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Identification and Classification of Bacterial Plasmids

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INTRODUCTION

Identification and classification has always been an important aspect of the study of living organisms. This statement applies not only to higher plants and animals, but also to microorganisms, including viruses and, in recent years, plasmids. To be able to identify and classify an organism is essential for studying its distribution in nature and its relationship to other organisms. One can raise the question whether or not plasmids are living organisms, since they depend completely on their hosts and since they consist only of nucleic acid. The same kind of question has been raised previously about viruses. If we accept the definition proposed in the consideration of viruses that "an organism is the unit element of a continuous lineage with an individual evolutionary history" (72), we can consider plasmids to be living organisms in spite of their simple structure. This view is in agreement with Datta (33), who contended that plasmids, along with bacteriophages and certain transposons, should be considered as belonging to a family of primitive organisms with vertical and horizontal replicative dissemination. The unifying theme among these molecules is their replicative dissemination, either intra- or intercellular.

Plasmids are circular double-stranded deoxyribonucleic acid (DNA) molecules that replicate autonomously in a host cell. They vary in length from a few to several hundred kilobase pairs. They contain genes that are essential for plasmids maintenance functions, such as the initiation and control of replication. Some contain genes that control traits ensuring stable inheritance, such as equipartitioning during cell division or conjugal transfer. Many plasmids contain genes that are useful not only to themselves, but also to their host. Examples are genes controlling drug resistance, degradation of organic compounds, and virulence factors, including the production of toxins. These types of genes are frequently located within transposons, and this has created a great deal of variation and flexibility in the constitution of plasmids.

Identification and classification should be based on genetic traits that are universally present and are constant. These criteria are best met by traits concerned with plasmid maintenance, especially replication control. Among plasmids, differences are found for replication control and this can be recognized by studying incompatibility relationships. Plasmids with the same replication control are incompatible, whereas plasmids with different replication controls are usually compatible (see below).

Identification and classification of plasmids are especially important in medicine, because genes for clinically important traits, such as drug resistance and virulence factors, are frequently present in plasmids. The recognition of the type of virulence plasmid or resistance (R) plasmid present in a pathogen can be instrumental in tracing the source and spread of an infection, and it may also serve in establishing a diagnosis. Besides these practical uses, there is another, more basic, use, the tracing of genetic relatedness and of evolutionary origins.

HISTORY OF PLASMID CLASSIFICATION

Classification of plasmids became important at the end of the 1950s after the discovery of R plasmids and the recognition of their wide distribution. Prior to that, studies on plasmids had been confined largely to the F plasmid (F stands for fertility) and its function in gene transfer and to colicinogenic (Col) plasmids. The multiple resistance of R plasmids was recognized by their ability to transfer several drug resistance genes en bloc by conjugation. The first criterion to be used for plasmid classification was related to conjugal transfer. Most R plasmids are transferred at low frequencies, whereas the F plasmid is transferred at a high frequency. It was found that some R plasmids when present in the same host cell inhibit the transfer of the F plasmid, while others do not. Watanabe used this difference as a means of dividing plasmids into fi^+ (fertility inhibition plus) and fi^- (149).

Subsequently, it was shown, mainly through the work of the Meynells and Datta (84), that there was a correlation between the fi status of a plasmid and the type of sex pili produced. In fi^+ strains, pili structurally and immunologically related to those determined by the F plasmid were present, whereas in fi^- strains pili related to those determined by the ColI plasmid were present. Accordingly, plasmids were designated as F-like and I-like.

As the studies on plasmids expanded during the 1960s, some plasmids were found to be nonconjugative and not to inhibit conjugal transfer. Therefore, they could not be classified as fi^+ or fi^-. Moreover, besides F-like and I-like pili, new types of pili were discovered (31). These developments made the classification schemes based on fi^+ and fi^- or F-like and I-like inadequate.

PLASMID INCOMPATIBILITY

A property which is universally inherited by plasmids and which is more suitable for classification is incompatibility. This is the inability of two plasmids to be propagated stably in the same cell line. Incompatibility is a manifestation of relatedness: the sharing of common elements involved in plasmid replication control or equipartitioning (for reviews, see references 52, 94, and 95).

Historically, plasmid incompatibility was first described for the F plasmid in the early 1960s (74, 115), although its existence was implicit earlier from the observation that in male *Escherichia coli* strains the F plasmid exists either in the autonomous state in F^+ strains or in the integrated state in Hfr strains, but not in both states simultaneously. In the late 1960s, experimental evidence was provided for the notion that, in transconjugant cells carrying integrated and autonomous F plasmids, the replication of the autonomous F plasmid was inhibited (40). Since the copy number of the F plasmid is low (1 to 2 copies per chromosome), this inhibition was interpreted to be due to the normal mechanism of copy number control. It was assumed that this copy number control mechanism is expressed by integrated as well as autonomous F plasmids.

A formal scheme of classification based on incompatibility was developed in the early 1970s, mainly by Datta and Hedges (35). Plasmids incompatible with each other were assigned to the same incompatibility group. Subsequent work in several laboratories established the validity of incompatibility grouping. At present, about 30 incompatibility groups are recognized among plasmids of enteric bacteria and 7 are recognized among staphylococcal plasmids. The use of incompatibility grouping for classification has been reviewed by Datta (32). A list of plasmids arranged according to incompatibility groups is provided in a book entitled *DNA Insertion Elements, Plasmids, and Episomes* (19).

Operationally, testing for incompatibility involves introduction (by conjugation, transduction, or transformation) of a plasmid into a strain carrying another plasmid. The two plasmids must have different genetic markers in order to

follow their segregation. Selection is usually carried out for the entering plasmid, and the progeny are examined for the continued presence of the resident plasmid. If the resident plasmid is eliminated, the two plasmids are said to be incompatible and are assigned to the same incompatibility group. One of us (P.L.B.) has recently described in detail practical aspects of incompatibility testing (7).

Although incompatibility has been generally useful for the classification of plasmids, certain complications have been recognized. These are of two kinds, technical and methodological. The former can arise because the plasmid to be tested does not contain a suitable marker gene or is not transmissible by the known means of plasmid transfer. Another technical obstacle is surface exclusion, which is due to inhibition of entry of the donor plasmid and which may be difficult to distinguish from incompatibility. Some of these technical difficulties have been resolved by the construction of a series of reference miniplasmids belonging to different incompatibility groups and containing a gene for galactose utilization (36). These miniplasmids, which are not conjugation proficient, are used as recipients in crosses with the plasmid to be classified. Problems related to surface exclusion are eliminated and incompatibility is revealed by the appearance of Gal^- segregants on suitable indicator plates.

Methodological limitations are of a more basic nature and arise mainly from the kinds and numbers of replication control systems and other incompatibility determinants present in a plasmid. Before discussing these limitations, it will be useful to briefly review what is known about basic replicons of plasmids. The review will also serve for the following discussion of "replicon typing," a new method for plasmid identification, based on hybridization with specific DNA probes containing replication control genes.

BASIC REPLICONS, AN INTERLUDE

The autonomous replication of plasmids takes place in a controlled manner such that a plasmid in a given host under given growth conditions is maintained with a defined average number of copies per cell. Plasmids themselves determine the means for correcting deviations from their characteristic copy numbers. All plasmids studied so far control their own replication by specifying a negative feedback loop (for reviews, see references 92 and 94). The genes and sites required for autonomous replication and its control constitute the basic replicons of plasmids. They generally consist of an origin of replication, "*cop*" and "*inc*" genes involved in the control of the initiation of replication, and (in most cases) "*rep*" genes encoding proteins required for replication and its control. Basic replicons, usually 2 to 3 kilobases (kb) in length, when ligated in vitro to a suitable selective marker, form miniplasmids which replicate autonomously with the same characteristics as the parental plasmid.

So far, only a small fraction of the known plasmids have been studied in detail (for reviews, see references 25, 58, 93, 117, 139), but a pattern has emerged in which most of the basic replicons fall into one of two types with respect to the manner in which they are controlled.

One type uses a small countertranscript ribonucleic acid (RNA) molecule as the main inhibitor in the control of initiation of replication (38, 89, 108, 131, 144). The target of these RNAs is an overlapping RNA transcribed from the opposite strand which is required as a primer, or as a messenger for a Rep protein, for the initiation of replication (62, 131, 143). The inhibitory effect is due to the formation of an RNA-RNA duplex between the countertranscript RNA and the complementary sequence of the target RNA. This RNA-RNA duplex is initiated by base pairing between complementary unpaired loops that are formed in both RNAs by secondary folding (17, 63, 67).

This type of control, called the inhibitor-target mechanism (94), is found in basic replicons of some small plasmids, such as ColE1 and its relatives (104), in the staphylococcal plasmid pT181 and its relatives (61, 96), and in basic replicons of large conjugative plasmids belonging to IncF incompatibility groups (9, 93, 110, 113).

In the case of ColE1-like plasmids, the countertranscript RNA acts by preventing the processing of the preprimer RNA by ribonuclease H (53). In the case of the large conjugative plasmids and pT181, the countertranscript RNA binds to the leader sequence of a messenger RNA and prevents the translation of a Rep protein which is required and rate limiting for replication (61, 131). In all three systems, the countertranscript RNA is able to act in *trans* and is responsible for the expression of an incompatibility phenotype.

It should be noted that in this control mechanism the colinearity of the inhibitor and the target leads to a high potential for genetic variation of the control mechanism and to the derivation of new incompatibility groups. Since the inhibitor and the target are transcribed (in opposite directions) from the same DNA segment, all base-pair changes in this DNA segment alter the countertranscript RNA and the target RNA in a complementary manner, which preserves the pairing of the two RNA molecules. As a consequence, some of these base changes do not affect the functioning of the replication control circuit but rather change its specificity: the mutant inhibitor does not recognize the wild-type target and vice versa. This can eventually lead to new incompatibility groups if all downstream functions involved in initiation of replication are *cis* acting.

This situation is exemplified by ColE1 and R1. In these plasmids, the downstream functions (the primer RNA in ColE1 and the Rep protein in R1) are *cis*-acting (or preferentially *cis*-acting) molecules (78, 97). In these cases, changes in the specificity of the countertranscript RNA create new incompatibility groups (17, 63, 118). On the contrary, in the case of the pT181 plasmid, the countertranscript RNA inhibits the synthesis of a Rep protein which acts in *cis* and in *trans*. In this case, even if two plasmid molecules have messenger RNA inhibitors of different specificities, they will still have a common Rep protein pool and therefore remain incompatible (51, 96).

Besides the countertranscript RNA, the ColE1 and R1 plasmids code for a protein which modulates the control of replication. For ColE1, the gene for this protein is called *rop* or *rom* (22, 126); for the IncFII plasmids, it is called *copB* (85) or *repA2* (39, 71). It should be mentioned that mathematical formulations for the countertranscript type of replication control have been proposed by Nordström (92) and Womble and Rownd (152).

