# PLASMID-MEDIATED AEROBACTIN IRON UPTAKE SYSTEMS

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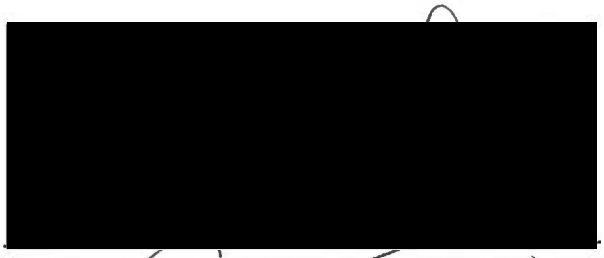

(Professor in Charge of Thesis)

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To Jorge and Lidia Crosa

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## ABBREVIATIONS

| | |
|---|---|
| ATCC | American Type Culture Collection |
| bp | base pair |
| ColV | colicin V; also refers to colicin V-encoding plasmids |
| DNA | deoxyribonucleic acid |
| *E. coli* | *Escherichia coli* |
| Fur | the ferric uptake regulator protein |
| IS*1* | insertion sequence #1 |
| kb | kilobase pair |
| kd | kilodalton |
| PAGE | polyacrylamide gel electrophoresis |
| REPI | replication region #1, in reference to the ColV plasmid pColV-K30 |
| REPII | replication region #2, in reference to the ColV plasmid pColV-K30 |
| SDS | sodium dodecyl sulfate |

# ABSTRACT

Aerobactin iron uptake systems as mediated by the plasmids and chromosomes of enteric bacteria are important for the invasiveness of these bacteria, that is, the ability of these organisms to invade the bloodstream and potentially spread throughout the animal. The fact that the aerobactin iron uptake system as encoded by the E. coli plasmid pColV-K30 is also found encoded by the plasmids and chromosomes of several other enteric species suggests that the genes for this system are mobile. Also it was previously found that inverted copies of the insertion sequence IS1 flanked the aerobactin system genes in pColV-K30, suggesting the possibility of transposition of the aerobactin system. To gain insight into the possible mechanisms of genetic mobility, several ColV plasmids were surveyed regarding the internal and flanking sequences of this aerobactin iron uptake system. It was found that inverted copies of the insertion sequence IS1 were conserved in these plasmids, as well as the internal sequences specific for the synthesis of the components of the aerobactin iron uptake system. An upstream "flanking" replication region called REPI was also absolutely conserved in these ColV plasmids, regardless of the presence of the aerobactin system genes. The downstream flanking region was not conserved, including another replication region, REPII, which was encoded by only some plasmids, and in variable locations. The inference from these results is that the REPI region could have been instrumental in the preservation and spread of the aerobactin system genes in these ColV plasmids of E. coli , and the REPI-aerobactin system linkage explains why this system is only found among these large IncFI plasmids.

In the course of the ColV plasmid survey, two ColV plasmids were mapped entirely, based on restriction enzyme sites and landmark phenotypic characteristics such as the colicin V gene, transfer region, and repeated sequences.

Described herein is the discovery of a novel genetic system encoding for the production of the same iron-chelating siderophore aerobactin by another enteric species, Klebsiella ozaenae 62-1, formerly called Aerobacter aerogenes 62-1. This new genetic system was cloned and found to be plasmid-mediated. The internal aerobactin system sequences were only weakly homologous to the aerobactin system genes as encoded by E. coli ColV plasmids. These new aerobactin system genes were not flanked by copies of IS1 and this Klebsiella plasmid had no sequences homologous to REPI. The finding of this novel system encoded by a plasmid which is not of the incompatiblity group IncFI supports the correlation of the "E. coli aerobactin system" with the IncFI-specific replication region REPI in the ColV plasmids.

Taxonomic criteria for the respeciation of Aerobacter aerogenes 62-1 to Klebsiella ozaenae 62-1 is included as the fourth manuscript of this thesis.

