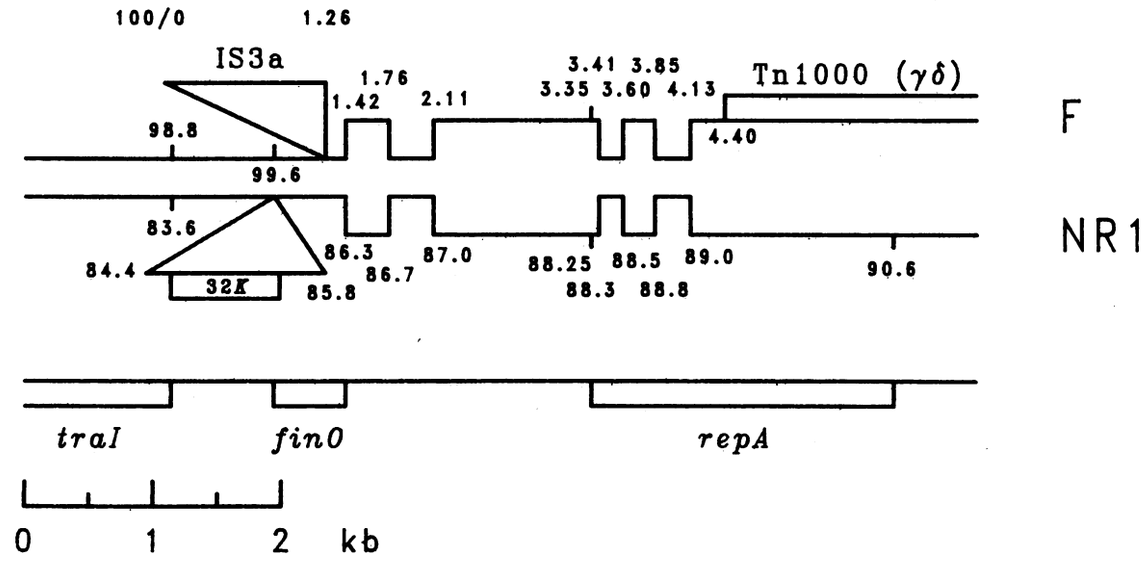

FIG. 5. Comparison of the *finO* and *repA* regions of plasmids NR1 and F. The diagram represents an inferred heteroduplex between the equivalent regions of the two plasmids, as presented by Sharp et al. (158). The details result from a more recent heteroduplex analysis of this region (90) as well as restriction mapping (23) and direct nucleotide sequencing of the *finO* (94, 193) and *repA* (6, 123, 136) and RepFIC (153) regions of both NR1 and F. The kilobase scale for F is derived from Willetts and Skurray (183) with details from Yoshioka et al. (193) and Saadi et al. (153). F has IS*3* inserted into *finO* and Tn*1000* inserted into *repA1*. NR1 has an additional 1.4 kb of DNA between *traI* and *finO*, which carries a gene encoding a 32-kilodalton protein (23, 31).

RepFIC and *repA* of F and NR1, respectively (153); and (xvii) an insertion in F of Tn*1000* at position 89.3 kb of NR1 (4.4 kb of F), which is in the *repA1* gene of RepFIC (153). These differences result in a calculated size of 100 kb for F, in agreement with the map of Willetts and Skurray (183).

Based on a reinterpretation of published data, an inferred heteroduplex between the *finO* and *repA*/RepFIC regions of NR1 and F is presented in Fig. 5. In earlier analyses of these data (72, 90, 158), a 1.4-kb nonhomologous loop was assigned, apparently incorrectly, to NR1 downstream of the site of IS*3* insertion in F. However, the distances from *finO* to *repA* in F and NR1 are the same (23, 153, 193). In addition, R6 (and therefore NR1, which is indistinguishable from R6 in this region) contains a gene for a 32-kilodalton protein upstream of *finO* (23). The *32K* gene is of unknown function and is not present in F (72, 183).

The F DNA clockwise from Tn*1000* does not include the remainder of the *repA1* gene or replication origin of RepFIC (153), which suggests that they were lost during a Tn*1000*-mediated deletion event in some ancestor of F. Some other plasmids related to F have a complete functional RepFIC replicon (12, 26a), and it seems likely that there was some common ancestor to this whole group of plasmids, including IncFII plasmids. The vestigial RepFIC replicon of F is nonfunctional, although the *repA2* and *inc*RNA genes that remain appear to be still functional (12, 153). However, the nucleotide sequences of these genes in RepFIC are different from those of the *repA* region of NR1 (153). Therefore, F and NR1 are compatible (27). Interestingly, the remnants of the *repA1* gene of RepFIC of F, the complete *repA1* gene of the RepFIC replicon of plasmid P307, and the *repA1* gene of IncFII plasmids are totally nonhomologous (153). This sug-