The second group of basic replicons uses a series of direct DNA repeats, each about 20 base pairs (bp) long, as the main incompatibility determinant (replication inhibitor). These replicons show similarities in regard to the location of the repeats near the origin of replication and near a *rep* gene and in regard to the size of the Rep protein (29 to 38 kilodaltons).

This type of replicon structure is found in plasmids F (RepFIA replicon), P1, R6K, RK2, pSC101, and Rts1 (for reviews, see references 25, 41, 54, 58, 70, 117, 139). It has been proposed that the repeats limit the replication rate by binding the *trans*-acting Rep protein which is required and

rate limiting for initiation of replication (26, 70, 90, 98, 142, 148).

This hypothesis is based on the following observations: for several replicons, the Rep protein was shown to bind physically to the repeats; it was also shown to be autoregulated. The repeats express incompatibility without any indication of the synthesis of a diffusible product; mutations leading to increased copy number and decreased incompatibility have been located in the repeats and in the *rep* gene (1, 2, 11, 44, 57, 59, 107, 125, 127). In this model, the Rep protein could either interact with its own operator for autoregulation or bind to the repeats, which could lead to either the sequestration of the Rep protein or initiation of replication. However, the two regulatory circuits that have been proposed to render the Rep protein rate limiting for replication (autoregulation and sequestration) appear to be mutually exclusive, since the autoregulatory circuit is expected to replace the Rep protein molecules as they become captured by the repeats.

In an attempt to solve the autoregulation-sequestration dilemma, Trawick and Kline, in their studies on F replication (145), proposed to separate the initiation and autoregulation functions of the Rep protein by postulating two different forms of this protein, one formed irreversibly from the other. This proposition has been formulated in mathematical terms (153). Recently, evidence against this model has been presented by Chattoraj et al. for P1 replication (27). These authors have proposed another model involving simultaneous binding of the Rep protein to the Rep promoter and to the repeats. In this case, the sequestered Rep protein, though unavailable for replication, is still available for promoter repression. Evidence for this model was provided by showing in electromicrographs the formation of a DNA loop formed in the presence of bound Rep protein by the intervening DNA between the Rep promoter site and the region containing the repeats.

It will be of interest to see how generally applicable this model is to other replicons controlled by repeats. In the case of plasmid R6K, the situation appears to be more complicated than that proposed by the model (99).

INCOMPATIBILITY (CONTINUED)

To return to the discussion of shortcomings of incompatibility tests for plasmid classification, there are two situations in which such tests may give misleading information about the presence of related replicons. The first is the presence of several replicons in a plasmid, a situation common in plasmids belonging to IncF groups (9). When a multireplicon plasmid is used as a resident plasmid and is challenged by an incoming plasmid containing one of its constituent replicons, it is expected not be displaced, because the presence of a second functional replicon will take over its replication and thus prevent its loss from the cell. In multireplicon plasmids, potential incompatibility determinants are thus suppressed and combinations of replicons result in the formation of single incompatibility groups. The second situation arises from genetic changes, already alluded to above, that affect both the replication inhibitor and its target and thus lead to an altered incompatibility determinant. Under such circumstances, closely related replicons are assigned to different incompatibility groups, resulting in the creation of an unnecessarily complicated classification scheme. This situation is found in plasmids belonging to IncF groups, as well as in plasmids of several other incompatibility groups. In the following, an example for each of the two types of complications is described.

Plasmid pCG86, a conjugative plasmid with genes for enterotoxin production and drug resistance (79), contains two basic replicons, RepFIIA/FIC and RepFIB (64). In incompatibility tests it was found to be compatible with IncFI plasmids, and when used as a donor it displaced resident IncFII plasmids, such as R100; it was therefore assigned to group IncFII. However, a naturally occurring deletion mutant of pCG86 which had lost the replicon RepFIIA/FIC was found to be incompatible with IncFI plasmids, but not with IncFII plasmids, thus revealing a hidden incompatibility determinant.

The second situation leading to complications in plasmid classification can be illustrated by a group of basic replicons present in conjugative plasmids and having a countertranscript RNA control mechanism, such as the basic replicon RepFIIA in the IncFII plasmid R1. Basic replicons with this type of control mechanism are widely distributed among the six F-type incompatibility groups, among I-type incompatibility groups, and among plasmids belonging to incompatibility groups com9, B/O, K, and Z. The DNA sequences of some of the countertranscript RNA genes have been determined and the corresponding RNAs are shown in Table 3. They differ from each other in relatively few bases, but these differences are enough to make them mutually compatible, except for the FIC replicons of plasmids P307 and F. In some of these plasmids (IncFII and pINV plasmids), only one replicon is present, so that the assignment to different incompatibility groups must be due to differences in this replicon. Conversely, some of the I-type plasmids originally assigned to different incompatibility groups show cross-incompatibility (12). It follows from these examples that here the use of incompatibility for classification created too many incompatibility groups. Similar examples of such inconsistencies are cited by Datta (32).

It is because of these complications that we have developed the classification scheme based on replicon typing, described below. In this scheme, specific DNA probes are used to test for the presence of basic replicons by DNA-DNA hybridization. In addition to being a more direct test for replicons than incompatibility grouping, it is also technically simpler and less time-consuming.

REPLICON TYPING

Choice and Development of a Bank of rep Probes

To undertake the establishment of a bank of replicon probes, we cloned in high copy number plasmid vectors restriction endonuclease fragments derived from 19 different basic replicons. These clones were screened for their ability to express incompatibility towards the parental plasmids or miniplasmids used in their construction. Thus, in all cases, the probes carry at least one of the incompatibility determinants associated with replication or partition functions. Whenever possible, loci involved in plasmid copy number control were chosen rather than partition loci, since they are universally present in plasmids. For replicons with an unknown mechanism of replication, this distinction has yet to be established by genetic analysis.

The probes isolated so far range in size from 304 to 2,250 bp (see Table 1). Since the majority of the known incompatibility loci do not exceed 100 bp, it follows that these probes include genes or parts of genes that map outside the incompatibility determinants. The consequences of this fact will be discussed in the section dealing with the specificity of the probes.

TABLE 1. Construction of a bank of rep probes

Plasmid	Probe	Origin of probe	Inc group	Probe size (bp)	Restriction site(s) for excision of the probe	Antibiotic resistance of the vector
pULB2154	repFIA	F	IncFI	917	*Eco*RI	Tc
pULB2404	repFIB	P307	IncFI	1,202	*Pst*I	Tc
pULB2440	repFIC	P307	IncFI	967	*Eco*RI + *Hin*dIII	Ap
pULB2401	repFIIA	R1*drd-19*	IncFII	543	*Pst*I	Tc
pULB2422	rep9	pIP71	com9	539	*Pst*I	Tc
pULB2428	repI1	R64*drd-11*	IncI1	1,100	*Eco*RI + *Pst*I	Tc
pULB2406	repB/O	pMU700	IncB/O	1,600	*Pst*I	Tc
pULB2439	repK	R387	IncK	1,000	*Bam*HI + *Sal*I	Ap
pULB2436	repHI1	TR6	IncHI1	2,250	*Eco*RI + *Hin*dIII	Ap
pULB2433	repH12	TP116	IncHI2	1,800	*Eco*RI	Ap, Tc
pULB2423	repL/M	pMU407.1	IncL/M	800	*Pst*I	Tc
pULB2432	repN	R46	IncN	1,000	*Eco*RI	Ap, Tc
pULB2420	repP	RK2	IncP	750	*Hae*II	Km
pULB2424	repQ	R1162	IncQ	357	*Eco*RI	Ap, Tc
pULB2425	repT	Rts1	IncT	304	*Eco*RI + *Bam*HI	Ap
pULB2429	repU	RA3	IncU	950	*Eco*RI	Tc
pULB2426	repW	RSa	IncW	1,150	*Eco*RI	Tc
pULB2405	repX	R6K	IncX	942	*Hin*dIII	Ap
pULB2410	repY	P1	IncY	1,245	*Hin*dIII	Ap

Table 1 describes the plasmids that carry the replicon probes. The references to the original plasmids, the vectors used, and the precise steps followed in the construction of the plasmids carrying replicon probes are listed in Appendix II.

Cloning of sequenced rep probes carrying incompatibility loci involved in copy number control. Incompatibility loci associated with the control of initiation of replication have been located and sequenced for the following 10 basic replicons: FIA, FIC, 9, FIIA, K, P, Q, T, X, and Y. In these cases, the obvious route was to choose from the sequence data suitable restriction sites adjacent to the incompatibility determinants and to clone the fragments in high-copy-number vectors in such a way that ligation restored usable restriction sites at each side of the probe. Three plasmids carrying incompatibility determinants had already been constructed in other laboratories and were used directly as sources of probes. They are pCT7 (repP), pTW703 (repT), and pALA13 (repY). Plasmids carrying the other seven rep probes with known nucleotide sequences (repFIA, repFIC, rep9, repFIIA, repK, repQ, and repX) were constructed by cloning restriction fragments from available miniplasmids carrying the corresponding basic replicons.

Cloning of partly sequenced rep probes carrying incompatibility loci probably involved in copy number control. Miniplasmids with mapped incompatibility loci were available to

TABLE 2. Analysis of the specificity of rep probes

Plasmid	rep probe																		
	FI			9	FIIA	HI		I1	B/O	K	L/M	N	P	Q	T	U	W	X	Y
	A	B	C			1	2												
pULB2154 (repFIA)	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
pULB2404 (repFIB)	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
pULB2440 (repFIC)	−	−	+	+	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−
pULB2401 (repFIIA)	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
pULB2422 (rep9)	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
pULB2450 (repHI1)	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
pULB2433 (repHI2)	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
pULB2428 (repI1)	−	−	+	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
pULB2406 (repB/O)	−	−	+	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
pULB2439 (repK)	−	−	+	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
pULB2423 (repL/M)	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
pULB2432 (repN)	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
pULB2420 (repP)	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
pULB2424 (repQ)	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
pULB2425 (repT)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
pULB2429 (repU)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
pULB2426 (repW)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
pULB2405 (repX)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
pULB2410 (repY)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+

clone probes for repFIB, repI1, repB/O, and repW. For these probes, the loci supposed to be involved in copy number control had been partly sequenced. We cloned these loci and identified the resulting plasmids by screening for their ability to express incompatibility towards the parental miniplasmids.

Cloning of rep probes carrying unsequenced incompatibility loci. Plasmids carrying probes repHI1, repHI2, repL/M, repN, and repU were obtained by cloning fragments from restriction enzyme digests of representative plasmids of the corresponding incompatibility groups and screening for those able to express incompatibility towards the corresponding parental plasmid. Further analysis is now necessary to determine the genetic structure of the cloned fragments and, particularly, whether the cloned incompatibility determinants are associated with copy number control or equipartitioning functions. Note that, besides these 19 available probes, several others should be cloned so as to provide for a more complete typing of plasmids among the family *Enterobacteriaceae*. It would require the additional isolation of probes for replicons representative of the IncA/C, IncD, IncHI3, IncHII, IncI2, IncJ, and IncV incompatibility groups.

