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Composite IS*1* Elements Encoding Hydroxamate-Mediated Iron Uptake in FI*me* Plasmids from Epidemic *Salmonella* spp.

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Eleven FI*me* plasmids representative of those identified in epidemic strains of *Salmonella wien* and *Salmonella typhimurium* isolated in North Africa, Europe, and the Middle East have been examined for the presence of determinants of toxigenicity, adherence, and iron-sequestering mechanisms. Chemical and genetic data indicated that all plasmids code for a hydroxamate-mediated iron assimilation system. Detailed analysis of derivative plasmids and cloned fragments of FI*me* plasmid pZM61 demonstrated that the general genetic and structural organization of the DNA region containing the genes for hydroxamate biosynthesis and cloacin DF13 receptor was virtually identical to that described for the aerobactin-mediated iron uptake system of pColV-K30. This DNA region is part of a composite element that is 16.7 kilobases long and carries its IS*1* modules as inverted repeats. A very similar element is present in either orientation in all nine FI*me* plasmids analyzed.

From 1969 to 1980 considerable clinical and epidemiological evidence indicated that the emergence and prevalence of some epidemic strains of human *Salmonella* spp. might be correlated to the acquisition of FI*me* plasmids. In such studies the best-followed strains have been *Salmonella wien* in North Africa and Europe (5, 17, 18), *Salmonella typhimurium* phage type 208 or derivatives of it mainly in the Middle East (1), and *S. typhimurium* phage type 66/122, or derivatives of it in Southeast Asia, including several states of India, and the Middle East (26). Almost all isolates of the three *Salmonella* spp. strains in those wide geographical areas carried an FI*me* R plasmid. FI*me* plasmids have been also identified in clinical strains of *Salmonella johannesburg* in Hong Kong (6) and in isolates of *Salmonella typhi*, *Salmonella oranienburg*, *Salmonella newport*, *Salmonella heidelberg*, and *Escherichia coli* in Algeria, Indonesia, Zimbabwe, United States, and Brazil (1). They are the largest subgroup of IncFI plasmids, at least as far as salmonellas are concerned.

FI*me* plasmids are conjugative or defective conjugative multiple drug resistance plasmids ranging in size from 100 to 180 kilobase pairs (kb) (1, 5, 6, 18, 26). They are incompatible with IncFI plasmids and the MP10 plasmid (or derivatives of it) isolated from *S. typhimurium* phage type 36 (1). FI*me* plasmids isolated from different bacterial strains, in different countries, and in different years show high DNA homology (36) as well as characteristic restriction enzyme patterns and specific variations in their structure (5; M. Nicoletti, B. Colonna, M. Casalino, and F. Maimone, manuscript in preparation).

To understand the possible reasons for the correlation between this class of plasmids and the epidemiological behavior of *Salmonella* spp. strains we decided to investigate at the molecular level whether individual genes or whole genetic systems on these plasmids could be directly involved in determining pathogenicity. We screened strains carrying representative FI*me* plasmids for production of heat-stable (ST) and heat-labile (LT) enterotoxins, biosynthesis of colonization factor antigens, and iron-sequestering mechanisms (9, 10, 21, 22).

In this paper we report the genetic and physical identification of a plasmid-encoded, hydroxamate-mediated iron assimilation system and the location of essential genes of this system on a large composite element flanked by inverted repeats of IS*1*. Furthermore we report the presence in opposite orientations of this genetic element on different FI*me* plasmids in *S. wien* and *S. typhimurium* phage type 208.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* K-12 strains normally used in genetic experiments were ZM46 (18), a nalidixic acid-resistant mutant of CSH26 *ara* Δ(*lac pro*) *thi*, and strain 803 *met gal lac hsdS*. *E. coli* strains RW193 F⁻ *thi proC leuB trpE lacY rpsL galK ara entA403 mtl xyl azi tsx supE44* and RWB18, a *fepA* mutant of RW193, were kindly provided by J. B. Neilands.

Plasmid pJN21, CloDF13::Tn*901*, was obtained from G. Cesareni. Reference and vector plasmids were F′ *lac pro*, R100, R1 *drd-19*, pBR322, and pACYC184. The FI*me* plasmids are listed in Table 1.

Media and chemicals. Rich and minimal growth media included LB medium (19), brain heart infusion, Trypticase soy broth (BBL Microbiology Systems), Minca medium (7), and M9 medium (19). The following antibiotics were used: ampicillin, 30 μg/ml; chloramphenicol, 25 μg/ml; gentamicin, 10 μg/ml; kanamycin, 25 μg/ml; mercurochrome, 150 μg/ml; nalidixic acid, 40 μg/ml; rifampin, 100 μg/ml; spectinomycin, 10 μg/ml; streptomycin, 10 μg/ml; sulfonamide, 600 μg/ml in minimal medium; and tetracycline, 5 μg/ml. When required, α,α′-dypiridyl (150 μM) or $FeCl_3 \cdot 6H_2O$ (30 or 50 μM) was added in M9 minimal medium to reduce or increase the availability of iron. Freeze-dried preparations of ovotransferrin, characterized by electrophoresis and iron-binding capacity, were kindly supplied by the Institute of Chemistry, Faculty of Medicine, University of Rome. Purified lactoferrin from human colostrum was purchased from United States Biochemical Corp.

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TABLE 1. Representative naturally occurring FI*me* R plasmids chosen for genetic analysis of pathogenicity characters

Plasmid	Original host strain	Origin, yr	Relevant phenotype[a]	Molecular size (kb)	Source and reference
pZM3	*S. wien* 20	Algeria, 1970	Ap Cm Hg Km Sm Sp Su Tc Clo^s Tra$^+$	165	L. Le Minor, Institut Pasteur, Paris, and this laboratory[b]
pIP174	*S. wien* 144	France, 1971	Ap Cm Sm Sp Su Tc Clo^s Tra$^+$		G. Gerbaud, Institut Pasteur, Paris
pIP180	*S. wien* Horn	France, 1974	Ap Cm Gm Hg Km Sm Sp Su Tc Clo^s Tra$^+$		G. Gerbaud
pZM61	*S. wien* WZM6	Italy, 1974	Ap Cm Hg Km Tc Clo^s Tra$^-$	145	(5, 18)
NTP101	*S. typhimurium* type 208, 14M6407	England, 1974	Ap Cm Hg Sm Sp Su Tc Clo^s Tra$^-$	135	B. Rowe, Central Public Health Laboratory, London (1, 36)
TP181	*S. typhimurium* type 208, 15M3557	Iran, 1975	Ap Cm Hg Km Sm Sp Su Tc Clo^s Tra$^+$	165	B. Rowe
pZM33	*S. wien* WZM3	Italy, 1976	Ap Cm Hg Km Tc Clo^s Tra$^+$	150	(5, 18)
pZM111	*S. wien* WZM11	Italy, 1978	Ap Cm Gm Hg Km Clo^s Tra$^+$	130	(5)
pZM141	*S. wien* WZM14	Italy, 1979	Ap Cm Hg Km Tc Clo^s Tra$^+$	150	(5)
pZM151	*S. wien* WZM15	Italy, 1979	Ap Cm Hg Km Tc Clo^s Tra$^+$	155	(5)
pZM171	*S. wien* WZM17	Italy, 1980	Ap Cm Hg Km Clo^s Tra$^+$	145	(5)