Sequencing analysis of the putative promoter region of the Klebsiella ozaenae 62-1 aerobactin system genes reveals the presence of two open reading frames, one of which presumably is the 5' end of the first enzyme gene in this aerobactin system "operon." The other open reading frame is upstream and encodes the 3' end of a cryptic protein

which appears to be nonetheless a conserved protein upstream from both of the aerobactin systems' genes. A sequence was found in the putative Klebsiella promoter region between these open reading frames, and between the -10 and -35 regions, that fits the consensus sequence for the binding site of the Fur protein. (The Fur protein is a negative regulator found in E. coli which represses transcription from iron-sensitive promotors.) This finding suggests that there is a similar sort of regulation in Klebsiella, using a Fur-like negative regulator. Supporting this suggestion is the observation that down-regulation is accomplished in the Klebsiella cloned aerobactin system in the E. coli background under conditions of high iron concentration. Thus, in spite of the considerable divergence which spans several genes in the aerobactin system genotype, there may be a conservation of some of the basic features of iron regulation of these systems. However, the expression of each system under conditions of low iron concentration appears to require factors that are unique to each bacterial species, because the production of the siderophore by the Klebsiella system, when the system was cloned and put into an E. coli background, was not equal to such production in the native Klebsiella strain.

# Introduction

Aerobactin is a chelator of iron, that is, a siderophore. Most siderophores are low molecular weight compounds, as is aerobactin at 565 daltons. This siderophore is produced and secreted by strains of enteric bacteria, such as E. coli, Salmonella, Shigella, Klebsiella, Enterobacter, and Yersinia. Most of these strains are invasive pathogens which means they can enter the bloodstream and be carried throughout the vertebrate host. There are several reasons why invasive bacteria need to produce aerobactin. First is the chemistry of iron. At physiological pH and oxygen level, iron has a strong tendancy to polymerize as the insoluable compound ferric hydroxide. This form of iron is unavailable to bacteria. However, too much iron can be a problem to bacteria, since iron is the catalytic generator of oxidizing radicals. So iron uptake must be strictly regulated by the bacterial molecular machinery. Second, the vertebrate host has several ways to keep the iron concentration too low to sustain bacterial growth. There is the phenomenon of "nutritional immunity," in which the host, when stressed by infection, will withdraw circulating iron and store a higher per cent in the liver and spleen. Also, the circulating form of iron is as bound to transferrin, which is usually only about 30% saturated. Transferrin-bound iron is not accessible to bacteria unless they produce a siderophore which can sequester this iron from transferrin. Third, all bacteria (except Lactobacilli) require nutritional iron to be the cofactor of essential enzymes. Fourth, aerobactin has been found to be better at supplying iron from transferrin than other siderophores such as enterobactin, although it is known that the latter has a higher binding affinity constant for iron. There are several reasons for this, such as the stability and reuseability of aerobactin.

The widespread use of antibacterial agents and host factors have selected for the genetic mobility of virulence factors. This mobility often takes the form of transferrable plasmids. The work described in this thesis involves plasmid-mediated aerobactin iron uptake systems. These systems include the genes and proteins necessary for the synthesis of the siderophore aerobactin, and the synthesis of the outer membrane receptor which binds ferric-aerobactin. The first aerobactin system investigated is encoded by the ColV plasmids of E. coli. Two of these ColV plasmids were restriction-mapped in the process of completing this study of ColV-mediated aerobactin iron uptake. Later a newly discovered aerobactin system is described, one encoded by a plasmid found in the strain from which aerobactin was first discovered. This strain was originally called Aerobacter aerogenes 62-1, but, since the speciation was outdated and not rechecked since the discovery of aerobactin in 1969, taxonomic criteria were applied and the organism was found to give excellent identification as a Klebsiella ozaenae. The most recent data obtained regarding this new Klebsiella aerobactin iron uptake system is included as an appendix to paper #3, in which the promoter region is analyzed by sequencing and by comparing the resultant sequence to that of the published promoter region of the aerobactin system of E. coli.