Analysis of Specificity of rep Probes

To analyze the specificity of the probes, we hybridized each of them with bacterial genomes of plasmid-free bacteria, with plasmids carrying each of the probes, and with the miniplasmids from which the probes were isolated. We observed that none of the probes hybridized with the DNA of the plasmid-free strains; 13 (FIA, FIB, L/M, N, P, Q, T, U, W, X, Y, HI1, and HI2) hybridized only with the plasmids carrying their own sequence (see Table 2) and with the miniplasmid used for their construction. The results support the conclusion that these 13 rep probes are specific for the replicon type of miniplasmid from which they are obtained (with the reservation that we do not have probes for all existing replicons).

Two sets of rep probes share regions of similarity since they cross-hybridize (Table 2): these are repFIIA, rep9, and repFIC, on the one hand, and repFIC, repI1, repB/O, and repK on the other hand. To understand the cause of this behavior, it will be helpful to consider RepFIIA of plasmids R100 and R1, the best-analyzed replicons of this series. Their genetic elements are: the *inc* (= *copA* in R1) gene that codes for the countertranscript RNA and that is involved in the control of plasmid copy number and in the expression of the IncFII incompatibility phenotype, and the *repA1* (*repA* in R1) and *repA2* (= *copB* in R1) genes that respectively code for the initiator RepA1 protein and the repressor RepA2 protein. The latter is involved in a secondary copy number control circuit. Recent nucleotide sequence analyses revealed that probes for repFIIA, rep9, repFIC, repI1, and repB/O all contain regions of significant similarities with the *inc* locus of the RepFIIA replicon of R100. Table 3 shows a comparison of the nucleotide sequences of the major stem-and-loop structures of the countertranscript RNA molecules encoded by these *inc* genes. The analysis suggests that these genes are phylogenetically related and seem to have been derived from a common ancestor by single-base-pair substitutions that created new incompatibility specificities.

When extending the comparison of the sequences to the left and to the right of the *inc* locus in probes repFIIA and repFIC, exchangeable DNA segments (modules) consisting of genes analogous to the *repA2* and *repA1* genes are

TABLE 3. Alignment of the nucleotide sequences of the major stem-and-loop structures predicted for analogous *inc* RNAs of different plasmids[a]

Structure	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Loop	25	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
1. FIC (P307)	C	C	C	C	G	G	U	A	A	U	C	U	U	-	C	U	C	-	C	-	U	C	G	U	-	CGCCAA	-	A	C	U	A	-	G	A	-	U	G	-	A	A	G	A	U	U	A	U	C	G	G	G	U
2. FIC (F)	U	C	C	C	G	G	U	A	A	U	C	U	U	-	C	U	C	-	C	-	U	C	G	U	-	CGCCAA	-	A	C	U	A	-	G	A	-	U	G	-	A	A	G	A	U	U	A	U	C	G	G	G	G
3. FIIA	C	C	C	C	C	G	G	U	A	A	U	C	U	U	-	-	U	-	U	C	G	U	A	C	U	-	C	G	C	C	A	A	-	G	U	U	-	A	G	-	A	U	U	A	U	C	G	G	G	G	G
4. com9	C	C	C	C	G	G	U	A	A	U	C	U	U	-	C	-	-	U	C	-	A	G	U	-	-	CGCCAA	-	A	C	U	U	-	G	A	C	-	G	-	A	A	G	A	U	U	A	U	C	G	G	G	G
5. ColV	C	C	C	C	G	G	U	A	A	U	C	U	U	-	C	U	-	U	C	G	C	A	G	U	-	CGCCAA	-	A	G	U	U	-	-	A	A	G	-	A	A	G	A	U	U	A	U	C	G	G	G	G
6. Inv	C	C	C	C	G	G	U	A	A	U	C	U	U	U	C	U	-	-	G	-	A	C	U	-	-	CGCCAA	-	A	G	U	U	-	-	A	A	G	-	A	A	G	A	U	U	A	U	C	G	G	G	G
7. I1	C	C	C	C	C	A	C	U	A	A	U	C	U	U	U	U	C	-	G	U	C	G	U	-	-	UCGCCAA	G	-	-	U	U	C	G	U	A	A	G	-	A	A	A	G	A	U	U	G	G	G	G	G
8. Iγ	C	C	C	C	C	A	U	A	A	U	C	U	U	U	U	U	C	-	C	G	U	-	C	-	-	CGCCAA	G	-	-	U	U	C	G	U	C	-	G	A	A	A	G	A	U	U	G	G	G	G	G
9. B/O	C	C	C	C	C	A	U	U	A	U	U	U	U	U	U	U	G	-	C	G	U	-	U	C	G	CGCCAA	G	-	-	U	U	C	G	A	G	-	G	A	A	A	A	A	U	A	G	G	G	G	G
10. Consensus	C	C	C	G	G	U	A	A	U	C	U	U	U	C	U	C	U	C	G	U	A	G	U	C	G	CGCCAA	G	A	C	U	U	C	G	A	C	A	G	A	A	A	A	G	A	U	A	U	C	G	G	G	G
																										U																								

[a] The stem is made up of nucleotides 1 to 25 (ascending branch) and 25 to 1 (descending branch). The alignments were made so that the nucleotides at each position and the complementarity between nucleotides in the stem are conserved as much as possible. The consensus sequence (bottom line) was derived from analogous *inc* RNA nucleotide sequences of the following plasmids: 1, P307 (IncFI, RepFIC replicon)—nucleotide sequence described in Saadi et al. (113); 2, F (IncFI, RepFIC replicon)—nucleotide sequence described Saadi et al. (113); 3, R*ldrd-10* (IncFII, RepFIIA replicon)—nucleotide sequence described in Rosen et al. (108); 4, pIP71 (com9)—nucleotide sequence from Bex and Couturier, unpublished data; 5, ColV2-K94 (incFI, Rep1 replicon)—nucleotide sequence described in Weber and Palchaudhuri (151); 6, pWR110—nucleotide sequence from Maas, unpublished results; 7, R64-11 (IncI1)—nucleotide sequence from Nikoletti et al. (91); 8, R621a (IncIγ)—nucleotide sequence from Nikoletti et al (91); and 9, pMU720 (IncB/O)—nucleotide sequence from Nikoletti et al. (91).

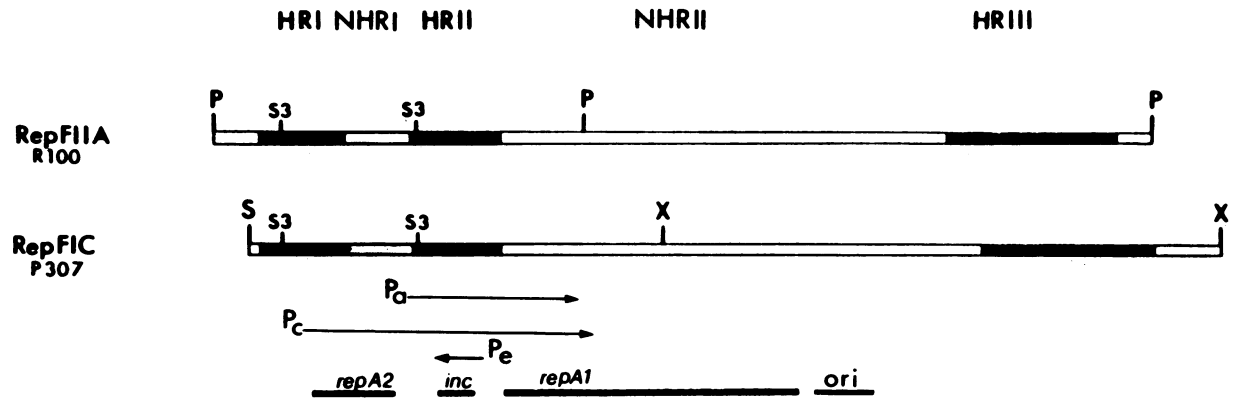

FIG. 1. Schematic presentation of replicons RepFIC and RepFIIA. The corresponding functional regions are aligned, and the genes are shown at the bottom. Symbols: solid boxes, complete homology; open boxes, no homology. P, *Pst*I; S, *Sma*I; S3, *Sau*3A; X, *Xho*I. Adapted from the paper by Saadi et al. (113).

observed (113), but corresponding modules sometimes have little or no sequence homology (Fig. 1). Nucleotide sequence analysis revealed that the fragment of the *repA1* gene present in rep9 is similar to the *repA1* gene of repFIIA but has no significant similarity to the *repA1* gene of repFIC. Furthermore, sequences similar to the *repA1* gene of RepFIC are present in probes repI1, repB/O, and repK. Thus, the replicons corresponding to the six probes repFIIA, rep9, repFIC, repI1, repB/O, and repK seem to be phylogenetically related. Their cross-hybridization in two sets can be explained by the divergence of their *inc* loci and by the different combinations of modules containing the *repA2* and *repA1* loci.

The implications of these findings for replicon typing are that replicons hybridizing with one of the six rep probes repFIIA, rep9, repFIC, repI1, repB/O, or repK should be considered members of one replicon family, which we call the RepFIC family (formerly RepFIIA family; 113). At present, we define a family of replicons as the group of replicons which hybridize with a defined replicon DNA probe or probes that are closely related. Hybridization is performed at a stringency allowing 15 to 20% mismatch. These hybridization conditions imply that a family of replicons will contain related replicons which share a DNA fragment highly similar (>80%) to part or all of the DNA fragment used as the replicon probe. They may include replicons with highly similar regions interspersed by sequence-divergent regions, but will not include phylogenetically related replicons with mismatches homogeneously distributed all along their DNA sequence (>20%). This is the present limit of our technique, but a looser relationship could easily be recognized by reducing the hybridization stringency or by DNA sequence analysis.

More precise identification of the members of the RepFIC family could be achieved by using probes internal to the *repA2* and *repA1* genes. This will lead to a classification scheme of plasmids based, first, on the identification of the common evolutionary lineage (*inc* region) and, second, on the composition of the individual modules.

Distribution of Replicons Having Similarity to the Available rep Probes among Representative Plasmids Belonging to 27 Currently Recognized Incompatibility Groups (Table 4)

In the preceding section, we have shown that the rep probes define different types of replicons. In this section, we examine the distribution of these types among representative plasmids belonging to 27 incompatibility groups and compare incompatibility grouping with replicon typing.

A collection of 120 strains carrying representative plasmids from 27 incompatibility groups were lysed and their DNA was bound to filters, as described in Appendix I. The filters were hybridized with the 19 available rep probes labeled with [α-^{32}P]deoxycytidine triphosphate by nick translation. As controls, we included on each filter the strains carrying the miniplasmids from which each probe sequence had been isolated, the vector plasmids, and the plasmid-free strains used as hosts.