[a] Abbreviations indicate the following resistances: Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin, Hg, mercuric ion; Km, kanamycin; Sm, streptomycin, Sp, spectinomycin; Su, sulfonamide; and Tc, tetracycline. Other abbreviations: Clo^s, susceptibility to cloacin DF13; Tra$^+$, conjugative; Tra$^-$, defective conjugative. Molecular lengths of pZM3, pZM61, pZM33, pZM141, and pZM151 were determined by single and double digestions of plasmid DNA with the restriction enzymes *Eco*RI, *Hin*dIII, *Bam*HI, *Sal*I, and *Xho*I; those of NTP101, TP181, pZM111, and pZM171 were determined by single digestions with *Eco*RI, *Hin*dIII, and *Bam*HI. In all cases they were approximated to the nearest 5-kb value. The molecular sizes of pIP174 and pIP180 were not calculated.

[b] The *S. wien* strain 20/70 was provided by L. Le Minor; the plasmid pZM3 was isolated in this laboratory.

Genetic procedures. Techniques for conjugation, transformation, incompatibility testing, and isolation of mutant and recombinant plasmids were as previously described (18). The susceptibility of strains to cloacin DF13 was determined on plates sprayed with cloacin DF13 purified from strain 803(pJN21) by the method of Krone et al. (15). The effect of iron-binding proteins on bacterial growth at 37°C was tested in brain heart infusion broth. This medium (containing 36 μM iron as determined by atomic absorption spectroscopy) was supplemented with 50 mM sodium bicarbonate and 60 μM ovotransferrin or 60 μM lactoferrin and inoculated at a density of 10^6 bacteria per ml. The growth rate was assayed by periodic measurement of culture turbidity and plating on brain heart infusion agar.

Enterotoxin and colonization factor tests. LT was detected by both the Chinese hamster ovary (CHO) cell (12) and African green monkey kidney (Vero) cell (29) assays. The production of ST was detected by the infant mouse assay (8). Synthesis of colonization factor antigens (CFA) was assayed by hemagglutination tests with type A human, bovine, and guinea pig blood in the presence and absence of mannose (9) and by slide agglutination with specific anti-CFA sera. Rabbit antisera prepared with purified antigens CFA/I, CFA/II, and CFA/III (7) were kindly supplied by A. Darfeuille.

Chemical assays for siderophores. The presence of hydroxamate-type compounds was estimated by both the ferric perchlorate assay of Atkin et al. (3) and the Csàky method with a modification of the hydrolysis conditions (11). The concentration of hydroxylamine-nitrogen groups was estimated by using a standard curve prepared from known quantities of hydroxylamine-hydrochloride (30). The method of Arnow (2) was used for the detection of cathecols.

Isolation, cleavage, and molecular cloning of DNA. Plasmid DNA was isolated from crude lysates by polyethylene glycol precipitation (13). Covalently closed circular DNA was purified by ethidium bromide-cesium chloride equilibrium centrifugation. Small-scale plasmid DNA preparation by alkaline lysis and phage DNA purifications were performed as described previously (19). Restriction enzymes and T4 ligase were purchased from New England Biolabs and Boehringer Mannheim and used as recommended by the suppliers. Fragments from partial or complete digestions were cloned (19) into vector plasmids pACYC184 and pBR322.

DNA gel electrophoresis. Restriction DNA fragments were separated by gel electrophoresis on 0.7 to 1% agarose gels in 36 mM Tris-hydrochloride (pH 8.5)–30 mM NaH_2PO_4–1 mM EDTA or 10% polyacrylamide slab gels (acrylamide/bisacrylamide ratio-, 29:1) in Tris-borate buffer. The molecular lengths of DNA fragments were estimated by using restriction endonuclease fragments of known molecular size from digests of λ, R1 *drd-19*, and pBR322.

Southern blot analysis. DNA fragments from agarose gel were denatured and transferred to nitrocellulose filters by the method of Southern (28). λ::IS*1*, λ::IS*2*, and λ::Tn*10* DNA was labeled with [α-^{32}P]dATP by nick translation and used as IS probes in filter hybridization as described by Maniatis et al. (19). The hybridization solution consisted of a buffer containing 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate), 0.01 M EDTA, 0.5% sodium dodecyl sulfate, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, and 0.1% bovine serum albumin. Hybridizations were performed at 68°C for 18 h. Filters were washed in 2× SSC–0.5% sodium dodecyl sulfate for 5 min, 2× SSC–0.1% sodium dodecyl sulfate for 15 min, and 0.1× SSC–0.5% sodium dodecyl sulfate for 2 h at 68°C. ^{32}P-labeled DNA of wild-type λ phage without transposable elements was used as a negative control probe. Bacteriophages λ::IS*1*, λ::IS*2*, and λ::Tn*10* were kindly provided by P. Starlinger.

In situ colony hybridization. Hybridization to score colonies for the presence of sequences homologous to the LT

and ST probes was carried out as described by Moseley et al. (21). The LT probe was a 850-base-pair *Hin*dIII fragment of plasmid pWD299 containing the *eltB* gene and a portion of the *eltA* gene (21). The ST-P probe, prepared from plasmid pRIT10036, consisted of a 157-base-pair *Hin*fI fragment containing a portion of an ST gene of porcine origin (21). The ST-H probe consisted of a 216-base-pair *Hpa*II subfragment derived from the 850-base-pair *Eco*RI-*Hin*dIII fragment of plasmid pSLM004 (21). Positive and negative control colonies were included on the same filter. Plasmids pWD299, pRIT10036, and pSLM004 were kindly provided by S. Falkow.

