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Lack of Homology Between the Iron Transport Regions of Two Virulence-Linked Bacterial Plasmids

MARY A. WALTER,[1] ALBRECHT BINDEREIF,[2] J. B. NEILANDS,[2] AND JORGE H. CROSA[1]*

Department of Microbiology and Immunology, School of Medicine, Oregon Health Sciences University, Portland, Oregon 97201[1]; and Department of Biochemistry, University of California at Berkeley, Berkeley, California 94720[2]

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Two plasmids involved in bacterial virulence, the *Escherichia coli* plasmid pColV-K30 and the *Vibrio anguillarum* plasmid pJM1, have been studied with respect to the iron sequestering systems mediated by these two plasmids. Bioassay results show that the two systems are not related functionally because specific iron uptake-deficient mutants in each system cannot be cross-fed by the heterologous bacteria using culture supernatants from iron-proficient strains containing wild-type plasmids. DNA hybridization studies show an extensive lack of homology between regions involved in iron sequestration in both plasmids.

In recent years, the virulence of two types of bacteria belonging to different ecological niches has been demonstrated to be due to the presence of specific plasmid classes (6, 15). It was found that a significant proportion of *Escherichia coli* causing bacteremias in humans and domestic animals harbor a ColV plasmid. Cured strains showed decreased virulence in the mouse model (21). By using another vertebrate system, a salmonid fish, it was found that the virulence of highly pathogenic strains of the marine fish pathogen *Vibrio anguillarum* was correlated with the possession of the 47-megadalton plasmid pJM1. Curing of this plasmid caused an attenuation of virulence in this marine bacterium (5). Until recently, it was not known which properties of these two plasmids were responsible for virulence, although it was obvious that the diseases caused by both *E. coli* and *V. anguillarum* are of a similar nature in the sense that they both are characterized by the establishment of a fulminant septicemia. Therefore, in considering the possible mechanisms by which these two organisms can cause disease, their ability to grow and be disseminated in the host vertebrate blood was investigated as a potential factor of virulence. One component that is essential for bacterial growth is iron. However, free iron is not readily available in the vertebrate host, being present mainly intracellularly (20). Circulating iron is bound by high-affinity iron binding proteins, such as transferrin in serum and lactoferrin in secretions (2a). These proteins are unsaturated in normal hosts and thus limit the availability of iron for any invading pathogens. Thus, microorganisms have had to develop mechanisms whereby the otherwise unavailable iron could be assimilated by the invading bacteria. Recent work (3, 4, 21) demonstrated that the pColV-K30 and the pJM1 plasmids coded for novel iron transport systems which are highly efficient in obtaining iron from complexes of iron-transferrin. In vivo experiments corroborated the essential function coded for on these plasmids; therefore, these plasmid-mediated iron transport systems were demonstrated to be an important component of the virulence repertoire of these two bacteria. Because of the similar functions coded for by pJM1 and pColV-K30 plasmids in nonrelated bacterial species, we decided to investigate these two plasmid-mediated iron transport systems by using both a functional and a molecular approach.

Iron uptake-deficient mutants were recently characterized in both the pColV-K30 and in the pJM1 systems by the use of a physiological bioassay, which defined the existence of at least two important components for iron transport (18, 22). One is a diffusible substance which may play a role as a siderophore (12, 13), and the other is a nondiffusible component which may be a membrane receptor for iron-siderophore complexes (10). In the case of the pColV-K30 system, the siderophore was identified as the hydroxamate aerobactin (19) and the membrane receptor was presumed to be an outer membrane protein of 74,000 daltons (11).