The 19 replicon probes were isolated from plasmids belonging to 17 incompatibility groups. Three of them, repFIA (142), repFIB (64), and repFIC (112), are derived from three replicons found in plasmids of the IncFI group. The other 16 probes, replicons FIIA, 9, I1, B/O, K, HI1, HI2, L/M, N, P, Q, T, U, W, X, and Y, were isolated from plasmids belonging to the corresponding incompatibility groups.

In the collection of 120 strains used in this analysis, 90 carry plasmids belonging to 16 incompatibility groups for which probes are available (IncB/O, IncFI, IncFII, IncHI1, IncHI2, IncI1, IncK, IncL/M, IncN, IncP, IncT, IncU, IncW, IncX, IncY, and com9) and 30 strains carry plasmids belonging to 11 incompatibility groups from which probes have not been isolated so far (IncA/C, IncD, IncFIV, IncFV/FO, IncFVI, IncHI3, IncHII, IncI2, IncI, IncJ, and IncV).

Replicon typing of plasmids belonging to incompatibility groups with available probes. Plasmids belonging to incompatibility groups for which rep probes are available are listed first in Table 4. The first observation which emerges from the colony hybridization data is that, among the 90 plasmids tested, the majority (86 strains) hybridize at least with the probe corresponding to their incompatibility group. The four plasmids that do not hybridize with the probe corresponding to their incompatibility group belong to group IncX (plasmids R485, TP228, TP231, and pHH1187). This problem will be discussed below.

Among these 86 plasmids, two classes can be distinguished. Class I is made up of plasmids that hybridize with a single probe (Ia) or probes derived from one replicon family (Ib). Class II is composed of plasmids that hybridize with several probes derived from different types of replicons. These two classes will be discussed separately.

Class Ia. Plasmids hybridizing with a single probe. The great majority of plasmids belonging to 7 of the 16 incompatibility groups for which probes are available (IncL/M, N,

TABLE 4. Hybridization of replicon probes to plasmids to known incompatibility groups[a]

Incompatibility group and plasmid	Drug resistance[c]	rep probes[b]															
		FI			FII	HI		I1	L/M	N	P	Q	T	U	W	X	Y
		A	B	C	A	1	2										
IncL/M (=com7)																	
R471a	ApHg	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
R69-2	ApKm	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
R69	ApKmTcHg	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
pIP171	KmSu	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
pIP135	GmSmSuTcHg	−	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−
R446b	SmTc	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
pTH1	ApCmGmKmSmSpSuTcTpHg	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
IncN (=com2)																	
R46	ApSmSpSuTc	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
RPC3	KmPmSm	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
pIP113	Tc	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
N3	SmSpSuTcHg	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
N3T	Tc	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
R390	ApCmSmSpSUTc	−	−	−	−	−	−	p	−	+	−	−	−	−	−	−	−
IncP																	
RP1	ApKmTc	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
RP4	ApKmTc	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
R702	KmSmSpSuTcHg	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
R751	Tp	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
R906	ApSmSpSuHg	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
IncT																	
R402	ApSmSp	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Rts1	Km	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
R394	ApKm	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−
R401	ApSmHg	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
IncU																	
R1460	KmSmSpSuTc	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
RA3	CmSmSpSu	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
IncW																	
RS-a	CmGmKmSmSpSuTm	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
pIP100b	KmSu	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
pIP339	KmSu	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
R388	SuTp	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
R7K	ApSmSp	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
pIP356	Cm	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
IncY																	
MIP231	Tc	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
P1Cm	Cm	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
IncB/O (=com10)																	
RRIP185	KmTp	−	−	p	p	−	−	p	−	−	−	−	−	−	−	−	−
R16	ApSmSpSuTc	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
R16-1	SmSpSu	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
R723	CmSmSuTc	−	−	p	−	−	−	p	−	−	−	−	−	−	−	−	−
IncFII																	
R1*drd-19*	ApCmKmSmSpSu	−	−	+	+	−	−	p	−	−	−	−	−	−	−	−	−
pIP24	Tc	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
pIP187	SmTc	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
pIP100	CmKmSu	−	−	+	+	−	−	−	−	−	−	−	−	−	+	−	−
R1	ApCmKmSmSpSu	−	−	+	+	−	−	p	−	−	−	−	−	−	−	−	−
R1-16	Km	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
R136	Tc	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
R222Jap	CmFaSmSpSuTcHg	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
R494	ApKmTcHg	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
R538-1	CmSmSpSuHg	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−

Continued on following page

TABLE 4—*Continued*

Incompatibility group and plasmid	Drug resistance[c]	rep probes[b]															
		FI			FII	HI		I1	L/M	N	P	Q	T	U	W	X	Y
		A	B	C	A	1	2										
IncI1 (=incIα, = com1)																	
RIP112	KmNmPm	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
RIP186	SmTp	−	−	p	−	−	−	p	−	−	−	−	−	−	−	−	−
R144*drd-3*	Km	−	−	+	−	−	−	+	−	+	−	−	−	−	−	−	−
R64	SmTc	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
R144	KmTc	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
R483	SmSpTp	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
R648	ApKmSm	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
JR66a	KmSm	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
IncK																	
R387	CmSm	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−
com9																	
RIP71	ApCmSmSpSuTc	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
IncFI																	
F′*lac*		+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
pIP180	ApCmKmSmSuTc	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
pIP174	ApCmSmSuTc	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
R386	Tc	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
R453	ApCmSmSpSuTcHg	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
pHH507	Tc	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
R773	SmTcAsaAsi	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
R455	ApCmSmSpSuTcHg	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
R456	ApCmSmSPSuTc	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
pIP162-1	ApCmSmSu	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
pIP162-2	ApTc	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
RGN238	ApCmSmSuTc	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
ColV		−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
ColV2-K94		−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
ColV3-K30		−	+	p	p	−	−	p	−	−	−	−	−	−	−	−	−
pIP234		+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
P307		−	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−
IncHI1																	
TP123	CmSmSu	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
pIP522	CmSmSu	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
TR6	CmSmSu	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
pIP523	Tc	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
R27	Tc	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
R726	CmSmSpSuTc	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
IncH12 (=incS)																	
R477	CmKmSmSuTcHgTe	−	−	−	−	p	+	−	−	−	−	−	−	−	−	−	−
R478	CmKmTcHgTe	−	−	−	−	p	+	−	−	−	−	−	−	−	−	−	−
R826	ApCmGmKmSmTcHgTe	−	−	−	−	p	+	−	−	−	−	−	−	−	−	−	−
TP116	CmSmSu	−	−	−	−	p	+	−	−	−	−	−	−	−	−	−	−
pIP235		−	−	−	−	p	+	−	−	−	−	−	−	−	−	−	−
pSD114		−	−	−	−	p	+	−	−	+	−	−	−	−	−	−	−
IncX																	
R6K	ApSm	−	−	−	−	−	−	p	−	−	−	−	−	−	−	+	−
R485	Su	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
TP228	KmSmSuTc	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
TP231	ApCmTc	−	−	−	−	−	−	p	−	−	−	−	−	−	−	−	−
pHH1187	SmSuTp	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
IncA/C (= com6)																	
pIP55	ApCmGmKmSuTmHg	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
pIP55-1	Su	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
pIP218	CmKmSu	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
pIP216	CmKmSu	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
R16a	ApKmSu	−	−	−	−	−	−	−	−	−	−	p	−	−	−	−	−

Continued on following page

TABLE 4—*Continued*

Incompatibility group and plasmid	Drug resistance[c]	rep probes[b]															
		FI			FII	HI		I1	L/M	N	P	Q	T	U	W	X	Y
		A	B	C	A	1	2										
RA1	SuTc	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
pIP40a	ApKmSuHg	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
R666	Su	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
R707	ApCmKmSmSuTcHg	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
R714b	KmSm	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
R807	ApSuTcHg	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
P-*lac*	Su	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
pHH1350	SuTcHg	–	–	–	p	–	–	–	–	–	–	–	–	–	–	–	–
IncD																	
R711b	Km	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IncFIV																	
R124*drd-2*	Tc	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
IncFV/FO																	
Folac		–	–	p	p	–	–	–	–	–	–	–	–	–	–	–	–
IncFVI																	
pSU104		–	–	+	+	–	–	p	–	–	–	–	–	–	–	–	–
IncHI3																	
MIP233	SuTe	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IncHII																	
pHH1508a	SmSpTpTe	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IncI2 (=incIδ)																	
R175	Ap	–	–	p	p	–	–	p	–	–	–	–	–	–	–	–	–
TP114	Km	–	–	–	–	–	–	p	–	–	–	–	–	–	–	–	–
MIP241		–	–	+	p	–	–	–	–	–	–	–	–	–	–	–	–
pHH721	SmSpTp	–	–	–	–	–	–	p	–	–	–	–	–	–	–	–	–
R821a	Ap	–	–	–	–	–	–	p	–	–	–	–	–	–	–	–	–
IncIγ																	
R621a	Tc	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–
R621a-1a	ApCmKm	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–
IncJ																	
R391	KmNmHg	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
R391-3b-1	SmSpTpHg	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
IncV																	
R753	ApCmSmSu	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
R905	CmSmSu	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] The strains were obtained mostly from the National Collection of Type Cultures (Central Public Health Laboratory, London, England), from the collection of the Institut Pasteur, Paris, France (Y. A. Chabbert, P. Courvalin, G. Gerbaud, and L. Le Minor), and from the collection of Stanford University, Stanford, Calif. (E. Lederberg). The results were obtained according to the procedure outlined in the legend to Fig. A1.

[b] +, Strong hybridization with the probe (black spots on the radiograph); p, weak hybridization; –, absence of hybridization. Probes repFIIA and rep9, on the one hand, and probes repI1, repB/O, and repK, on the other hand, gave similar or identical results. Only the results with probes repFIIA and repI1 are reported.

[c] Abbreviations for drug resistance markers: Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; Tc, tetracycline; Sm, streptomycin; Su, sulfonamides; Sp, spectinomycin; Gm, gentamycin; Hg, mercuric ions; Asa, arsenate; Asi, arsenite; Fa, fusidic acid; Te, tellurium; Tp, trimethoprim; Tm, tobramycin; Nm, neomycin; Pm, paramomycin.

P, T, U, W, and Y) hybridize only with the specific probe of their corresponding incompatibility group (29 of 32 plasmids tested). We refer to this group as class Ia. This class of plasmids is exemplified by plasmids of the IncP (five plasmids tested) and IncW (six plasmids tested) groups. A detailed analysis of the homology found in the region involved in replication has been carried out for 10 IncP plasmids (27, 120). It revealed that the direct repeats in the origin region (the *incP* incompatibility determinant) are highly conserved in all IncP plasmids. In addition, the observed pattern of hybridization with other regions, such as the origin of conjugal transfer (*oriT*) (154), the G+C-rich region of the replication origin, and the *trfA* gene (28), led to the distinction of two subgroups among IncP plasmids, IncPγ and IncPπ. The use of the repP probe that carries the repeat sequences is consequently the proper choice for identifying all IncP plasmids. Further subdivision of IncP plasmids could be achieved by determining the pattern of hybridization with probes for the above-mentioned regions of the plasmid (*oriT*, G+C-rich region of the replication origin, or *trfA* gene).