# Literature Review

Time Line

| | AEROBACTIN HISTORY (AFTER 1981, Non-ColV AEROBACTIN) | COLV HISTORY (AFTER 1981, ColV AEROBACTIN) |
|---|---|---|
| 1987 | *K ozaenae 62-1 promoter sequenced, found distinct;<br>*A. aerogenes 62-1 actually Klebsiella ozaenae 62-1;<br>*A. aerogenes 62-1 aerobactin system cloned, found distinct | *Two ColV plasmids entirely mapped<br>ColV aerobactin operator-promoter Fur-footprinted;<br>ColV REPI characterized |
| 1986 | Aerobactin found produced by Yersinia sp. & K. pneumoniae;<br>Aerobactin further distinguished from enterobactin | ColV aerobactin biosynthetic pathway proposed;<br>*IS1/REPI conserved among prototypic ColV plasmids |
| 1985 | | 3 groups identify genes & peptides of aerob. system |
| 1984 | Aerobactin genes found on chromosomes of E. coli & Shigella & Salmonella R plasmids;<br>In vitro synthesis of aerobactin w/ A. aerogenes 62-1 cell-free extracts | Operator-promoter mapped, sequence<br>IS1, REP regions flank ColV aerobactin system |
| 1983 | | Aerobactin system cloned from ColV plasmid |
| 1982 | Chemical synthesis of aerobactin | Fur protein regulates ColV aerobactin;<br>74 Kd protein is OMP for ferric aerobactin |
| 1981 | Aerobactin of A. aerogenes 62-1 = Aerobactin of ColV plasmids | Other virulence factors found encoded by ColVs |
| 1980 | | ColV virulence correlated to aerobactin iron uptake system |
| 1979 | | Tn-mutagenesis of ColV does not affect pure culture virulence |
| 1977 | In vitro conversion of lysine to hydroxylysine | |
| 1976 | | ColV+ strains outgrow ColV- strains in mixed infections;<br>Col/phage receptors = iron uptake system receptors |
| 1969 | Aerobactin discovered in Aerobacter aerogenes 62-1 supernatants | |
| 1962 | | ColV found transferrable (therefore a plasmid) |
| 1949 | | ColV production correlated with pathogenicity |

The historical context for the work described in this thesis is presented diagrammatically in the Time Line of the previous page. This Time Line shows the separate histories of the siderophore aerobactin and "antibiotic" colicin V, how the two histories merge, and how the two subjects later become distinct. Chronologically, the first discovery was in 1949 in France when P. Fredericq and E. Joiris (12) noticed a correlation between pathogenicity and the ability of bacterial cells to produce colicin V. (Colicin V is an antibacterial factor produced by nonsusceptible bacteria which kills the susceptible bacteria.) The history of the siderophore aerobactin began twenty years later in 1969, when this dihydroxamate iron chelator was discovered, isolated, purified, and the chemical structure determined by F. Gibson and D. Magrath in Australia (13). The two subjects remained separate until 1981, since aerobactin was discovered in the culture supernatants of an organism called Aerobacter aerogenes 62-1, while the colicin V genotype was found to be carried by plasmids of E. coli.

Between 1969 and 1981, however, six important experiments were done. The first five of these are: 1) In 1962, it was demonstrated in Argentina by Nagel de Zwaig, Anton, and Puig (19) that the colicin V phenotype was transferrable and therefore plasmid-mediated; 2) In the analysis of the constituents of the aerobactin molecule, Viswanatha et al. (18) achieved for the first time the in vitro conversion of lysine to $N^6$-hydroxylysine, the first step in the (chemical) synthesis of aerobactin, using cell-free extracts of Aerobacter aerogenes 62-1; 3) it was shown by V. Braun et al. (4) that phage and colicin receptors are commonly components of iron uptake systems; 4) it was confirmed by

Smith and Huggins (26) that the ColV, F-like plasmid was transferrable, and that if a mixed infection was accomplished using a transfer-deficient ColV-carrying strain (B188) and a strain without the ColV plasmid, a proportionally higher number of ColV-carrying cells would subsequently be recovered from the infected animal; they also noticed that a higher proportion of ColV-resistant cells (over the ColV-sensitive cells) would be recovered, depending on the physiological site of recovery; and 5) It was found by transposon mutagenesis by Quackenbush and Falkow (25) that cells carrying a ColV plasmid with a mutated colicin V structural gene were at least as virulent as the nonmutant ColV-carrying parent strain.