RESULTS

Epidemiological data and choice of representative FI*me* plasmids. At present enough is known about *S. wien* to describe in detail its sudden emergence, prevalence, and disappearance in a few European countries throughout the 1970s. In this period the numbers of *S. wien* isolates were at least 6,000 in France, 12,100 in Yugoslavia, and 8,800 in Italy; the highest incidences in human salmonella isolations in a year where 32, 59, and 28%, respectively. The epidemics displayed the same pattern, and the peak was reached in France in 1973, in Yugoslavia in 1975, and in Italy in 1976. It is worth stressing that in Western Europe from 1951 to 1969 this serotype appeared only in sporadic cases of salmonellosis, and the isolates in these years were antibiotic susceptible (17). After 1969, over 80% of clinical *S. wien* isolates showed multiple drug resistance. An extensive survey for drug resistance of more than 1,600 *S. wien* strains isolated in Italy from 1974 to 1980 indicated that the most common resistance pattern was ampicillin, chloramphenicol, kanamycin, streptomycin, sulfonamide, and tetracycline. Complete characterization of the plasmid content of 39 strains belonging to the main drug resistance groups (5, 18; unpublished data) has led us to identify the most widely distributed class of FI*me* R plasmids and some of its more frequent variants in the different phases of the epidemic history of *S. wien* in Italy. The origin and phenotypic properties of six FI*me* plasmids chosen on this basis as representative of the Italian epidemic are reported in Table 1. In addition, since multiply antibiotic-resistant strains of *S. wien* were first observed in Algeria in 1969 to 1970 and the first widespread epidemic in Western Europe occurred in France, we included one Algerian FI*me* plasmid isolated in 1970 and two FI*me* plasmids isolated early and in the middle of the French epidemic in the investigation (Table 1). Finally, the two plasmids NTP101 and TP181 are generally considered prototypes of the FI*me* plasmids in epidemic *S. typhimurium* phage type 208.

Screening for FI*me* plasmid-mediated virulence factors. The presence and phenotypic expression of plasmid genes throughout this investigation were studied in *E. coli* K-12 strains.

Strains bearing plasmids pZM3, pZM61, and pZM33 were examined for production of ST and LT toxins by the suckling mouse assay and the CHO and Vero cell assay. No typical reaction was observed. The absence of DNA sequences encoding for ST or LT toxins was confirmed by colony hybridization experiments with standard LT, ST-H, and ST-P probes.

The production of external fimbrial proteins CFA/I, CFA/II, and CFA/III (7, 9) on strains carrying plasmids pZM3, pZM61, and pZM33 was tested by hemagglutination with human, bovine, and guinea pig erythrocytes and more precisely by slide agglutination with anti-CFA/I, −CFA/II, and −CFA/III sera. In all three cases no positive reaction was detectable.

The iron-binding protein ovotransferrin (10) at 60 μM in brain heart infusion broth reduced the number of cell divisions of *E. coli* K-12 strains ZM46 and 803 two to five times in comparison with the growth of the same *E. coli* strains carrying plasmids pZM3, pZM61, pZM33, and pZM111. Likewise the addition of human lactoferrin (10) at 60 μM inhibited the growth of strains 803 and 803 (R100), but inhibited the growth of strain 803(pZM61) much less. These data suggested that FI*me* plasmids could be involved in iron-sequestering mechanisms.

E. coli strains RW193 (*entA*) and RWB18 (*entA fepA*), both blocked in enterochelin synthesis and the latter also defective in the 81-kilodalton outer membrane receptor for ferric enterochelin, did not grow on minimal agar containing 150 μM α,α′-dipyridyl. When plasmid pZM33 was transferred into these strains, they were able to form colonies on the same selective medium.

Strains 803(pZM61) and RWB18(pZM33) grown in M9 minimal medium (with and without 150 μM α,α′-dipyridyl) were tested for the ability to synthesize hydroxamate siderophores. Each culture supernatant was found to contain hydroxamate-type compounds by both the Csàky method and the ferric perchlorate assay; no detectable amount of hydroxamate was observed in the 803 and RWB18 supernatants used as controls. The addition of iron to the medium strongly repressed hydroxamate production.

In *Enterobacter cloacae* (33) and *E. coli* bearing ColV plasmids (4) the receptor for ferric aerobactin (the hydroxamate siderophore more commonly found in pathogenic enterobacteria) is an outer membrane protein that also serves as the binding site for the bacteriocin cloacin DF13. The 11 FI*me* plasmids of Table 1 all conferred susceptibility to cloacin DF13 to *E. coli* strain 803.

Taken together, these results indicated that a hydroxamate-mediated iron assimilation system was present on FI*me* plasmids from epidemic strains of *S. wien* and *S. typhimurium*.

Identification of the iron uptake DNA region and its IS-like elements on pZM61. To identify the DNA region containing the gene complex for the iron uptake system of pZM61 plasmid, we examined 13 derivative plasmids of pZM61 by digestion with restriction endonuclease *Eco*RI. The derivative plasmids were three recombinant plasmids with F′*lac pro*, four deletion mutants, and six miniplasmids obtained by partial digestion with restriction endonuclease *Eco*RI. These plasmids had been isolated for general studies on the structure of FI*me* plasmids (M. Nicoletti, M. Casalino, and F. Maimone, manuscript in preparation) and had different phenotypes in biosynthesis of hydroxamate-type siderophores, susceptibility to cloacin DF13, drug resistance, replication, and K-MP10 incompatibility. After preliminary screening we concentrated the cleavage analysis on the following three plasmids: an *Eco*RI miniplasmid (pZM826) that determined synthesis of hydroxamate (Hds^+) and cloacin DF13 receptor (Clo^s) at levels comparable to those of the original plasmid pZM61 (including its regulation by iron), an Hds^- Clo^r pZM61 × F′ *lac pro* recombinant plasmid (pZM401) isolated in *E. coli* K-12 CSH26, and an Hds^- Clo^r tetracycline-susceptible deletion mutant (pZM560) isolated in *S. wien* WZM6. The restriction patterns of pZM61 and pZM826 (both Hds^+ Clo^s) compared with those of pZM401 and pZM560 (both Hds^- Clo^r) indicated the possible *Eco*RI fragments involved in the plasmid-mediated iron assimilation system (Table 2). They were the three fragments of 6.2, 8.3, and 12.3 kb

TABLE 2. *Eco*RI-generated DNA fragments of plasmids pZM61, pZM401, pZM560, and pZM826

Plasmid	Description	Phenotype[a]	*Eco*RI restriction fragments[b] (kb)																Molecular size (kb)
pZM61	Original F1*me* plasmid	Hds^+ Clo^s	20.8	20.5	15.6	**12.3**	9.8	8.6	**8.3**	8.0	7.2	**6.2**	5.0	5.0	3.5	3.5	2.85	2.8	2.5	1.35	1.3	1.25	146.4
pZM401	Recombinant pZM61 × F′ *lac pro*	Hds^- Clo^r	21.3[c]	20.8	15.6			9.4	8.6	8.0	7.5	7.2	5.0		3.8	3.5	3.5	2.85	2.8		1.35	1.3	1.25	123.8
pZM560	Deletion mutant of pZM61	Hds^- Clo^r	21.3[c]		15.6		11.5	9.8	8.6	8.0			5.0	5.0			2.85	2.8	2.5		1.3	1.25	95.5
pZM826	*Eco*RI miniplasmid of pZM61	Hds^+ Clo^s	20.8			**12.3**	9.8		**8.3**			**6.2**				3.5		2.8			1.3		65

[a] Hds^+ and Hds^-, Presence and absence of hydroxamate synthesis assay in *E. coli* K-12 supernatants; Clo^s and Clo^r, susceptibility and resistance to cloacin DF13.
[b] DNA fragments of less than 0.2 kb were not detectable under the conditions used in these experiments.
[c] The 21.3-kb fragment is the 20.5-kb fragment of pZM61 containing an insertion of a 0.8-kb IS-like element (unpublished data).