The three exceptions to the monoreplicon state of the

plasmids in these seven incompatibility groups are plasmids pIP135 (IncL/M), R390 (IncN), and R394 (IncT). They are plasmids which contain a second replicon or replicon remnant and actually belong to the second class of plasmids (see below). That plasmid R394 is a cointegrate of an IncT and an IncN plasmid has been reported (47).

As far as our analysis has been carried out, a perfect correlation between incompatibility grouping and replicon typing is observed in this class of plasmids. For these plasmids, the replicon typing technique offers only a technical advantage over incompatibility grouping.

Class Ib. Plasmids hybridizing with probes from one replicon family. In our case this refers to probes repFIC, repFIIA, rep9, repI1, repB/O, and repK, which are fragments of replicons belonging to what we defined as the RepFIC family. As pointed out above, these replicons seem to be phylogenetically related. They contain an *inc*-like gene showing sequence similarities with the *inc* gene of plasmid R100 but with different incompatibility specificities, and they seem to contain modules made of different *repA2*- and *repA1*-like genes.

Plasmids listed in Table 4 that appear to fall into this category belong to the five incompatibility groups B/O, FII, I1, K, and Com9. Several points can be made about the response of these plasmids to the repFIC probe.

(i) The repFIC probe obtained from plasmid P307 (IncFI, multireplicon; see below) cross-hybridizes with all other RepFIC family replicon probes. We observed that all of the 24 plasmids belonging to the five incompatibility groups hybridized with this probe. Thus, replicon typing with the repFIC probe puts all plasmids belonging to the five incompatibility groups into one group. This unification is supported by the nucleotide sequence analysis of some of these replicons (Table 3) and suggests that they are all built on the same basic plan and presumably evolved from a common ancestor.

(ii) Replicon typing clearly identifies two subgroups among plasmids belonging to these incompatibility groups, according to their ability to hybridize with two groups of probes that separately cross-hybridize with probe repFIC: probe repFIIA (and the cross-hybridizing rep9 probe), on the one hand, and the repI1 (and the cross-hybridizing repB/O and repK probes), on the other hand.

Plasmids that hybridize with probe repFIIA (subgroup 1). Plasmids that hybridize with probe repFIIA include plasmids of the com9 group (1 plasmid tested) and 9 of the 10 plasmids of the IncFII group, which all hybridize only to the FIC and FIIA probes. The IncFII plasmid pIP100 shows, in addition, hybridization to the repW probe. This plasmid is apparently a multireplicon plasmid since its deleted derivative, pIP100a, which no longer hybridizes with the repFIIA probe, replicates autonomously and expresses the IncW incompatibility phenotype. In this regard, it should be noted that Bergquist et al. (9) observed that most plasmids of the IncFII group hybridized weakly to a broad repFIB probe. The information in Table 4 was obtained with a more precisely defined probe and such weak hybridization is not apparent. Plasmids of other IncF groups (IncFV/FO and IncFVI) could also belong to subgroup 1 since they carry sequences similar to probes repFIC and repFIIA. However, the answer rests on the isolation and analysis of the incompatibility determinants of plasmids belonging to these groups. Results indicating that there is a great deal of similarity between the replication regions of IncFII and IncFIII plasmids have already been reported (3).

As mentioned, P307 is the only plasmid of subgroup 1 that shows strong hybridization with probes identifying subgroup 2 (repI1, repB/O, and repK): this is, in fact, expected since repFIC, obtained from P307, cross-hybridizes with these probes. A few other plasmids of these groups show weak hybridization with probes repI1, repB/O, and repK: these are R1 and R1*drd-19* (IncFII) and pSU104 (IncFVI).

Plasmids that hybridize with probe repI1 (subgroup 2). Plasmids that hybridize with probe repI1 are plasmids of the IncB/O (four plasmids tested), IncI1 (eight plasmids tested), and IncK (one plasmid tested) groups. Plasmids of the IncI (two plasmids tested) and IncZ groups (P. Bergquist, unpublished observations) could also belong to this subgroup. Indeed, nucleotide sequence analysis of the RepI replicon revealed sequence similarities with RepB/O and RepI1 replicons at the level of an *inc*-like locus (S. Nikoletti, personal communication).

(iii) The classification of class Ib plasmids into two subgroups is not arbitrary but seems to have evolutionary significance. Homologies in the morphology of pili have been recognized for plasmids belonging to incompatibility groups IncI1, IncIγ, IncB/O, and IncK (thin flexible pili), on the one hand, and plasmids belonging to groups IncFII and com9 (thick flexible), on the other hand (15). In addition, plasmids of the IncI1, IncIγ, IncB/O, and IncK groups determine one pilus serotype, whereas IncI2 plasmids determine another pilus serotype. It is interesting that IncI2 plasmids (five plasmids tested) hybridize only weakly with probes of the repFIC family.

In conclusion, incompatibility leads to the classification of class Ib plasmids into different subgroups, although they carry closely related replicons. It was mentioned above that during evolution replicons of the inhibitor-target mechanism with new incompatibility specificities can result from a few base changes. For instance, the RepI replicon of plasmid ColV2-K94 and the RepFIIA replicon of plasmid R1 present highly similar sequences, yet they are mutually compatible due to only six base changes in the *inc* region (151). In this class of plasmids the incompatibility phenotype should not be used as a criterion for classification since it creates artificial groups. In contrast, the replicon typing test leads to the pooling of all of these plasmids with related replicons in the same family. More detailed identification can then be achieved by using short probes within the replication control genes.

Class II. Plasmids hybridizing with several probes derived from different types of replicons. In contrast to plasmids of the 12 incompatibility groups described above, another group of plasmids (IncFI and IncHI1) show hybridization to probes derived from several replicons which display different replication strategies. For example, the IncFI group plasmids are notable for their possession of more than one replicon. The data presented in Table 4 complement the detailed description of the occurrence of three basic replicons (RepFIA, RepFIB, and RepFIC) among plasmids of the IncF groups (7, 8). The presence of more than one replicon has been established for R386, ColV2-K94, P307, pSU316, and R124 (3, 20, 21, 100, 106, 150, 151).

Several points also are apparent from Table 4. (i) Plasmids of the HI1 group have been reported to express one-way incompatibility towards the F plasmid (109, 121). There is a short region of similarity shared by IncFI1 plasmids and F which includes the initiator E gene and repeat sequences of the repFIA replicon of F (109, 134; D. Saul, D. Lane, and P. L. Bergquist, Mol. Microbiol., in press). Replicon typing of IncHI plasmids confirms this conclusion (six plasmids tested): five of the six IncHI plasmids hybridize with the

TABLE 5. Hybridization of replicon probes with plasmids belonging to ETEC and non-ETEC *E. coli* strains

Replicon probe	% of strains[a] showing positive hybridization with the indicated rep probe	
	ETEC ($STaP^+$ $K99^+$)	Non-ETEC ($STaP^-$ $K99^-$)
repFIA	89.6	31.9
repFIB	96.6	58.6
repFIC	100	72.4
repHI2	1.7	0
repI1	28.4	20.8
repN	4.3	4.3
repP	31	13.8
repQ	0.9	14.9
repX	6.9	1.7
repY	0	1.7
repL/M	0	0
repT	0	0
repU	0	0
repW	0	0

[a] ETEC and non-ETEC strains were from the collection of the National Animal Disease Center, Ames, Iowa, and the collection of the Faculty of Veterinary Medicine of the University of Liege, Liege, Belgium. The STaP enterotoxin gene probe is described by Lathe et al. (65), and the K99 adhesin gene probe is described by Mainil et al. (76). The results were obtained according to the procedure described in the legend to Fig. A1. A total of 116 ETEC strains and 116 non-ETEC strains were tested.

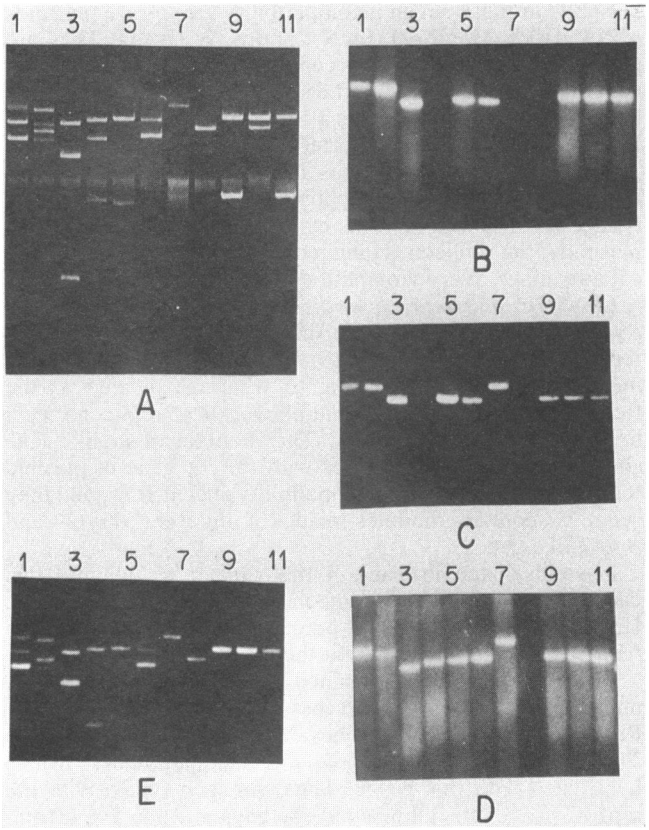

FIG. 2. Hybridization of plasmids isolated from ETEC strains with replicon and virulence gene probes. (A) Agarose gel (0.6%) electrophoresis showing the profile of plasmids isolated by the method of Kado and Liu (55). Radioautograph of this agarose gel after hybridization by the technique of Dalbadie-McFarland et al. (30) with probe for the STaP gene (B) (a probe for the K99 gene gave identical results), with probe repFIA (C), with probe repFIB (D), and with probe repFIC (E). Tracks 1, 2, 3, 5, 6, 9, 10, and 11 show plasmids isolated from ETEC strains. Track 4 shows plasmids isolated from a non-ETEC strain. Tracks 7 and 8 show test plasmids F'*lac* and R1*drd-19*, respectively.

RepFIA probe that carries the repeat sequences and part of the E gene. The sixth one, a tetracycline-sensitive deleted mutant of the IncHI1 plasmid pIP166 (pIP522), is compatible with F (23) and has lost the region of similarity with the E-gene sequence (134).