To characterize the systems functionally, we decided to study the ability of supernatants from iron-limited cultures of *E. coli* carrying pColV-K30 and *V. anguillarum* harboring pJM1 to cross-feed iron uptake-deficient mutants of these bacteria characterized as being deficient in the production of the specific plasmid-mediated siderophore (iuc). In most experiments we used culture supernatants containing the plasmid-mediated siderophores, although in some cases we also used purified aerobactin. Aerobactin could only cross-feed iuc mutants from the *E. coli* strain carrying the pColV-K30-1 plasmid, but it could not cross-feed iuc mutants of *V. anguillarum* containing the pJHC-91 plasmid (Table 1). Culture supernatants from *V. anguillarum* cannot cross-feed the *E. coli* iuc mutant. Thus, it appears that from a functional standpoint the pJM1 and pColV-K30 plasmid-mediated iron transport systems are not related. To determine whether the two systems are related at the plasmid DNA level, we decided to perform Southern blot hybridizations (16) of gels containing restriction endonuclease-cleaved pColV-K30 and pJM1 DNA, using as radioactive probes plasmid clones carrying plasmid DNA regions involved in iron transport. Thus, we used the plasmid pABN1 (2) carrying the pColV-K30 iron-transport genes and the plasmid pJHC-W1 harboring pJM1 iron transport regions (18). The pColV-K30 iron uptake regions hybridized only with restriction endonuclease-cleaved pColV-K30 DNA (Fig. 1A and B), whereas the clone containing pJM1 iron uptake regions hybridized only with restriction endonuclease-cleaved pJM1 DNA and not with restriction endonuclease-cleaved pColV-K30 DNA (Fig. 1C and D). These hybridization results clearly show that the iron transport regions of these two plasmids are not homologous under our assay conditions.

We should conclude from the experiments in this paper that there is no appreciable homology either functionally or at the DNA level between these two plasmid-mediated iron transport systems. From an evolutionary standpoint it is intriguing that both of these iron transport systems are

* Corresponding author.

TABLE 1. Bioassay with culture supernatants from plasmid-carrying, iron-proficient strains[a]

Strain used in lawns	Genotype	Plasmid	Cross-feeding with supernatants from:	
			E. coli LG1315	*V. anguillarum* 775
E. coli LG1522	*iuc*	pCo1V-K30-1	+	−
V. anguillarum 775::Tn*1*-5	*iuc*	pJHC-91	−	+

[a] The ability of supernatants to support the growth of the *E. coli*(pCo1V-K30-1) and *V. anguillarum*(pJHC-91) iron uptake-deficient strains in iron-depleted medium was tested by impregnating a sterile filter disk with 10 μl of supernatant from the growth of the wild-type, iron-proficient strains *E. coli* LG1315(pCo1V-K30) and *V. anguillarum* 775(pJM1). pCo1V-K30-1 (*iuc*), a derivative of pCo1V-K30, has a mutation in genes concerned with aerobactin synthesis (22), whereas pJHC-91 (*iuc*), a derivative of pJM1, has a mutation in genes concerned with the biosynthesis of the putative *V. anguillarum* siderophore (18). Supernatants were obtained from strains growing in M9 minimal medium (14) supplemented with glucose, Casamino Acids (Difco Laboratories), and a non-assimilable iron chelator EDDA (ethylendiamine-di-*o*-hydroxyphenyl acetic acid) at 10 μM. After centrifugation of the cells, the supernatants were removed and sterilized by filtration. Disks were added to bioassay plates containing 10 μM EDDA in minimal medium and 0.1 ml of an overnight culture of 10^8 cells per ml of the lawn strain of bacteria under iron stress (the iuc mutants), and the desired 10 μl of sterile culture supernatant was applied. A positive result for cross-feeding is represented as a halo of growth around the applied disk. This bioassay is able to categorize strains as those that can be cross-fed and those able to cross-feed.

required for virulence and are mediated by plasmids. Although it may appear that a lack of similarity in iron sequestration regions in plasmids carried by heterologous hosts is expected, there is precedence for interaction between heterologous systems at both a functional and a genetic level. For example, an important iron transport system in *E. coli* possesses an outer membrane receptor, the *tonA* protein, that can recognize a siderophore, ferrichrome, synthesized by the fungus *Ustilago sphaerogena*, which is not phylogenetically related to *E. coli*. Recent cross-feeding bioassay findings have shown that the addition of a *V. anguillarum* culture or supernatant can reverse the growth inhibition under iron limitation conditions of the human pathogen *Vibrio fluvialis*, suggesting some identity among iron uptake components (1). At the genetic level, *E. coli* is known to exchange genetic information by conjugation with other phylogenetically unrelated bacteria, such as *Vibrio* sp. (9), including *V. anguillarum* 775, the strain used in this work (5). In addition, it was shown that conjugation can occur between the fish pathogen *V. anguillarum* and the human pathogen *Vibrio parahaemolyticus* (8). Therefore, it is conceivable that the conjugative ColV plasmid or DNA regions from this plasmid, such as the iron transport system, could be transferred to a *V. anguillarum* host. There is also precedence for the mobility of pJM1 and pColV sequences between genomes and bacteria. Workers in our laboratory have recently demonstrated that certain virulent strains of *V. anguillarum*, which do not possess a pJM1-type plasmid, are iron proficient and do possess chromosomal DNA sequences that are homologous to pJM1 plasmid DNA sequences (17). This mobility of plasmid DNA sequences is also found with the pColV plasmid iron regions, since the genes for aerobactin synthesis have been found on plasmids from other enteric bacteria such as *Arizona hinshawii* and *Aerobacter aerogenes* (S. McDougall and J. B. Neilands, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K186, p. 208). Nevertheless, our results do not substantiate the presence of pColV iron transport regions in pJM1. At the DNA level, under the conditions used to perform our hybridization experiments, we would expect to detect even somewhat distant se-