(boldface values in Table 2). Figure 1 (lanes a, b, and c) shows *Eco*RI digests of pZM61, pZM563, and pZM560, respectively. Plasmid pZM563 was an Hds^+ Clo^s kanamycin-susceptible deletion mutant belonging to the group of the pZM61 derivatives analyzed in the preliminary survey.

We performed single and double digestions of pZM61, pZM826, pZM401, and pZM560 and compared the lengths of the generated fragments. From this comparison and the analysis of subclones of pZM826 (see Fig. 4A) we were able to locate the *Eco*RI, *Hin*dIII, *Bam*HI, and *Sal*I cleavage sites on the 26.8-kb DNA region represented by the three identified *Eco*RI fragments (see Fig. 4B). Examples of double digests of pZM401, pZM560, and pZM61 are shown in Fig. 1.

To determine whether possible insertion sequences were present on the 26.8-kb region, single and double digests of pZM61 and pZM826 plasmid DNA were probed for IS*1*, IS*2*, and IS*10* sequences in Southern hybridization experiments with ^{32}P-labeled λ::IS*1*, λ::IS*2*, and λ::Tn*10* DNA, respectively, as probes under stringent conditions. The results demonstrated that in the 26.8-kb region there were two copies of the six IS*1*-like elements contained in pZM61 (Fig. 1, lanes i and i′) and no copies of the IS*2* and IS*10* elements

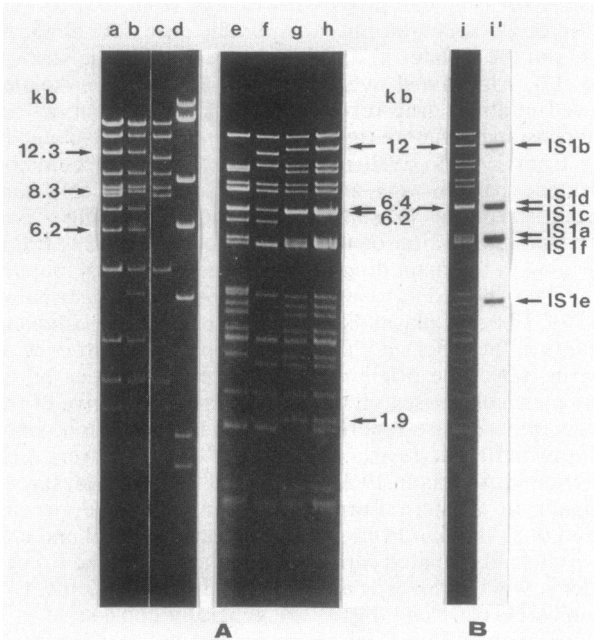

FIG. 1. (A) Agarose gel electrophoresis of single and double restriction endonuclease digests of pZM61 and its derivatives: *Eco*RI digests of pZM61 (a) (molecular lengths are given in Table 2), pZM563 (b), and pZM560 (c) (Table 2); λ and *Hin*dIII-digested λ DNA as molecular size markers (d); and *Eco*RI and *Sal*I double digests of pZM401 (e), pZM560 (f), pZM563 (g), and pZM61 (h). The arrows show the position of each restriction fragment (in kb) associated with the plasmid DNA region involved in the hydroxamate-mediated iron assimilation system. The band marked 6.2 and 6.4 kb contains two 6.4-kb fragments in lane e, a single 6.4-kb fragment in lane f, and four fragments (three of 6.4 kb and one of 6.2 kb) in lanes g and h. DNA fragments less than 0.6 kb are not shown in these photographs. (B) Autoradiogram of *Eco*RI-*Sal*I doubly digested pZM61 DNA (i) hybridized with ^{32}P-labeled λ::IS*1* DNA as an IS*1* probe. The designation of the six IS*1*-like elements were assigned on the basis of their location on the general genetic and physical map of pZM61 (unpublished data). The arrows on the left of lane i indicate the two restriction fragments containing IS*1*b and IS*1*c (Fig. 4B).

(data not shown). No hybridization between pZM61 sequences and ^{32}P-labeled DNA of wild-type λ was observed. An IS*1* element (IS*1*b) was located in the 3.5-kb *Bam*HI subfragment of the 12.3-kb *Eco*RI fragment (Fig. 2, lanes a and a'), and the other copy (IS*1*c) was located in the 2.9-kb *Eco*RI-*Hin*dIII subfragment of the 6.2-kb *Eco*RI fragment (Fig. 3, lanes a and a'; Fig. 4B).

Functional analysis and mapping of IS*1* elements. To investigate the organization of the genes responsible for the synthesis of hydroxamate-type siderophores and cloacin DF13 receptor, we analyzed the phenotypic properties exhibited by various cloned fragments obtained from pZM826 miniplasmid. The main restriction fragments inserted into the plasmid vector pACYC184 were the *Eco*RI fragments of 12.3, 8.3, and 6.2 kb, the DNA segments containing both the 8.3- and 6.2-kb *Eco*RI fragments, the *Hin*dIII fragments of 11.3 and 9.9 kb, and the *Bam*HI-*Hin*dIII fragment of 6 kb. The specific portion of the entire DNA region contained in each clone is depicted in Fig. 4A. Table 3 shows the phenotypes expressed by the different clones. The hybrid plasmids pZM1000 (containing the 8.3- and 6.2-kb *Eco*RI fragments), pZM1001 (containing the 8.3-kb *Eco*RI fragment), pZM1300, and pZM1301 (containing the 9.9-kb *Hin*dIII fragment in both orientations) specified synthesis of hydroxamate. These data indicated that structural genes associated with hydroxamate biosynthesis were located in the 7-kb *Hin*dIII-*Eco*RI segment (Fig. 4B and C). Furthermore, hydroxamate expression occurred by both plasmids pZM1300 and pZM1301, and the expression was repressed in excess iron (Table 3). These features suggested that some regulatory sequences were also present in the *Hin*dIII-*Eco*RI segment. Bacterial clones carrying the hybrid plasmids pZM1000 and pZM1565 (containing the 6-kb *Bam*HI-*Hin*dIII fragment; Fig. 4A) were Clo^s, whereas strains carrying plasmids pZM1001 and pZM1002 (containing the 6.2- and 8.3-kb *Eco*RI fragments separately) were Clo^r. This finding suggested that the *Eco*RI site was within the gene for the cloacin DF13 receptor. Such a genetic organization of DNA sequences for the iron uptake system of pZM61 greatly resembled that for the aerobactin-mediated iron assimilation system of the pColV-K30 plasmid (4, 15). This similarity was confirmed by comparing the restriction map (including the additional *Ava*I and *Pvu*II cutting sites) of the critical region of pZM61 to that of pColV-K30 as reported by Bindereif and Neilands (4). The two physical maps for six restriction enzymes are virtually identical (Fig. 4C; for pColV-K30 see the cloned fragment in pABN5 plasmid in Fig. 1 of reference 4).