(ii) Plasmids of the IncHI2 group (six plasmids tested) all hybridize strongly with the repHI2 probe and weakly with the repHI1 probe. These data suggest the presence of similar replication or partition elements between IncHI1 and IncHI2 plasmids. However, to obtain a clear answer, further analysis of the nature of the elements shared by the two groups of plasmids is necessary.

In conclusion, data presented in Table 4 show that the multireplicon status of plasmids of class II can be identified clearly by replicon typing providing all corresponding replicon probes are available. On the contrary (see Introduction), the possession of more than one replicon often leads to unpredictable incompatibility phenotypes. Thus, for class II plasmids, incompatibility grouping is not a suitable method of classification and replicon typing gives a clearer identification. As expected from these observations, there is no obvious way to correlate incompatibility grouping with replicon typing of this class of plasmids.

Problem of IncX plasmids. The repX probe that contains the γ origin and part of the π gene of plasmid R6K hybridizes with plasmid R6K but does not hybridize with four other IncX plasmids tested. This is the only serious problem encountered among the 17 incompatibility groups for which probes are available. In fact, the basis of the incompatibility phenotype between different IncX plasmids is poorly understood, as demonstrated for autonomously replicating derivatives of R6K and R485 (128). Here, no cross-complementation between the R6K Rep protein and the R485 origins or the presumptive R485 Rep protein and any of the three R6K origins was observed. Replicon typing identifies such replicons as belonging to different types and leads to the classification of R6K and R485 (as well as plasmids TP228, TP231, and pHH1187) into different groups (Table 4). Note that two of these IncX plasmids (R485 and pHH1187) hybridize with probe repQ and would thus be associated, at this point of the analysis, with plasmids of the RepQ replicon type.

Replicon typing applied to plasmids belonging to incompatibility groups for which no probes are available. Among 30 plasmids of the 11 incompatibility groups with no available probes, 7 belonging to the five groups IncD, IncHI3, IncHII, IncJ, and IncV do not hybridize with the 19 replicon probes. Five plasmids that are members of the three groups IncFV/FO, IncFVI, and IncIγ have been tentatively associated with subgroups 1 and 2 of class Ib plasmids since they all hybridize with probes of the repFIC family. In contrast to IncIγ plasmids, IncI2 plasmids (five plasmids tested) hybridize only weakly with probes of the repFIC family and are awaiting the isolation of a repI2 probe for further classification. Finally, among the 13 IncA/C plasmids, 6 do not hybridize with any available probes, 2 hybridize with probes of the repFIC family, and 5 hybridize with the repQ probe. The latter positive hybridizations seem to reflect the presence of secondary or vestigial replicons in IncA/C plasmids, although this point has to be confirmed by isolating a repA/C probe.

Partition probes. Hybridization tests have also been per-

formed with a probe carrying partition loci associated with replicon RepFIA of IncFI plasmid F (data not shown). The probe contains part of the *sopB* and *sopC* loci: its nucleotide sequence is located between coordinates 1,961 (*Pvu*II site) and 3,778 (*Eco*RI site) as described by Mori et al. (88). Our results showed that most of the IncFI plasmids (14 of 17 plasmids tested) hybridize strongly with this probe. In addition, most of the IncB/O (3 of 4), IncI1 (7 of 8), and IncIγ (2 of 2) plasmids hybridize weakly with this probe. These plasmids seem to carry partition loci that diverged from a common ancestor. Thus, partition probes can be used for detailed identification of plasmids.

Replicon Typing of Virulence Plasmids Isolated from Bovine Enterotoxigenic *E. coli* (ETEC) Strains

Bacterial strains of medical importance often contain plasmids which render them pathogenic and multiresistant to antibiotics. Therefore, it is of interest to medical and veterinary microbiologists to identify these plasmids. The availability of the replicon typing technique makes such analysis feasible. Colony hybridization with the replicon probes enables one to type large numbers of strains and to have an overview of the replicon types present in a collection of pathogenic strains. Replicon typing of plasmid DNA separated by agarose gel electrophoresis allows one to identify the replicon types carried by each plasmid. For comparison, such typing may also be carried out with probes derived from virulence and from antibiotic resistance genes.

Here we give an example of such an analysis. We applied replicon typing to a collection of 232 *E. coli* strains isolated from calves with enteric and systemic diseases (J. Mainil, F. Bex, P. Dreze, M. Kaeckenbeek, and M. Couturier, manuscript in preparation). This collection contains two groups of strains: ETEC that hybridize with a probe for the heat-stable enterotoxin STaP gene (65) and a K99 adhesin gene probe (76) (116 isolates), and non-ETEC strains which show no detectable hybridization with these probes (116 isolates).

The results of the colony hybridizations in Table 5 show that in STaP$^+$ K99$^+$ ETEC isolates the number of strains hybridizing with rep probe repFIA or repFIB or repFIC or all three reaches 100%. In non-ETEC strains, sequence similarities with these replicon probes was observed, but at a lower frequency. This finding suggests a correlation between the virulence genes and plasmid(s) of the IncF groups.

To go further in our analysis, the plasmid DNA contents of 18 STaP$^+$ K99$^+$ *E. coli* isolates were separated by agarose gel electrophoresis and hybridized to STaP, K99, repFIA, repFIB, and repFIC radiolabeled probes. The results in Fig. 2 show that the ETEC isolates harbor one to four plasmids, that the virulence genes (STaP and K99) are located in a single plasmid in each isolate, and that the virulence plasmids have replicon profiles typical of the IncF groups plasmids (Table 4). The triple repFIA-repFIB-repFIC profile was the most common (15 of 18 virulence plasmids tested).

This result agrees with previous analyses which showed that in members of the family *Enterobacteriaceae* many virulence plasmids belong to the IncF groups. These plasmids were shown to express incompatibility towards IncF plasmids or to share DNA sequence similarities with IncF plasmids (for review, see reference 122) or both. Examples are virulence plasmids encoding enterotoxin formation (heat stable and heat labile), aerobactin formation (high-affinity transport of iron), invasiveness, and serum resistance (5, 9, 101, 103, 114, 123, 124).

In our experiment described in Table 5, we found that in most of the ETEC strains, the repFIC probe hybridizes with more than one plasmid. This demonstrates the widespread occurrence of the RepFIC replicon family in ETEC strains.

DISCUSSION

We have reviewed the ways in which plasmids have been classified, especially by arranging them into incompatibility groups, and we have described another scheme for plasmid classification based on replicon typing. It has been shown that for many incompatibility groups this grouping and replicon typing are equivalent, but for plasmids with multiple replicons or with similar replicons replicon typing is less equivocal and more specific for identification and classification. Moreover, replicon typing is technically simpler and faster to carry out than testing for incompatibility.

It is noteworthy that most plasmids of medical importance, such as plasmids with genes for toxin production, colonization factors, invasiveness, etc., belong to the IncF and IncI groups and frequently contain more than one basic replicon. For such plasmids, the use of replicon typing for identification is clearly of advantage.

The development of replicon typing has only begun, and there is much room for future improvements and refinements. Probes have not been constructed for all known incompatibility groups or for plasmids, such as pSC101 or ColE1, that have not been assigned to an incompatibility group. Existing probes need to be modified so that they contain only known replicon genes and no undefined sequences. Synthetic probes will be useful toward this end. The quality of replicon probes will presumably improve as our knowledge of basic replicons is expanded.

Of special interest is the design of probes for replicon families, such as the RepFIC family. These replicons contain regions of similarity interspersed with regions of divergence (113). By using segments from similar regions as probes, one tests for common features, whereas by using segments from dissimilar regions one tests for individuality. By this means, it becomes possible to divide replicons belonging to the same family into subgroups and thus to achieve a greater degree of differentiation.

Finally, we have confined ourselves for the present to probes derived from basic replicons, but it is of course possible to use DNA segments from other plasmid genes, especially genes for other maintenance functions, such as *par* genes. Development of such probes will lead to finer gradations and greater precision in the identification and classification of plasmids. In light of these considerations, the scheme presented in this review is not meant as a finished product, but as an impetus for the development of more precise and unambiguous schemes of plasmid classification in the future.

APPENDIX I

Technical details of the replicon typing test. Replicon typing is based on the possibility of revealing sequence relationships between plasmid replicons by nucleic acid hybridization methods. In this approach, the plasmid content of the strain to be tested (target nucleic acid) is immobilized on a solid matrix and hybridized with probes made from purified DNA fragments isolated from plasmid replicons and labeled by incorporation of radioactive or biotin-labeled nucleotides (for details of the probes used in replicon typing, see Appendix II). Before carrying out replicon typing, it is important to establish whether the strain to be tested contains one or several plasmids. Confusion has been caused in

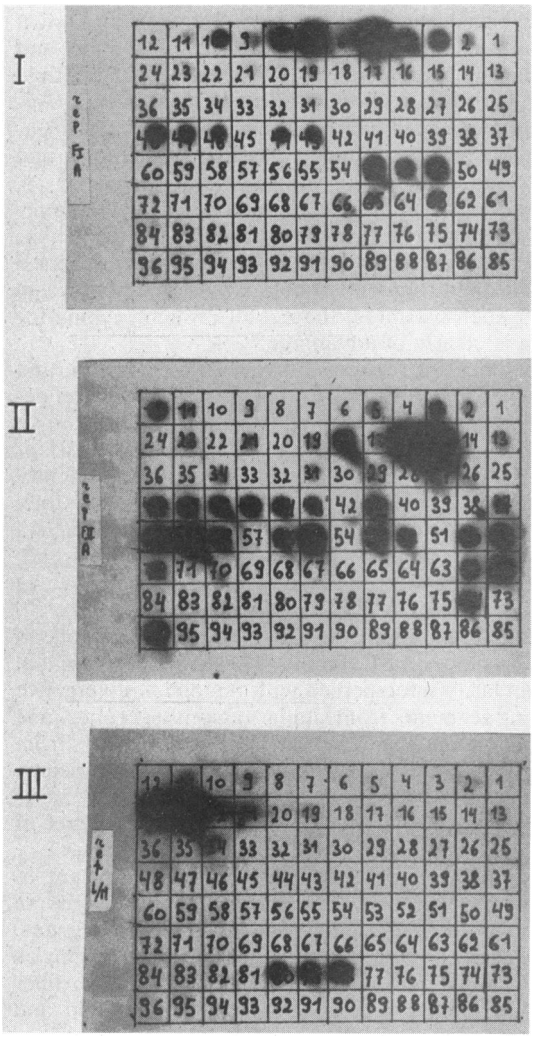

FIG. A1. Radioautograph of colony hybridization tests of bacterial strains carrying the plasmids listed in the table on the right. Colony arrays were printed from nutrient agar onto Whatman no. 541 filter paper. Each paper was processed for colony lysis and in situ denaturation of the nucleic acid content of each colony. Hybridization with radiolabeled probes and washing were carried out as described previously (43) under conditions that allowed 20% mismatch. Autoradiography on Kodak X-Omat AR film followed. I, repFIA probe; II, repFIIA probe; III, repL/M probe.