The implications of these last two findings appear to conflict. In the mixed infection study, one would conclude that there is a selective advantage, based on Darwiniam principles, in the carriage of the ColV plasmid, such that the ColV plasmid-carrying cells are able to outgrow the ColV-minus cells, and sometimes the ColV-sensitive cells. In the transposon study, one would conclude that there is no advantage for the cell in possessing the colicin V phenotype in terms of virulence. The possible conflict with these two conclusions is clarified with the point that in the latter study, the virulence assays were performed using pure cultures injected intraperitoneally into mice. The use of pure cultures rather than mixed is a standard approach but a more artificial one, and in this case it addresses one aspect of virulence but not the aspect of competitive advantage. The fact that the latter study has been more often quoted than the first demonstrates how a case may be prematurely considered closed although an appropriate experiment was not

applied. ("High-tech" experiments are not necessarily more conclusive than "low-tech" experiments.) The conclusion of the transposition mutant study should have been that the colicin V protein does not act as a toxin against eucaryotic cells, but the next question should have been the possible role of colicin V activity against procaryotic cells in virulence, using mixed infections. A facet of the broader view, that "the mark of a successful parasite is not its ability to cause diseae but rather its ability to survive and multiply" in the host that is attempting to eliminate it (11), might have been demonstrated by means of these studies, had the investigators followed through to the logical final experiment.

The sixth investigation of this era was in 1979, when P. H. Williams presented evidence for the activity of an efficient ColV plasmid-specified iron-sequestering system (33). The enhanced virulence of invasive strains of E. coli carrying such a ColV plasmid was shown to be due to this iron uptake system, by indirect but convincing studies analyzing the effect of administering iron in mixed infections of mice and the effect of added transferrin on bacterial growth in minimal media (33). Briefly, cells that do not carry ColV plasmids are equally as virulent as when they do carry the plasmid if iron is added to the inoculum in an in vivo experiment; likewise, they grow equally as well if iron is added to transferrin-chelated media. In 1980, S. Stuart et al. showed that the ColV plasmid-mediated iron uptake system involved a siderophore that is a hydroxamate (28), and, in 1981, Warner, Williams, Bindereif, and Neilands demonstrated by field desorption mass spectroscopy that this hydroxamate was aerobactin, and indeed identical

to the molecule secreted into culture supernatants by Aerobacter aerogenes 62-1 (32). Thus, the "real" virulence factor of the ColV plasmids is a nutrition-related factor, enabling the organism to grow in the hostile environment of the host's fluids as the host attempts to make the essential nutrient iron unavailable. Aerobactin-producing bacteria can therefore be successful parasites, able to survive and multiply, and, with their complement of other virulence factors, cause disease.

It is at this point in the Time Line that the two histories of aerobactin and ColV plasmids merge, since the two genotypes were found to reside on the same replicon. Also, since the location (plasmid vs. chromosome) of the aerobactin system genes in Aerobacter aerogenes 62-1 was at the time unknown, and perhaps presumed to be chromosomal, and probably since E. coli was the more familiar organism, the aerobactin iron uptake system was investigated primarily as encoded by ColV plasmids. The first exception to this generality was the investigation by T. Viswanatha, who continued to use the original organism Aerobacter aerogenes 62-1 to study the enzymes in the biosynthetic pathway leading to aerobactin. Ultimately he and his colleagues developed a completely in vitro system for the synthesis of aerobactin, using components derived from A. aerogenes 62-1 cultures, from which the relevant biosynthetic enzymes could be characterized (31). Later the aerobactin system biosynthetic enzymes and outer membrane receptor as encoded by the ColV plasmid were also found encoded by the chromosomes of E. coli K1 (30), of enteroinvasive E. coli (16), and of Shigella (15), and by Salmonella R plasmids (8).

After 1981, therefore, the two components of the Time Line deal with ColV plasmid-encoded aerobactin and "non-ColV"-encoded aerobactin. Before advancing along the Time Line, however, it should be mentioned that, by 1981, there were three other virulence factors found encoded by ColV plasmids, that is, increased serum survival (3, 27), resistance to phagocytosis (21), and intestinal epithelial cell adherence (7). These factors, as well as the aerobactin system (and probably colicin V), no doubt played a role in the increased pathogenicity that had been correlated with the presence of ColV plasmids in strains of E. coli since 1949. However, the data from these studies cannot be used to draw conclusions about the relative virulence potential of any given plasmid or if any set of factors are additive in any given model system. More than a dozen "different" ColV plasmids were used in these studies, although it is very likely that some of them are actually identical but of different nomenclature. In none of these studies was the plasmid "fingerprint" given in the form of a restriction pattern. Ideally, an experiment should be performed in which a "skeleton" ColV plasmid could be used to reconstruct various levels of virulence by sequentially inserting the genotypes of each virulence-related factor. This kind of experiment is now feasible, since two ColV plasmids have been entirely mapped: these maps are presented in the second manuscript contained in this thesis.