We have assumed that the structure of the two IS*1* elements flanking the genetic region involved in the iron assimilation system of pZM61 was the same as that de-

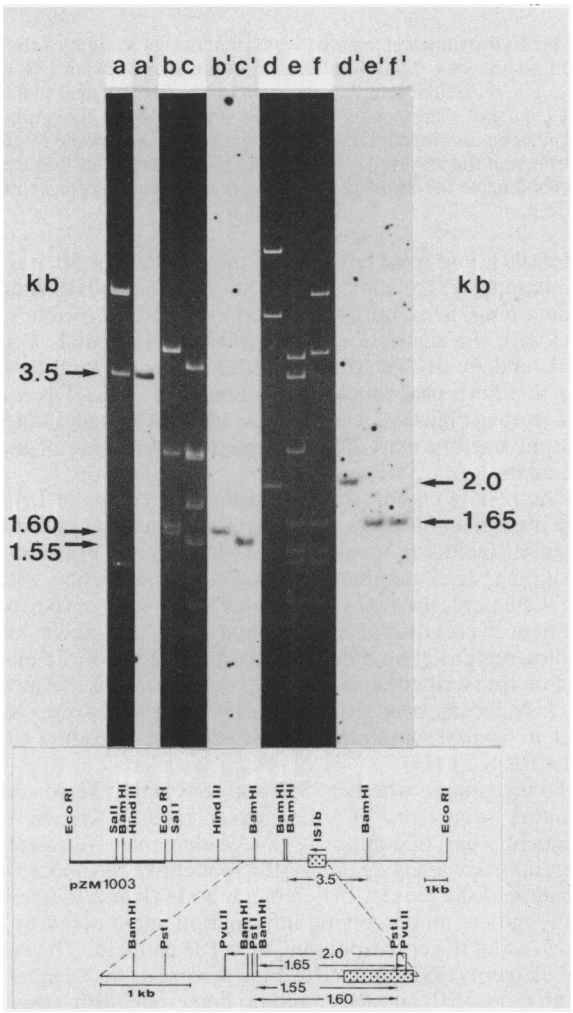

FIG. 2. Agarose gels and Southern blot hybridizations used to map IS*1*b: pZM1003 DNA digested with *Bam*HI (a, a'); *Eco*RI and *Pst*I (b, b'); *Eco*RI, *Bam*HI, and *Pst*I (c, c'); *Pvu*II (d, d'); *Eco*RI, *Bam*HI, and *Pvu*II (e, e'); *Bam*HI and *Pvu*II (f, f'). The autoradiograms were obtained after hybridization with ^{32}P-labeled λ::IS*1* DNA as an IS*1* probe. The arrows show the position of the restriction fragments (in kb) containing the IS*1* element or most of its sequence. Their locations are reported in the detailed part of the restriction map of pZM1003, which consists of the 12.3-kb *Eco*RI fragment of pZM61 linked to pACYC184 (heavy line).

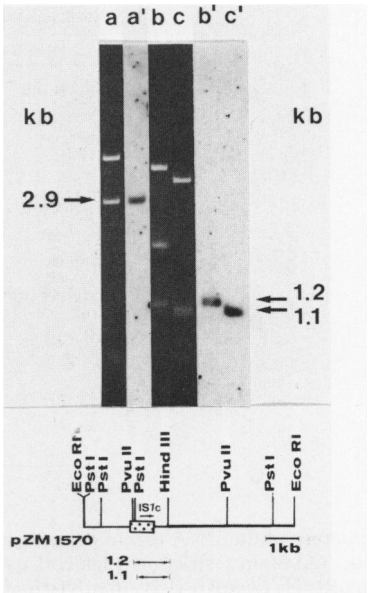

FIG. 3. Agarose gels and Southern blot hybridizations used to map IS*1*c: pZM1570 DNA doubly digested with *Eco*RI and *Hin*dIII (a, a'), *Pvu*II and *Hin*dIII (b, b'), and *Pst*I and *Hin*dIII (c, c'). The autoradiograms were obtained after hybridization with ^{32}P-labeled λ::IS*1* DNA as an IS*1* probe. The arrows show the position of the restriction fragments (in kb) containing the IS*1* element or most of its sequence. Their locations are reported in the schematic restriction map of pZM1570, which consists of the 2.9-kb *Eco*RI-*Hin*dIII fragment of pZM61 linked to the *Hin*dIII-*Eco*RI fragment of pBR322 (heavy line).

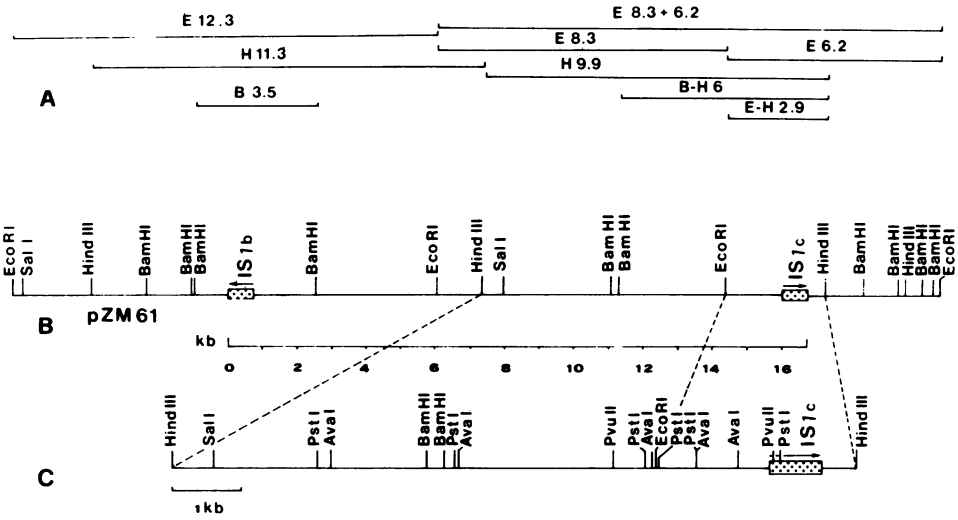

FIG. 4. Structure of the DNA region containing genetic information for the hydroxamate-mediated iron assimilation system of the FI*me* plasmid pZM61. (A) Main restriction fragments used for functional or structural analysis. They were cloned into pACYC184 or pBR322 (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; B-H, *Bam*HI-*Hin*dIII; E-H, *Eco*RI-*Hin*dIII). The molecular lengths are given in kb. (B) Physical map of the 26.8-kb region of pZM61 and location in it of the composite IS*1* element (16.7 kb). The gene complex for hydroxamate biosynthesis is contained in the 7-kb *Hin*dIII-*Eco*RI segment (kb coordinates 7.3 to 14.3). The gene for cloacin DF13 receptor either overlaps the *Eco*RI site (kb coordinate 14.3) or is between it and the inner end of IS*1*c. The arrows represent the inverted orientation of the IS*1* modules. (C) Detailed parts of the 9.9-kb *Hin*dIII fragment which show the DNA segment corresponding to the *Hin*dIII-*Eco*RI fragment encoding for aerobactin biosynthesis in the pColV-K30 plasmid (4) and the physical mapping of IS*1*c.