No. on filter	Plasmid	Characteristics
1		Plasmid-free strain C600 (4)
2	pKT279	Plasmid used as cloning vector for probes (133)
3	F'*lac*	IncFI group
4	pSC138	Mini-F-Ap^r; carries sequence of coordinates 40.1 to 49.5 kb on F map (140)
5	pULB2001	Mini-F cloned in pKT279 vector (10)
6	pBK53	Fragment of coordinates 46.13 to 49.5 kb of mini-F cloned in vector pBK50 (77)
7	pBK57	Fragment of coordinates 42.84 to 46.13 kb of mini-F cloned in vector pBK50 (77)
8	pULB2154	repFIA subclone (this paper)
9	pULB2403	Fragment of coordinates 47.7 to 49.5 kb of mini-F cloned in vector pKT279
10	pMU614	Mini-F-gal (36)
11	pULB2404	repFIB subclone (this paper)
12	P307	IncFI group
13	pWM114	Mini-FIB cloned in pBR322 (100)
14	pSS3928	Mini-FIB cloned in pBR322 (Maas et al. unpublished data)
15	pULB2400	Mini-FII cloned in pBR322
16	pULB2401	repFIIA subclone (this paper)
17		No plasmid
18	pMU610	Mini-FII-gal (36)
19	pULB2421	repI1 subclone
20	pMU605	Mini-I1 gal (36)
21	pULB2406	repB/O subclone
22	pMU602	Mini-B/O-gal (36)
23	pULB2423	repL/M subclone
24	pMU604	Mini-L/M-gal (36)
25	pULB2420	repP subclone
26	pMU612	Mini-P-gal (36)
27	pULB2424	repQ subclone
28	pMU608	Mini-Q-gal (36)
29	pULB2425	repT subclone
30	pMU607	Mini-T-gal (36)
31	pULB2405	repX subclone
32	pRK419	Mini-X-Km^r (129)
33	pMU609	Mini-X-gal (36)
34	pULB2410	repY subclone
35	pMU615	Mini-I2-gal (36)
36	pMU613	Mini-W-gal (36)
37	RIP185	IncB/O group
38	pIP55	IncC group
39	pIP55-1	IncC group
40	pIP218	IncC group
41	pIP216	IncC group
42	R16a	IncC group
43	pIP180	IncFI group
44	pIP174	IncFI group
45	R386	IncFI group
46	R453	IncFI group
47	R455	IncFI group
48	R456	IncFI group
49	pHH507	IncFI group
50	R773	IncFI group
51	pIP162-1	IncFI group
52	pIP162-2	IncFI group
53	RGN238	IncFI group
54		No plasmid
55	ColV	IncFI group
56	ColV2-K94	IncFI group
57	ColV3-K30	IncFI group
58	P307	IncFI group
59	R1*drd-19*	IncFII group
60	pIP214	IncFII group
61	pIP187	IncFII group
62	R124*drd-2*	IncFIV group
63	TP123	IncHI1 group
64	pIP522	IncHI1 group

Continued on following page

FIG. A1—*Continued*

No. on filter	Plasmid	Characteristics
65	pIP523	IncHI1 group
66	TR6	IncHI1 group
67	TP116	IncHI2 group
68	MIP233	IncHI3 group
69	RIP112	IncI1 group
70	RIP186	IncI1 group
71	R144*drd-3*	IncI1 group
72	R175	IncI2 group
73	TP114	IncI2 group
74	MIP241	IncI2 group
75	pHH721	IncI2 group
76	R391	IncJ group
77	R387	IncK group
78	R471a	IncL/M group
79	R69-2	IncL/M group
80	pIP171	IncL/M group
81	R46	IncN group
82	RPC3	IncN group
83	pIP113	IncN group
84	RP1	IncP group
85	RP4	IncP group
86	R478	IncHI2 group
87	R402	IncT group
88	R753	IncV group
89	RS-a	IncW group
90	pIP339	IncW group
91	IP100b	IncW group
92	R6K	IncX group
93	MIP231	IncY group
94	P1Cm	IncY group
95	R111	com8 group
96	pIP71	com9 group

the past by the presence of several plasmids in strains tested for incompatibility (for example, see reference 47). The plasmid contents of cells can be readily established by using a single-colony lysis technique (for example, see references 55 and 75) and electrophoresis on agarose gels. When more than one plasmid is found, the gel should be retained for replicon typing. It can be dried and used directly for hybridization with the replicon probes (30). In the analysis reported in Fig. A1, replicon typing was carried out with strains of members of the *Enterobacteriaceae* containing single plasmids representative of different incompatibility groups.

Colony lysis and immobilization of DNA on filters. The first step involves immobilization of the denatured nucleic acid obtained from lysed cells on solid supports such as nitrocellulose, nylon membranes, or high-wet-strength filter paper. The lysis and denaturation can be carried out directly on the support (43, 73) or in the test tube. In the latter case, the lysis step is followed by filtration of the lysate with a filtration manifold (75). Multiple filter replicas can be treated simultaneously, and once dried, they can be saved indefinitely. Steaming during the lysis procedure is very helpful for most isolates to increase bacterial lysis and to optimize the denaturation of the covalently closed circular plasmid DNA (73). Furthermore, hybridized filters can be stripped of their probes and reused.

Colony lysis is applicable to large-scale screening of bacteria. The liquid lysis method, which involves bacterial lysis and DNA denaturation prior to immobilization on nylon membranes, is particularly suited to relatively small numbers of samples which may have to be tested with more than one probe. This procedure allows adjustment of the number of cells lysed so that sufficient DNA is bound to the filter to allow the detection of replicons present in large, low-copy-number plasmids. For example, DNA equivalent to 0.6 ng of a 100-kb plasmid DNA (4×10^6 cells; plasmid copy number = 1) can be detected readily with a radioactive probe labeled to a specific activity of 5×10^7 dpm/μg (10^5 dpm/ml of hybridization solution).

Purification and labeling of probes. The probes are purified in the following way. DNA from the hybrid plasmid carrying the probe sequence is digested with a suitable restriction enzyme and electrophoresed in agarose gels. The probe DNA fragment can then be recovered from the agarose gel by a number of suitable methods (e.g., electroelution, melting and solubilization of low-melting agarose, electrobinding to diethylaminoethyl-cellulose membrane followed by extraction). Then the probe DNA is labeled by nick translation, using α-^{32}P-labeled nucleotides, using the method described by Rigby et al. (105).

Hybridization of the immobilized DNA with the labeled probes. To hybridize the immobilized DNA with the labeled probes, we have chosen to use conditions that allow 15 to 20% mismatch (Fig. A1). The choice of conditions for hybridization is discussed in the review by Meinkoth and Wahl (80).

Nick-translational labeling of probe DNA with biotin and colorimetric detection of hybridization. In our hands, biotin labeling of probe DNA is somewhat less sensitive than using radioactive probes and is confined to liquid lysis procedures. By using dot-blotted total DNA as described earlier, the limit of detection is 1.3 ng of plasmid DNA (M_r = 60,000) and is limited by background hybridization to the dots. We find it important to dot-blot the DNA to nitrocellulose membranes if the biotin system is used. The amount of DNA required to enable detection of a single copy plasmid of 60 megadaltons is equivalent to about 8×10^6 cells total and is well within the practical limitations (2×10^8 cells) of filtering onto the membrane. We have used the BluGene nonradioactive nucleic acid detection system supplied by Bethesda Research Laboratories. Nick translation is carried out as described previously (105), except that biotin-11–deoxyuridine triphosphate is used in the reaction mixture. At the completion of the reaction, the biotinylated DNA is isolated by passage through a Sephadex G50 column. Prehybridization and hybridization are carried out as for radioactive probes.

APPENDIX II

Construction of a bank of hybrid plasmids carrying probes for replicons. Table 1 summarizes the different plasmids used in the construction of the subclones containing the replicon probes and the characteristics of the probes.

Below we describe in detail the steps followed in the construction of the subclones.

repFIA: *inc/rep* functions. pULB2154 was constructed by cloning a 917-bp *Hae*III fragment from the RepFIA replicon (*Eco*RI f5 fragment) of the IncFI plasmid F'*lac pro* (66) in the filled *Eco*RI site of vector pKT279 (133). The *Hae*III fragment has the coordinates 45.029 to 45.946 kb on the F map and was subcloned from pULB2001 (10), a recombinant plasmid composed of the *Eco*RI f5 fragment of plasmid F'*lac pro* cloned in vector pKT279. Its nucleotide sequence is described in Murotsu et al. (90) between coordinates 930 and 1,846. It contains the four repeated sequences of 19 bp of the *incB* locus and the first 695 bp of the 755-bp-long *E* gene that codes for the initiator (E) protein. The construction of pULB2154 creates *Eco*RI sites on each side of the cloned

fragment. pULB2154 was shown to express incompatibility towards the F'*lac pro* plasmid and towards pSC138, a RepFIA-Ap^r miniplasmid (140).

repFIB: *inc* functions. pULB2404 was constructed by cloning a 1,202-bp *Pst*I fragment from the RepFIB replicon (*Eco*RI E11 fragment) of the IncFI plasmid P307 (114) in the *Pst*I site of vector pBR322 (14). The 1,202-bp *Pst*I fragment was subcloned from pWM114 (R. Maas, unpublished data), a recombinant plasmid composed of the *Eco*RI E11 fragment of P307 cloned in vector pBR325. This fragment has been sequenced recently (Bergquist, unpublished experiments). Hybrid pULB2404 expresses incompatibility towards pSS3928, a RepFIB-Spc^r/Str^r miniplasmid derived from plasmid P307 (R. Maas, S. Saadi, and W. K. Maas, unpublished observations).

repFIC: *inc/rep* functions. pULB2440 was constructed by cloning a 967-bp *Ava*I fragment from the RepFIC replicon (*Eco*RI E7 fragment) of the IncFI plasmid P307 (114) in vector pUC12 (147). The *Ava*I fragment was purified from the PSS3945 miniplasmid (113). The fragment with filled sticky ends was cloned in the *Hin*cII site of vector pUC12. The nucleotide sequence of the 967-bp *Ava*I fragment is described in Saadi et al. (113) between coordinates 1 and 967. It contains a 257-bp open reading frame analogous to the *copB* gene of plasmid R1, a 91-bp sequence highly similar to the *copA* gene of plasmid R1 (nine mismatches), and 222 bp of the 587-bp-long analogous *repA* gene. *Eco*RI and *Hin*dIII sites located at each side of the cloned sequence in the pUC12 polylinker can be used to separate the probe from the vector. pULB2440 expresses incompatibility towards pSS3945, a RepFIC-Spc^r/Str^r miniplasmid derived from plasmid P307 (113).