Noteworthy post-1981 "ColV-aerobactin" findings include the defining of the low-iron inducible outer membrane 74 kd protein as the receptor for ferric-aerobactin and the down-regulation of the aerobactin system in high iron concentration by the global regulon fur. What expedited the

characterization of the aerobactin system operon was the cloning of this region from the ColV plasmid, which was first accomplished in the lab of J. B. Neilands in 1983 (1). Interest in the question of how the aerobactin system genes became ubiquitous among bacterial chromosomes and plasmids was kindled by the demonstration of inverted copies of the insertion sequence IS1 and of distinct replication regions flanking the aerobactin system genes in pColV-K30, the prototypic ColV plasmid (23). That is, is the aerobactin system one of the few non-antibiotic transposons? This question is addressed in the first manuscript of this thesis, and the results of these studies prompted the investigation of one of the flanking replication regions of the ColV plasmid (24). (Historic findings described in manuscripts contained in this thesis are indicated on the Time Line with asterisks.)

After cloning the pColV-K30 aerobactin system and concurrent with the studies on the aerobactin system DNA environment, three different groups independently attempted to characterize the constituent genes and encoded proteins acting as the enzymes in the biosynthetic pathway leading to the 565 dalton chelator aerobactin (6, 9, 14). The first to actually propose the pathway, with corresponding genetic loci that encoded enzyme #1, followed by enzyme #2, then #3, again came from the lab of J. B. Neilands (9). Briefly, the first step in the biosynthetic pathway leading to aerobactin is the hydroxylysine of lysine, followed by acetylation to form acetyl-hydroxylysine. Two moles of this compound are condensed with citrate to form the siderophore aerobactin. The site of chelation of ferric iron is believed to be the carboxyl moieties which

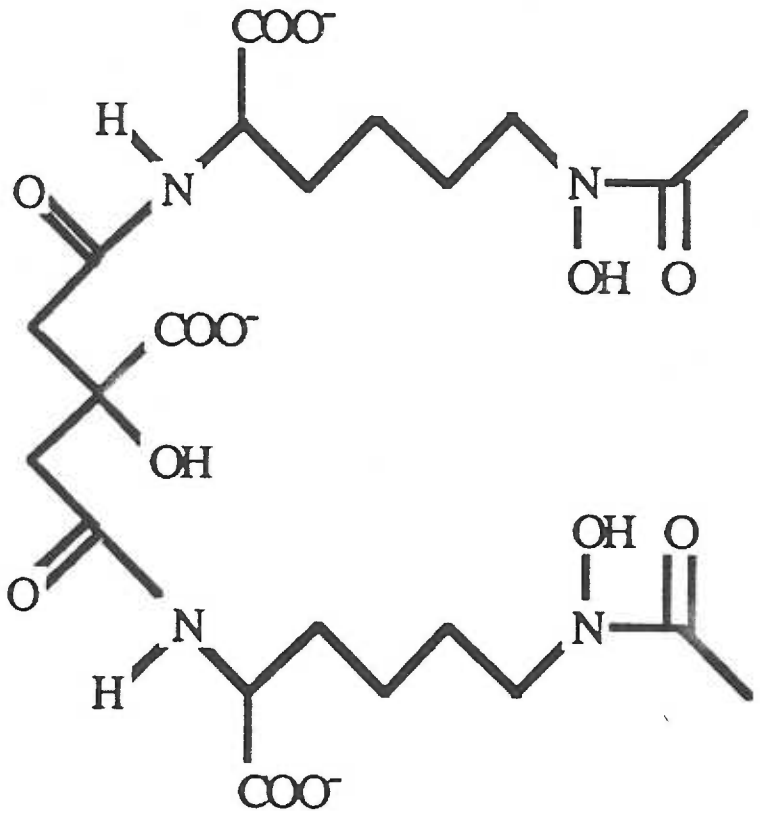
"surround" the iron (see the aerobactin molecule figure which follows this literature review).