scribed for the IS*1* element (IS*1*R) derived from the resistance plasmid R100 (23). To precisely locate IS*1*b and IS*1*c, we used the restriction endonucleases *Pvu*II and *Pst*I, which cut once in the IS*1* sequence. The cleavage sites are respectively situated 75 and 180 nucleotides from one end of the element (23). We analyzed single, double, and triple digests of the hybrid plasmids pZM1300, pZM1301, pZM1001, pZM1003, and pZM1303 and of the 3.5-kb *Bam*HI fragment (containing IS*1*b) and the 2.9-kb *Eco*RI-*Hin*dIII fragment (containing IS*1*c), both subcloned into pBR322 (Table 3, Fig. 4A) with the restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Pvu*II. The digested DNA was also hybridized by the Southern procedure with ^{32}P-labeled λ::IS*1* DNA as an IS*1* probe. Figures 2 and 3 show agarose gels and autoradiograms used to map the location and orientation of the IS*1* elements.

TABLE 3. Phenotypic properties expressed by pZM826 plasmid DNA fragments cloned into the plasmid vectors pACYC184 and pBR322

Plasmid	Cloned restriction fragments (kb)	Hds[a] (hydroxylamine-nitrogen in μM)		Clo[b]
		150 μM α,α'-dypiridyl	50 μM $FeCl_3$	
pZM1003	*Eco*RI (12.3)	−	−	R
pZM1000	*Eco*RI (8.3 + 6.2)	+ (80)	−	S
pZM1001	*Eco*RI (8.3)	+ (110)	−	R
pZM1002	*Eco*RI (6.2)	−	−	R
pZM1303	*Hin*dIII (11.3)	−	−	R
pZM1300[c]	*Hin*dIII (9.9)	+ (90)	−	S
pZM1301[c]	*Hin*dIII (9.9)	+ (200)	+ (60)	S
pZM1565	*Bam*HI-*Hin*dIII (6)	−	−	S
pACYC184	None	−	−	R
pZM1570	*Eco*RI-*Hin*dIII (2.9)	−	−	R
pZM1501	*Bam*HI (3.5)	−	−	R
pBR322	None	−	−	R

[a] Hds, Hydroxamate synthesis; +, presence of hydroxamate in supernatant from cultures of the host strain *E. coli* 803 grown to the stationary phase in M9 minimal medium with α,α'-dypiridyl or $FeCl_3$; −, no detectable hydroxamate. Concentrations of hydroxylamine-nitrogen groups were determined as described in the text.

[b] Clo, Susceptibility to cloacin DF13: S, susceptibility of the host strain *E. coli* 803; R, resistance.

[c] Plasmids pZM1300 and pZM1301 contained the 9.9-kb *Hin*dIII fragment in opposite orientations in the *Hin*dIII site of plasmid pACYC184. The higher levels of hydroxamate production exhibited by pZM1301 might be due to positive interaction with promoter sequences on the cloning vector pACYC184.

The results demonstrated that the two copies of IS*1* were repeated in an inverted orientation, and that the entire DNA segment including them was 16.7 kb in size (Fig. 4B). Still assuming strict homology of nucleotide sequence with the IS*1*R element, the two IS*1* modules at the ends of the 16.7-kb element were oriented in such a way that their longest coding regions should begin at the inner ends of the element and be transcribed outward. This orientation of IS*1* modules is classified as inverted-B (14). This is exactly opposite to that in Tn*1681*, a composite IS*1* element encoding the *hst* gene for ST I (27).

To investigate whether IS*1*b and IS*1*c might be considered flanking sequences of other DNA regions known to be frequently part of composite IS-like elements, we carried out a preliminary analysis on pZM61 to identify the approximate location of the closest IS*1* elements to IS*1*b and IS*1*c and get some indication on genetic information surrounded by IS*1*b or IS*1*c and the corresponding next IS*1* element. The nearest IS*1* elements (IS*1*a and IS*1*d, Fig. 1) were as far as more than 30 kb from IS*1*b and IS*1*c, and no detectable drug resistance gene was encoded within the internal regions (Nicoletti, Casalino, and Maimone, manuscript in preparation).

In conclusion the essential genetic information for the iron uptake system of pZM61 resides on an individually defined, composite 16.7-kb IS-like element flanked by inverted B repeats of IS*1*.

Distribution of IS*1*-hydroxamate regions in FI*me* plasmids. To determine the presence of IS*1*-hydroxamate elements on other FI*me* plasmids, we examined all of the plasmids in

Table 1, except pIP174 and pIP180, by digestion with *Eco*RI, *Hin*dIII, and *Bam*HI. The *Bam*HI-digested DNA of each plasmid was hybridized with the IS*1* probe. The single and double digests of plasmids pZM3, pZM33, pZM141, pZM151, and pZM171 contained *Eco*RI, *Hin*dIII, and *Bam*HI fragments and subfragments of the same size as the respective fragments and subfragments identified in the 26.8-kb DNA segment of pZM61. Restriction endonuclease profiles of these plasmids are shown in Fig. 5 and 6. As expected, the 3.5- and 7.1-kb *Bam*HI "junction" fragments (boxed values in Fig. 6) carried sequences that hybridized to the IS*1* probe. These results suggested that an IS*1*-hydroxamate region structurally similar to that in pZM61 was present in all five FI*me* plasmids.