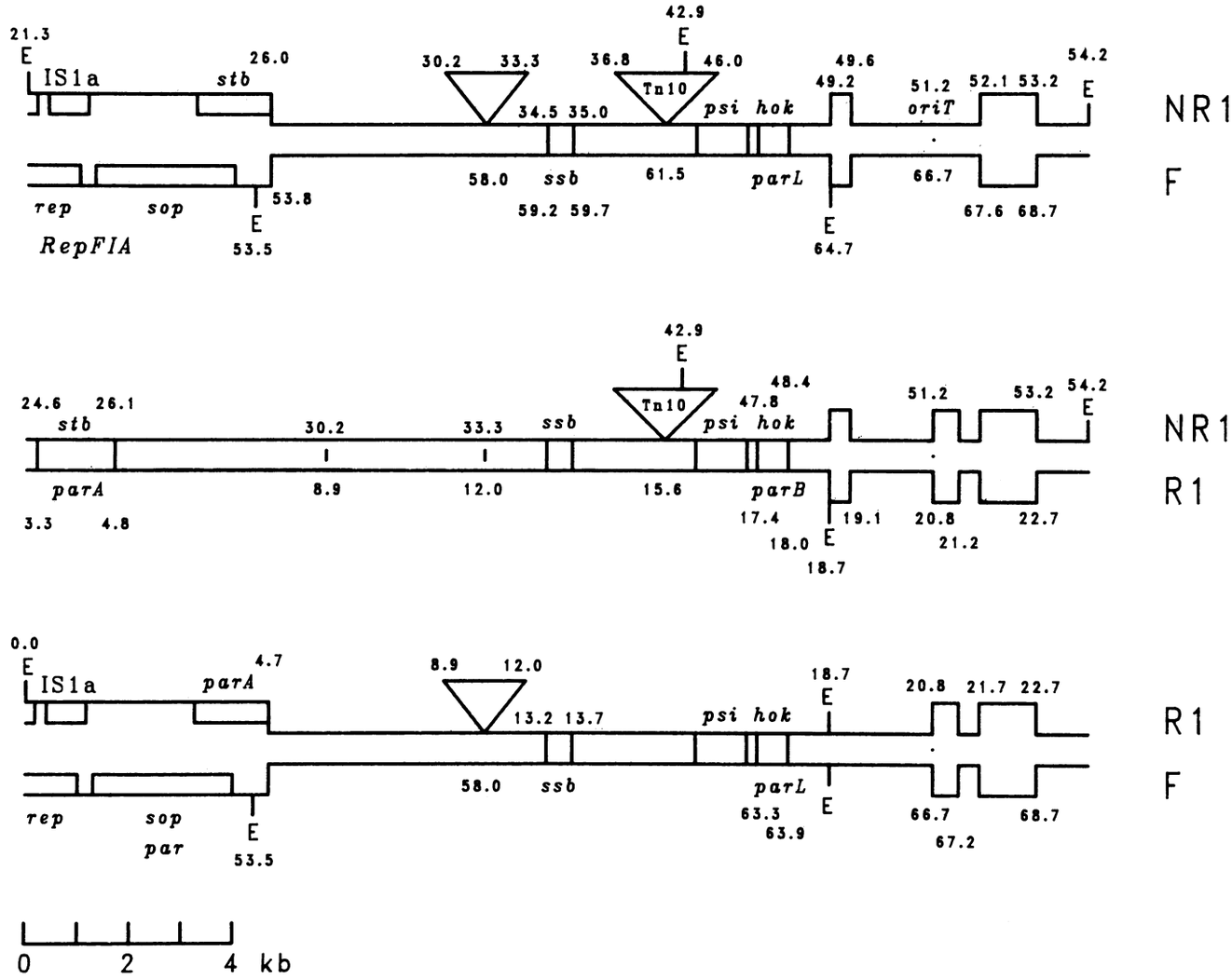

FIG. 6. Comparison of stability and origin of transfer regions of plasmids NR1, R1, and F. The diagrams represent inferred heteroduplexes between each pair of plasmids, as presented by Sharp et al. (158). The kilobase scale for F is derived from Willetts and Skurray (183) and that for NR1 is from Fig. 1. For R1 the numbering begins at the *Eco*RI site in *cat* (48). The heteroduplexes were inferred from the data in Sharp et al. (158) and modified according to the locations of the genes and functions in Table 2 and the nucleotide sequence data in Table 3. NR1 has Tn*10* inserted between *ssb* and *psi*, which separates them onto two different *Eco*RI fragments (A and D, respectively, of NR1). NR1 and R1 both have 3.1 kb of additional DNA between *stb* and *ssb*. In addition, there is a region of homology between R1 and F that is not shared with NR1 (coordinates 64.7 to 65.1 of F). This region contains an *Eco*RI site in R1 and F, which separates *hok* and *oriT* onto two different *Eco*RI fragments (f3 and f6, respectively, of F). The *Eco*RI sites are shown as E. The order of transfer of genes during conjugation is *oriT*, *hok*, *psi*, *ssb*, *stb*, etc. (184). Insertions are not drawn to scale.