The most recent studies on "ColV aerobactin" were the mapping and sequencing of the promoter region of the aerobactin operon, followed by DNaseI footprinting of the Fur protein, the negative regulator of the aerobactin system genes in high iron conditions, within this operator-promoter region (2,10). A 31 bp region of dyad symmetry was found to be the sequence protected by the Fur protein, and comparisons of this sequence and analogous Fur-regulated promoter regions resulted in the generation of a Fur consensus sequence which also comprises a perfect dyad. This data has been incorporated into the discussion of the Aerobacter aerogenes 62-1 putative operator-promoter.

The most recent studies on "non-ColV aerobactin" include the discovery of the production of aerobactin by certain Yersinia species (28) and by Klebsiella pneumoniae K1 and K2 (20). Also there has been a recent study addressing the question of why aerobactin, instead of the higher affinity siderophore enterobactin (commonly produced by enteric organisms, often simultaneously with aerobactin), is an important factor in invasive infections (34). It was found that aerobactin, probably because it is repeatedly reusable, efficiently stimulated bacterial growth at external concentrations 500-fold lower than those of enterobactin (34). It had previously been found that aerobactin outcompetes enterobactin when the iron is chelated by unsaturated levels of serum transferrin (5).

Studies recently done in our lab further distinguish the ColV from certain non-ColV aerobactin systems. The chromosomal determinants in E. coli K1 (30), in Shigella flexneri (15), and in enterinvasive E. coli (16) were found homologous to and of the same restriction pattern as the aerobactin biosynthetic genes of the pColV-K30 plasmid. It had been assumed that this was the case in the organism Aerobacter aerogenes 62-1, although the hybridization signals obtained with the E. coli plasmid and the A. aerogenes plasmid were not comparable, using an internal probe from the E. coli system (17). Clarification of this discrepancy led to the discovery of a distinct genetic system encoding aerobactin, and is the subject of the third manuscript of this thesis. In the process of this investigation, the speciation of Aerobacter aerogenes 62-1 became suspect, and led to its respeciation as a Klebsiella ozaenae, a species recognized as of 1980 (details are contained in the fourth manuscript of this thesis). The sequencing analysis of the "Klebsiella ozaenae" 62-1 promoter region is the subject of the appendix following the third paper. Comparing this sequence with the analogous region of the E. coli system further distinguishes the two systems, although the concept of the mechanism of regulation may be preserved. Sequencing the structural genes of the Klebsiella ozaenae aerobactin system is now in progress, and will define the differences we have found. The fact that both systems produce the identical aerobactin molecule (and presumably via the same biosynthetic pathway) predicts that certain areas should be conserved within the enzymes involved in each step of the pathway. Therefore, sequencing the genetic regions for both systems will provide information that could not be obtained by sequencing just one of the two genetic

systems, and provides a simple but tedious approach to the question of possible enzyme active sites.




AEROBACTIN

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Paper #1:

# DNA Environment of the Aerobactin Iron Uptake System Genes in Prototypic Plasmids

# ABSTRACT

The aerobactin iron uptake system genes in the prototypic plasmid pColV-K30 are flanked by inverted copies of insertion sequence IS1 and by two distinct replication regions. To address the question of how these flanking regions may facilitate the maintenance and spread of the aerobactin system among the plasmids and chromosomes of enteric species, we investigated the DNA environment of twelve ColV plasmids. We found that the aerobactin system-specific genes are conserved in every plasmid phenotypically positive for the aerobactin system. The upstream IS1 is also conserved and likewise its overlapping replication region (REPI). This replication region was cloned from several ColV plasmids and found to be functional by transforming these cloned derivatives into a polA bacterial host. In contrast, the downstream flanking region is variable. This includes the downstream copy of IS1 and the downstream replication region (REPII). We infer from these results that sequences in addition to the two flanking copies of IS1, in particular the upstream region including REPI, have been instrumental in the preservation and possible spread of aerobactin genes among ColV plasmids and other members of the FI incompatibility group.