In the case of plasmids NTP101, TP181, and pZM111, their digests contained the internal 8.3-kb *Eco*RI fragment, the internal 8.5- and 0.25-kb *Bam*HI fragments, and the external 1.3-, 0.1-, 0.95-, 0.7-, and 0.3-kb *Bam*HI fragments, but not the expected junction fragments. They showed 8.7- and 9.8-kb *Eco*RI fragments instead of the 12.3- and 6.2-kb fragments (Fig. 5B), 13.4- and 7.8-kb *Hin*dIII fragments instead of the 11.3- and 9.9-kb fragments (Fig. 5C), and 6.4- and 4.2-kb *Bam*HI fragments instead of the 3.5- and 7.1-kb fragments (Fig. 6). The sum of the sizes for each couple of corresponding fragments was the same. The simplest interpretation consistent with such restriction pattern is that the 26.8-kb segment was also present in these three plasmids, but the IS*1*-hydroxamate region was in inverted orientation in comparison with that in the other plasmids analyzed. The finding that the putative *Bam*HI junction fragments of 6.4 and 4.2 kb (boxed values in Fig. 6) contained sequences that hybridized to the IS*1* probe confirmed this interpretation.

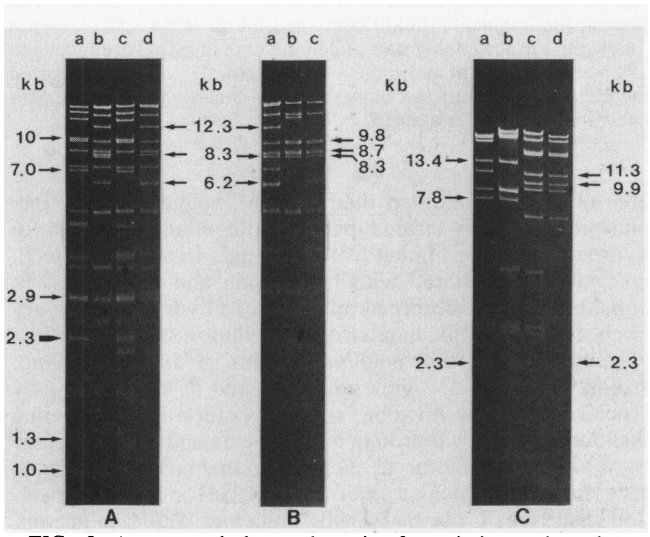

FIG. 5. Agarose gel electrophoresis of restriction endonuclease digests of FI*me* plasmids. (A) *Eco*RI and *Hin*dIII double digests of pZM61 and pZM33 (a, c) and *Eco*RI digests of pZM61 and pZM33 (b, d). (B) *Eco*RI digests of pZM141 (a), pZM111 (b), and NTP101 (c). (C) *Hin*dIII digests of NTP101 (a), TP181 (b), pZM3 (c), and pZM61 (d). The arrows show the position of each retriction fragment (in kb) characteristic of the 26.8-kb DNA segment containing the IS*1*-hydroxamate region. The band marked 2.9 kb in A is a triplet (2.8-, 2.85-, and 2.9-kb fragments) in lanes a and c and a doublet (2.8- and 2.85-kb fragments) in lanes b and d. The thick arrow indicates the doublet corresponding to the *Hin*dIII and *Eco*RI-*Hin*dIII fragments of 2.3 kb present in the 26.8-kb DNA segment (Fig. 4B). The bands marked 9.8 and 8.7 kb in B are doublets in lanes b and c and singlets in lane a.

We were dealing with independent, naturally occurring inversions of the entire IS*1*-hydroxamate element possibly due to homologous recombination between the flanking IS*1* elements.

The restriction maps in Fig. 6 represent the two physical organizations of the FI*me* plasmid region containing the IS*1*-hydroxamate element as deduced by the cleavage and Southern blot analyses.

DISCUSSION

In vitro and in vivo studies have demonstrated the key role of iron in microbe-host interactions. The capacity of microorganisms to compete with host iron-binding factors, such as transferrin in plasma and lactoferrin in milk and mucosal secretions, for the iron they need for growth and metabolism is an essential attribute of virulence (10).

The high-affinity system of iron acquisition expressed by many enteric bacteria is based on a cathecol-type siderophore, enterobactin (enterochelin), and specific transport proteins. In *E. coli* and *S. typhimurium* the genes involved in this system are located on the chromosome (22). In recent years an efficient enterobactin-independent iron transport system has been identified in enteric species with increasing frequency. It consists of a hydroxamate-type siderophore and corresponding transport functions (30, 34, 35). In addition to *Enterobacter* (*Aerobacter*) *aerogenes* (11) and *Enterobacter cloacae* (33), synthesis of hydroxamate siderophores has been observed in invasive strains of *E. coli* (30, 35), strains of *Salmonella arizona*, *Salmonella austin*, and *Salmonella memphis* (22, 34), *Shigella boydii* (16), and most of clinical isolates of *Shigella flexneri* (16, 24). The synthesized hydroxamate was identified as aerobactin, and the genes for its synthesis have been found on plasmids in *E. aerogenes* (20) and *E. coli* (30, 34, 35) strains and on the chromosome in an invasive *E. coli* strain (32), in *Salmonella* spp. (20), and in *Shigella* spp. (16) strains. In the cases examined, the aerobactin gene sequences shared considerable homology with those characterized in plasmid pColV-K30 (4, 20).

So far the more extensive and consistent studies on the role of hydroxamate-mediated iron uptake systems of enteric bacteria in the host-parasite interaction have been carried out in *E. coli* strains. A substantial percentage of *E. coli* isolates from cases of bacteremia and meningitis of humans and domestic animals harbor ColV plasmids. Experimental infections with wild-type and mutant plasmids have shown that the hydroxamate-mediated iron transport of ColV plasmids provides selective advantage to invading *E. coli* strains to survive and proliferate in the body fluids and tissues (35). Independently of colicinogeny, the capacity to promote iron-suppressible hydroxamate synthesis is widely distributed among *E. coli* strains in nature. This distribution appears not to be random, but related to severity of iron deprivation in specific environments such as human blood and feces of apparently healthy breast-fed babies (31).

From 1969 to 1980 three strains of *Salmonella* spp. (*S. wien*, *S. typhimurium* phage type 208, and *S. typhimurium* phage type 66/122) caused widespread epidemics in Europe, North Africa, the Middle East, and Southeast Asia, including India, with outbreaks occurring mostly in hospitals. The predominating clinical picture was gastroenteritis, but in many instances these epidemics showed a high incidence of septicemia or meningitis (1, 5, 17, 18, 26). A minor example was also represented by *S. johannesburg* in Hong Kong (6).