repFIIA: *inc/rep* functions. pULB2401 carries a 543-bp *Pst*I fragment from the IncFII plasmid R1*drd-19* (83). This *Pst*I fragment was subcloned in the *Pst*I site of vector pBR322 from pKN1562-Ap^r (86), a miniplasmid derived from the IncFII plasmid R1*drd-19*. The nucleotide sequence of the 543-bp *Pst*I fragment is described in Ryder et al. (111) between coordinates 76 and 619; it contains the last 19 bp of the 258-bp-long *copB* gene, the 91-bp sequence coding for the CopA RNA, and 226 bp of the 858-bp-long *repA* gene. pULB2401 was identified by its ability to express incompatibility towards plasmid R1*drd-19*.

rep9: *inc/rep* functions. pULB2422 was constructed by cloning a 539-bp *Pst*I fragment from the com9 plasmid pIP71 (116) in the *Pst*I site of pBR322, after initial cloning in the pMU601 miniplasmid (36). The nucleotide sequence of the 539-bp *Pst*I fragment has been determined by F. Bex and M. Couturier (unpublished results); it is highly similar to the 543-bp *Pst*I fragment of the IncFII plasmid R1*drd-19* that carries the *copA* gene (repFIIA probe). It contains 19 bp similar to the end of the *copB* gene of plasmid R1 (one mismatch), a sequence of 87 bp partly similar to the sequence which codes for the 91-bp CopA RNA in plasmid R1 (24 mismatches and four deletions of 1 bp each), and 230 bp which are highly similar to the first 230 bp of the *repA* gene in plasmid R1 (7 mismatches). pULB2422 expresses incompatibility towards pMU601. It is compatible with the IncFII plasmid R1*drd-19*.

repI1: *inc/rep* functions. pULB2428 carries a *Pst*I-*Sau*3A fragment, about 1.1 kb long, from the IncI1 plasmid R64*drd-11* (82). This fragment was subcloned from pMU605, a miniplasmid derived from R64*drd-11* (36). It was constructed by first cloning the 2.5-kb *Pst*I fragment carrying the *inc*I1 locus of pMU605 in the *Pst*I site of vector pBR322. This construction generated the hybrid plasmid pULB2421. Subsequently, a *Sau*3A fragment derived from pULB2421 was subcloned in the *Bam*HI site of vector pUC12; this construction is designated pULB2427. The cloned *Sau*3A fragment contains a *Pst*I site. The fragment flanked by this *Pst*I site and the *Eco*RI site located in the pUC12 polylinker was subcloned in the vector pBR322, which had been cleaved with enzymes *Pst*I and *Eco*RI, to generate pULB2428. This plasmid expresses incompatibility towards pMU605 as well as towards the IncI1 plasmid R186 (F. W. Goldstein, J. F. Agar, G. R. Gerbaud, and Y. A. Chabbert, Program Abstr. 15th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 169, 1975). Part of the *incI1* locus has been sequenced (Nikoletti et al., personal communication); it shows some sequence similarities in the *copA* gene of the IncFIIA plasmid R1.

repB/O: *inc/rep* functions. pULB2406 was constructed by cloning a *Pst*I fragment of 1.6 kb from the IncB/O plasmid pMU700 (45) in the *Pst*I site of pBR322. The *Pst*I fragment was subcloned from pMU602 (36), a miniplasmid derived from plasmid pMU700. The *inc*B/O locus is located on a 360-bp *Sau*3A fragment internal to this 1.6-kb *Pst*I fragment (13). It has been partly sequenced and shows sequence similarities to the *inc*I1 locus of the IncI1 miniplasmid pMU605 (Nikoletti et al., personal communication). Plasmid pULB2406 is incompatible with pMU602 and with the IncB/O plasmid R185 (Goldstein et al., 15th ICAAC); it is compatible with the IncI1 plasmid R186.

repK: *inc* functions. pULB2439 was constructed by cloning a 1-kb *Bam*HI-*Sal*I fragment from the IncK plasmid R387 (119) in pBR322 which had been cleaved with restriction enzymes *Bam*HI and *Sal*I. The 1-kb fragment was isolated by first cloning an 1,800-bp *Sal*I fragment of plasmid R387 that cross-hybridized with the repFIC probe isolated from hybrid pULB2440. This plasmid was identified by its ability to express incompatibility towards R387. The IncK locus has been sequenced (Couturier et al., unpublished results); it shows some sequence similarities with the *copA* gene of IncFIIA plasmid R1.

repHI1: *inc* functions. A 7-kb *Eco*RI fragment from the IncHI1 plasmid TR6 (102) was cloned in pBR322 (pULB2434). pULB2436 was constructed by recloning a 2.25-kb *Eco*RI-*Hin*dIII fragment from pULB2434 in pBR322 cut with *Eco*RI and *Hin*dIII. pULB2436 expresses incompatibility towards TR6. The function of the cloned *inc* locus is unknown.

repHI2: *inc* functions. pULB2433 was constructed by cloning an *Eco*RI fragment of 1.8 kb from the IncHI2 plasmid TP116 (16, 46) in the *Eco*RI site of pBR322. It was identified by its ability to express incompatibility towards TP116. The function of the cloned *inc* locus is unknown.

repL/M: *inc/rep* functions. pULB2423 was constructed by cloning an 800-bp *Pst*I fragment that carries the *incL/M* locus of pMU604 (36), a miniplasmid derived from the IncL/M plasmid pMU407.1 (37), in the *Pst*I site of pBR322. pUL2423 expresses incompatibility towards the IncL/M plasmid R69-2 (24). Sequence analysis of the 800-bp *Pst*I fragment is in progress (A. Tossens and M. Couturier, personal communication).

repN: *inc* functions. pULB2432 carries a 1-kb *Pvu*II fragment of the IncN R46 plasmid (34) cloned in the filled *Eco*RI site of pBR322. To construct this hybrid, a 6-kb *Bgl*II fragment of plasmid R46 was cloned in the *Bam*HI site of pUC12 to give pULB2430. This fragment has been shown to carry the replication function of plasmid R46 (18). Then, a 1-kb *Pvu*II fragment which was able to express incompatibility towards the IncN plasmid RPC3 was subcloned in the

filled *Eco*RI site of pBR322. This construction changes the *Pvu*II sites into *Eco*RI sites on each side of the cloned sequence. The function of the cloned *inc* locus is unknown.

repP: *inc/rep* functions. pULB2420 is plasmid pCT7 described in Thomas et al. (138). It carries a 750-bp *Hae*II fragment of the IncP plasmid RK2 (50). The nucleotide sequence of a 617-bp segment internal to this 750-bp *Hae*II fragment has been determined (130). It contains eight repeat sequences of 17 bp, five of which are part of the origin of replication of plasmid RK2 (29). The repeat sequences form an *inc* locus involved in IncP plasmid copy number control (137).

repQ: *inc/rep* functions. pULB2424 was constructed by cloning a 357-bp *Hae*III fragment from the IncQ plasmid R1162 (6) in the filled *Eco*RI site of vector pBR322. The *Hae*III fragment was subcloned from pMU608, a miniplasmid derived from R1162, in which the small *Pst*I fragment of R1162 was replaced by the *Pst*I Gal fragment of pRBD13 (36; J. Pittard, personal communication). The nucleotide sequence of the 357-bp *Hae*II fragment is described in Meyer et al. (81) between coordinates 216 and 572; this region contains three perfectly conserved direct repeats, part of which is repeated a fourth time. The repeats are part of the origin of replication (69) and are involved in expression of the *incQ* incompatibility (68). The construction creates *Eco*RI sites on each side of the probe sequence.

repT: *inc/rep* functions. pULB2425 is the hybrid plasmid pTW703 constructed as described in Terawaki and Itoh (135). It carries a 304-bp *Hae*II-*Sau*3A fragment of the IncT plasmid Rts1 (136). The nucleotide sequence of this fragment is reported in Kamio et al. (56) between coordinates 1,032 and 1,336; it contains three repeats of 21 bp (referred to as the *incII* locus) and the first 99 bp of the 836-bp *repA* gene. pULB2425 expresses incompatibility towards pMU607, a miniplasmid derived from Rts1 (36).

repU: *inc* functions. pULB2429 was constructed by cloning an *Eco*RI fragment, about 950 bp long, of the IncU plasmid RA3 (48) in the *Eco*RI site of vector pULB2130. pULB2130 is a derivative of pKT279 constructed by eliminating the *Eco*RI site of pKT279. The *Pst*I fragment of transposon Tn9 that carries the Cm^r marker was inserted in the *Pst*I site of the modified pKT279 vector. Insertions of fragments in the *Eco*RI site located in the chloramphenicol transacetylase gene of pULB2130 inactivate the Cm^r marker. pULB2439 was identified by its ability to express incompatibility towards RA3. The function of the cloned *inc* locus is unknown.

repW: *inc/rep* functions. pULB2426 carries a 1.15-kb *Hin*fI fragment of the IncW plasmid RSa (also known as pS-a) (48). This fragment has been partly sequenced (132). It contains the *oriV* site, three repeat sequences of 13 bp, and the major portion of the *repA* gene. The *Hin*fI fragment was subcloned from pMU613 (36), a miniplasmid derived from RSa. The sticky ends of the *Hin*fI fragment were filled, and the fragment was cloned in pULB2197 cleaved with *Sma*I. This vector is a derivative of pULB2130; it was constructed by insertion of two *Eco*RI-*Sma*I-*Bam*HI polylinkers in the *Eco*RI site of pULB2130. This construction allows the recovery of blunt-ended fragments cloned in the *Sma*I sites as *Eco*RI fragments. pULB2426 expresses incompatibility towards the IncW plasmids RSa and pIP339.

repX: *inc/rep* functions. pULB2405 was constructed by cloning a 942-bp *Hin*dIII fragment of the IncX plasmid R6K (60) in the *Hin*dIII site of pBR322. The nucleotide sequence of the 942-bp *Hin*dIII fragment is described in Stalker et al. (129) between coordinates 1 and 941. It contains eight repeat sequences of 22 bp (*inc* locus), the first seven being part of the γ origin and 455 bp of the 918-bp-long π gene. The *Hin*dIII fragment was subcloned from pRK419 (129), a miniplasmid derived from R6K. pULB2405 expresses incompatibility towards R6K. It is compatible with other plasmids of the IncX group such as R485 because it lacks a functional β origin of replication of R6K. Stalker and Helinski (128) have shown the β origin to be obligatory for the expression of incompatibility.

repY: *inc/rep* functions. pULB2410 is the subclone pALA 13, constructed as described in Abeles et al. (2). It carries a 1,245-bp *Hin*dIII fragment of the IncY plasmid P1 (49). The nucleotide sequence of the 1,245-bp *Hin*dIII fragment is described in Abeles et al. (2) between coordinates 610 and 1,855. It contains nine repeat sequences of 19 bp (*incA* locus) and the 860-bp-long *repA* gene.

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