# INTRODUCTION

The association of colicin V production and increased pathogenicity of E. coli strains was noted in 1949 (14) but not until 1979 did it become clear that factors other than colicin V production caused the increase in virulence when E. coli were injected into mice (27). ColV plasmids, encoding colicin V, are actually a heterogeneous group of IncF1 plasmids which may also encode virulence determinants such as: increased serum survival (4,23,28), resistance to phagocytosis (25), adherance to intestinal epithelial cells (10), and the aerobactin-mediated iron uptake system (6,32,34). In this work, we address the question of how the aerobactin iron uptake system came to be genetically determined on ColV plasmids. Interest in this question has been kindled by the recent demonstration of inverted copies of the insertion sequence IS1 (22,26) and of two distinct replication regions, REPI and REPII (26), flanking the aerobactin genes in the prototypic ColV plasmid pColV-K30. This, taken together with the discoveries of the same aerobactin iron uptake genes on the chromosomes of E. coli K1 (30) and of Shigella species (20), on Salmonella multiple drug resistance plasmids (11), and on a plasmid of Enterobacter aerogenes (22), suggest that the aerobactin operon is genetically mobile. However, in spite of this ubiquity in nature, the extrachromosomal aerobactin system genes have been found on plasmids, which, when the incompatibility group is known, are always IncFI (11,12,33). To gain insight into the reasons for these observations we surveyed the extent of genetic conservation surrounding the aerobactin operon in twelve ColV plasmids, and compare the resulting maps to that of IncFI Salmonella R plasmids. (These results were presented in part at the 85th Annual Meeting of the American Society of Microbiology, Las Vegas, Nevada, March 3rd to 8th, 1985.)

# MATERIALS AND METHODS

Bacterial strains and plasmids. Pertinent E. coli strains, plasmids and their relevant characteristics are listed in Table 1. E. coli 3478 and E. coli HB101 were used as recipients in transformation experiments, and E. coli C2110 and E. coli LE392 were used as recipients in the in vitro transductions.

Detection of aerobactin siderophore production and of the outer membrane receptor. Aerobactin production by bacteria harboring ColV plasmids was determined by a bioassay test (32). Presence of the aerobactin outer membrane receptor was assayed by the cloacin sensitivity test (26).

Analysis of plasmid DNA. Plasmid DNA preparation, restriction endonuclease and Southern blot analysis were carried out as previously described (26).

Molecular cloning of ColV plasmid DNA. To obtain clones which included flanking sequences as well as aerobactin specific sequences, ColV plasmid DNA was partially digested; cosmid vector pHC79 or pVK102 DNA was totally digested and added to the partially digested ColV plasmid DNA at an approximate molar ratio of 1:1. Ligated DNA was packaged in vitro using commercially available packaging systems (Amersham, Boston, Mass. and Promega Biotech, Madison, Wis.) and transduction was carried out according to the protocol recommended by Promega Biotech.

# RESULTS

Distribution of aerobactin genes among ColV plasmids. With the exception of the plasmid pColV-CA7V, the ColV plasmids studied herein (Table 1) all engender their bacterial host with the capacity to produce the siderophore aerobactin. The molecular weights of these plasmids, determined by gel electrophoresis (data not shown), ranged from the 80 kilobase pairs (kb) of pColV-B188 to the 180 kb of pColV-K229 (Table 2). However, restriction endonuclease digestion of these various ColV plasmids showed that some fragments appear to be shared (data not shown). On the basis of restriction enzyme patterns, pColV-K30 and pColV3-K30 appear to be identical plasmids; pColV-292, pColV-F70, and pColV-F54 also appear to be identical. To determine the extent of conservation of aerobactin-related and flanking sequences, we employed HindIII and HindIII-BamHI digests in Southern blot hybridization experiments. In the hybridization experiments shown in Fig. 2a and 3a, using the aerobactin-specific probe HB (Fig. 1), a 3.4 kb HindIII-BamHI fragment in all aerobactin-positive plasmids appeared universally conserved and is the equivalent of the pColV-K30's 3.4 kb HindIII-BamHI cloned sequence used here as probe HB (Fig.2a, lanes K through S, and Fig. 3a, lanes F through J). This same probe, which closely approximates the aerobactin siderophore genes (8,17), also demonstrated that these 3.4 kb HindIII-BamHI fragments are contained within HindIII fragments of molecular weights ranging from 14.5 to 45 kb (Fig. 2a, lanes A through I, and Fig. 3a, lanes A through E). Using the probe HB and other restriction enzymes (data not shown), we confirmed that the regions proximal to the siderophore genes were always conserved in aerobactin-positive ColV plasmids.