The great majority of clinical isolates of all these epidemic *Salmonella* spp. strains possessed a multiple drug resistance

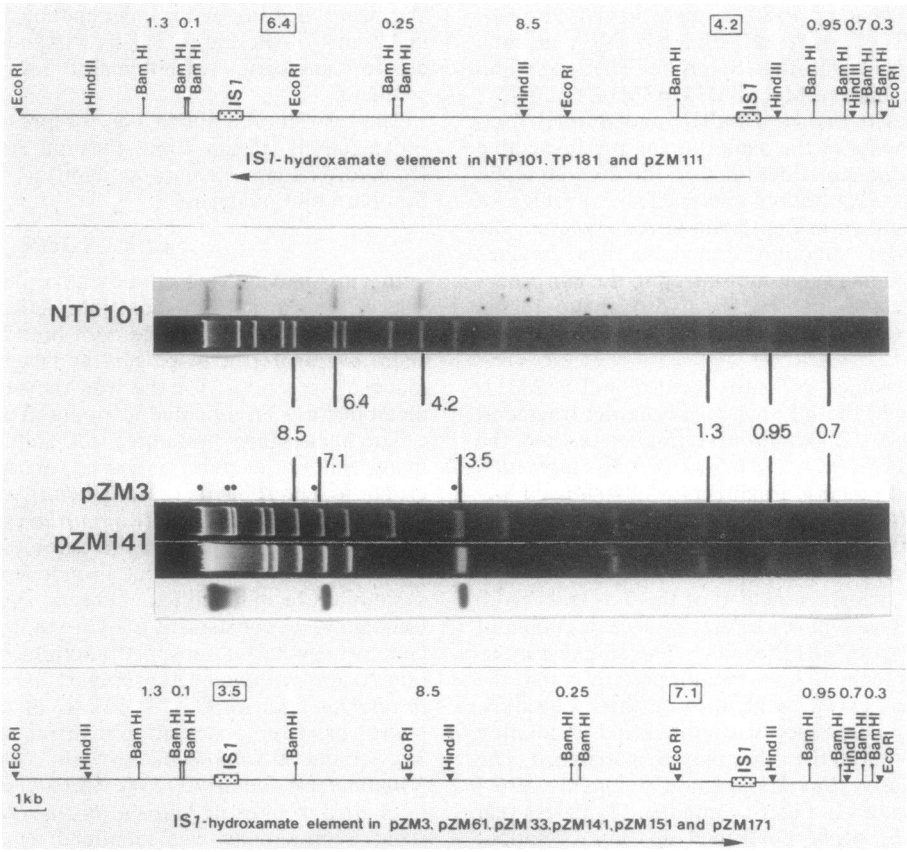

FIG. 6. Distribution and inversion of composite IS*1* elements encoding for hydroxamate-mediated iron uptake in FI*me* plasmids. The marked bands of the *Bam*HI digests of pZM141, pZM3, and NTP101 represent the fragments (in kb) contained in the 26.8-kb DNA region where the system is situated. The bands hybridizable to the IS*1* probe are shown in the autoradiograms and in the case of pZM3 are indicated (●). The locations of the *Bam*HI fragments (DNA fragments less than 0.5 kb are not shown) and the *Eco*RI and *Hin*dIII sites are reported in the schematic restriction maps. Boxed values denote the *Bam*HI junction fragments. The arrows show the two orientations of the element as deduced by the cleavage and Southern blot analysis for each of the nine FI*me* plasmids examined.

IncFI*me* plasmid of large size. This epidemiological characteristic suggested that such a plasmid encoded for virulence-enhancing factors.

In this study chemical, genetic, and structural data indicated that all 11 representative FI*me* plasmids under survey coded for a hydroxamate-mediated iron assimilation system. The detailed analysis of plasmid pZM61 demonstrated that the general genetic and physical organization of the critical DNA region encoding for this iron uptake system was virtually identical to that described for the aerobactin-mediated iron assimilation system of plasmid pColV-K30 (4, 20).

Although further investigation is required by means of in vivo experiments with *Salmonella* spp. strains, our results might explain some clinical and epidemiological features of infections due to *S. wien* and *S. typhimurium* phage types 208 and 66/122. A particularly interesting fact is the emergence of *S. wien* as a "new" epidemic serotype. Before 1969 the *S. wien* serotype occurred at very low frequency in human pathology, and the isolates tested were drug susceptible (17), which suggested that the FI*me* plasmid had not been acquired by *S. wien* strains at that time. Experiments in our laboratory showed that *S. wien* strains cured of the FI*me* plasmid lost the ability to synthesize hydroxamate (unpublished results). Moreover the high frequency of septicemia or meningitis in outbreaks caused by these *Salmonella* spp. strains, especially *S. typhimurium* phage types 66/122 and 208, or types related to them (1, 26), suggests that FI*me* plasmids in such strains play a role similar to that of hydroxamate-determining ColV plasmids in the invasive *E. coli* strains associated with bacteremia and meningitis. In conclusion, the widespread presence of a hydroxamate (very likely aerobactin)-mediated iron assimilation system on FI*me* plasmids in important epidemic strains of *Salmonella* spp. strongly supports the view that this kind of system may be crucial (35) to the invading enteric bacteria in overcoming the low availability of iron in human hosts and may therefore be a significant factor in increasing the basal pathogenic potential of a particular enteric strain. Second, the association of FI*me* R plasmids with epidemic *Salmonella* spp. strains exemplifies how the occurrence of drug resistance-virulence plasmids may be important in the emergence of strains with enhanced pathogenicity in environments where antibiotics are used.

Another intriguing finding of our investigation is that the FI*me* plasmid-encoded genes for hydroxamate biosynthesis and cloacin DF13 receptor are flanked by IS*1* elements. The genetic and physical analysis of derivative plasmids and cloned fragments of plasmid pZM61 demonstrates that these genes are located in a continuous 8.6-kb segment within a 16.7-kb DNA region that carries its IS*1* modules as inverted B repeats (Fig. 4B and C). The restriction enzyme and Southern blot hybridization patterns indicate that a very

similar element is present in either orientation in all of the nine FI*me* plasmids from *S. wien* and *S. typhimurium* phage type 208 (Fig. 6).

In related and independent reports IS*1* sequences have been also associated with the aerobactin region in plasmids pColV-K30 (16, 20, 25) and pSMN1 (20) and in the chromosome of *Shigella* spp. strains (16) and an *E. coli* K1 strain (32). The IS*1*-hydroxamate element characterized in pZM61 has the molecular size, the relative positions of *Bam*HI, *Eco*RI, and *Hin*dIII restriction sites, and IS*1* orientations virtually identical to those recently described for the IS*1*-aerobactin element in pColV-K30 (25). These data reinforce the hypothesis that a genetic unit such as a composite transposon may have been facilitating the dissemination of a common hydroxamate-mediated iron transport system among genomes of different species and genera of bacteria.

Presuming that the IS*1*-hydroxamate elements identified in FI*me* plasmids and pColV-K30 are not recently formed composite elements, but a genetic entity that has undergone evolutionary modifications to eliminate unnecessary or deleterious portions, the possible role of the 6.5 kb of DNA lying between the inner end of the left IS*1* module and the internal *Hin*dIII site (Fig. 4B) is an open question. It remains to be seen whether this region codes directly for regulatory genes or additional functions involved in the global efficiency of this important high-affinity system of microbial iron acquisition.

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