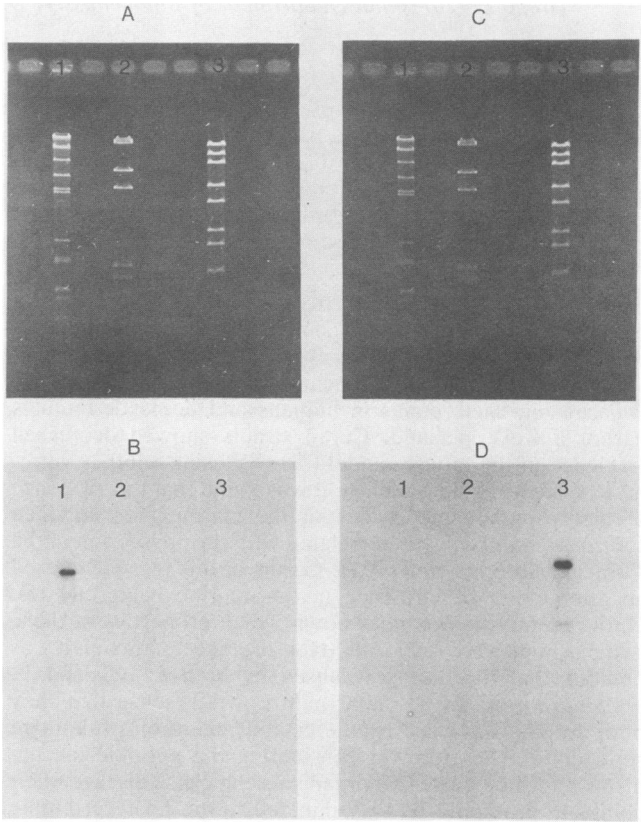

FIG. 1. Southern blot hybridization analysis of restriction endonuclease-cleaved pColV-K30 and pJM1 DNA. Plasmid DNA was cleaved with either *Hin*dIII or *Bam*HI restriction endonucleases (Bethesda Research Laboratories, Inc.) and electrophoresed in two identical 0.6% agarose gels in a Tris-borate buffer (89 mM Tris base, 2.5 mM disodium EDTA, 89 mM boric acid, pH 8.3) at 70 V for 3 h. The gels were removed, stained with ethidium bromide (1 μg/ml) and photographed. Restriction endonuclease-cleaved plasmid DNA was next transferred from the gels to nitrocellulose filters (Schleicher & Schuell, Inc.) by using the Southern blot technique (16). Nitrocellulose filters were next placed in plastic bags containing 5× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, Denhardt solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin [7]), 1 mM EDTA, and 0.1% sodium dodecyl sulfate. After 3 h at 37°C, the appropriate heat-denatured, ^{32}P-labeled plasmid DNA (10^6 cpm) was added and hybridization was carried out by incubation in the same preincubation solution with carrier salmon testes DNA at 100 μg/ml for 16 h at 37°C. Panels A and C are photographs of the duplicate 0.6% agarose gels showing restriction endonuclease-cleaved plasmid DNA. Lane 1: *Hin*dIII-cleaved pColV-K30; lane 2: *Hin*dIII-cleaved unheated lambda DNA standard; lane 3: *Bam*HI-cleaved pJM1 DNA. Panels B and D are photographs of the autoradiographs obtained when the gel blots described above were hybridized with ^{32}P-labeled plasmid DNA. Panel B shows the results for the hybridization with ^{32}P-labeled pABN1 DNA which carries pColV-K30 iron transport regions; panel D shows hybridization with ^{32}P-labeled pJHC-W1, a clone carrying iron transport regions of pJM1.

quences. Certainly, our results do not preclude the existence of topological homologies at sites involved in regulatory events of the plasmid-mediated iron transport genes. This possibility awaits further dissection of both iron transport regions by DNA sequencing experiments which are currently being pursued.

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