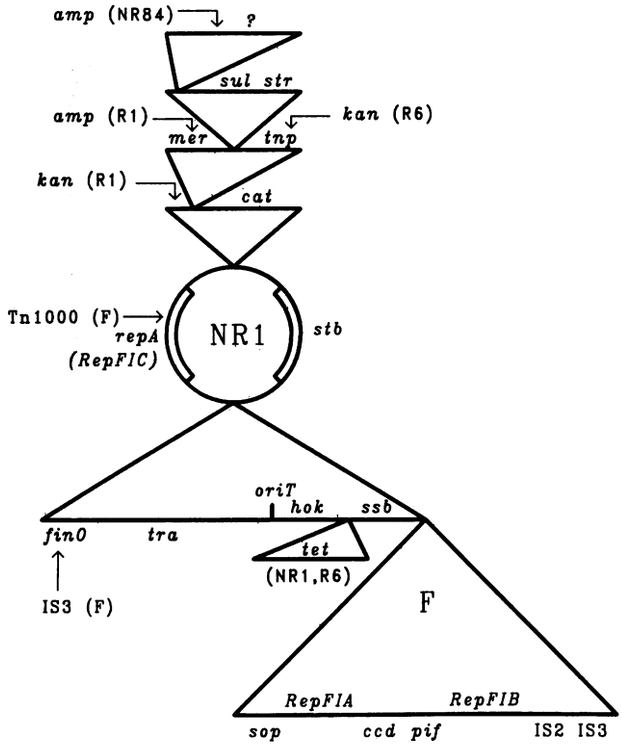

FIG. 7. Evolution of multiple antibiotic resistance plasmids. A possible scheme for the assembly of plasmid NR1 from a primordial plasmid that contained replication (*repA*) and stability (*stb*) functions is depicted. After obtaining transfer (*tra*) functions, it is presumed that the primordial conjugative plasmid acquired antibiotic resistance determinants through a series of transposition events. The functions encoded on the insert between *mer* and *sul* have yet to be elucidated. The sites of insertion of additional resistance elements in some related IncFII R plasmids also are indicated. In comparison with NR1, plasmid F contains a region of DNA that harbors two additional sets of replication functions, RepFIA and RepFIB. There is also an insertion in F of Tn*1000* in *repA* (RepFIC), which most likely was followed by a deletion clockwise from Tn*1000* through *stb*. F also contains an insertion of IS*3* in gene *finO*, which results in constitutive expression of the F *tra* operon. The figure is not drawn to scale.

gests that there may be multiple nonhomologous replication initiation proteins and replication origins that are encoded by otherwise similar plasmid replicons. F has two other functional replicons (12, 183), referred to as RepFIA and RepFIB (Fig. 1), for which there are no functional counterparts in NR1 (12, 26a, 158).

Inferred heteroduplexes between the *stb* through *oriT* loci of the leading regions of NR1, F, and R1 that are based on published data are presented in Fig. 6. Interestingly, the nonhomologous plasmid-partitioning functions of NR1 and F (*stb* and *sop*, respectively) are approximately coincident on the maps of these plasmids. The *Eco*RI site within Tn*10* of NR1 (and R6) separates the *stb* and *hok* loci onto different *Eco*RI fragments (*Eco*RI-A and *Eco*RI-D, respectively). In plasmid R1, these loci are both on *Eco*RI-A (48). Compared with R1 and F, NR1 contains a 0.42-kb substitution that eliminates the *Eco*RI site at 64.7 kb of F. Therefore, the *hok* and *oriT* loci of R1 and F are separated onto different *Eco*RI fragments (f3 and f6, respectively, of F), whereas they are both on *Eco*RI-D of NR1 (and R6).

CONCLUDING REMARKS

IncFII group conjugative plasmids have been found in collections of bacterial strains isolated prior to the clinical use of antibiotics (28). However, those plasmids do not carry antibiotic resistance genes (28). Considering the increasing use of antibiotics throughout the world during the 1950s, it is easy to speculate about the selection for multiple resistance plasmids that may have been previously assembled from various transposable elements. A scheme invoking the acquisition by a primordial plasmid of various combinations of antibiotic resistance genes through multiple transposition events is presented in Fig. 7. The subsequent infectious spread of multiple antibiotic resistance by conjugation between bacterial cells in an environment of strong antibiotic selection, such as exists in hospitals, is then easy to understand.

In comparing conjugative plasmids NR1 and F, it is not possible to determine from existing evidence whether the RepFIA and RepFIB replicons were acquired by F later in the evolutionary history of these plasmids, or whether these functions were subsequently deleted from the R-plasmid side of the family tree. However, it seems likely that some plasmid ancestral to F that contained all three replicons suffered an insertion of Tn*1000* in RepFIC, which was followed by deletion of the sequences clockwise from Tn*1000* through *stb*. Finally, the 1000-fold down-regulation of the *tra* operon by *finO* (in combination with *finP*) probably imparts an advantage to *finO*$^+$ plasmids such as NR1 by preventing an unnecessary drain on the metabolism of their host cells and by keeping their host cells resistant to the prevalent pilus-specific bacteriophages (183). However, the advantages to a bacterial geneticist of working with the *finO*::IS*3* mutant F plasmid are also obvious.

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