This region includes genes for the four enzymes needed to construct the hydroxamate siderophore aerobactin, and the structural genes for the 74K outer membrane receptor protein (8,17). The variation in HindIII fragment size must then reflect changes downstream from the aerobactin genes since the upstream region which encodes the aerobactin system is conserved among these plasmids.

Distribution of IS1 sequences among ColV plasmids. To assess the degree of conservation of the two flanking IS1 sequences within the various ColV plasmids, we probed the ColV plasmids with an IS1-specific probe, pBRG29. This plasmid is a pBR322 derivative containing the transposon Tn9 which is bordered by IS1 elements. Using as a negative control probe the pBR325 plasmid which, like Tn9, has the genes for chloramphenicol resistance, but, unlike Tn9, has no IS1 sequences, we could deduce that the ColV plasmid sequences hybridizing to pBRG29 but not to pBR325 were specific for IS1. The results of the hybridization with the IS1 probe are shown in Figures 2b and 3b. All signals seen in these autoradiographs are IS1 specific, with the exception of a small plasmid additionally present in B188 (15) which shows vector cross-reaction, since it is a ColE1-type plasmid (unpublished results). Since neither HindIII nor BamHI sites are found within the IS1 sequence, the minimum number of copies of IS1 could be counted (Table 2). Figure 2b, lanes A through I, and Fig. 3b, lanes A through E, show that the IS1-containing HindIII fragment upstream relative to the aerobactin genes is an 8.6 kb fragment which is held in common by all the aerobactin-positive plasmids. These figures also show that the downstream IS1 sequences were within HindIII fragments varying in size from 14.5 to 45 kb; these were the same fragments which also hybridized to the aerobactin siderophore probe HB. To more precisely map the location of this

IS1 sequence, presumed to be on the right flank of the aerobactin genes as seen in the pColV-K30 map, we used the restriction enzyme BstEII which splits the IS1 sequence approximately in half. Using the probe specific for IS1 and the probe BE (Fig. 1) we obtained the following results: the BstEII fragment containing both the 74K aerobactin receptor gene and the proximal half of the flanking IS1 sequence was 3.40 kb for pColV-K30, pColV-3K30, pColV-292, pColV-F70, pColV-F54, and pColV-K229, but was 3.50 kb for pColV-H247 and pColV-P72, and 3.47 kb for pColV-B188, pColV-K311, and pColV-K328 (data not shown). Therefore, although IS1 sequences flank the aerobactin operon in all aerobactin-positive plasmids, the downstream IS1 sequence varies slightly in distance from the 74K aerobactin receptor protein gene. Using the same strategy to map the upstream IS1 sequence, we found its location in all the aerobactin-positive plasmids to coincide with the map position of pColV-K30 (Fig. 1), within the upstream 8.6 kb HindIII fragment (Table 2).

Distribution of replication regions among ColV plasmids. To test whether one or both of the flanking replication regions of pColV-K30 were conserved in the other ColV plasmids, we probed with REPI and REPII. The REPI probe we used, ΔREPI, had the repeated sequence IS1 deleted (Fig. 1; J. Perez-Casal and J.H. Crosa, manuscript in preparation). The REPII probe DNA consists of a region beyond the rightward downstream IS1 with no overlap with that IS1 sequence (Fig. 1). With the ΔREPI probe the pattern of hybridization is uniform among all the aerobactin producing plasmids: in every case the homologous HindIII and HindIII-BamHI fragments within the ΔREPI region are identical to those of pColV-K30. This conservation of sequences is peculiar to the aerobactin-producing plasmids. In the aerobactin-negative plasmid pColV-CA7V (Fig. 2c, lanes F and P), the

conserved fragments are only those which are beyond the upstream IS1 sequence. The outcome of hybridization experiments using the RPII probe was quite different from those using the ΔREPI probe. REPII sequences are absent in the aerobactin-negative plasmid, but they are also absent in five aerobactin-positive plasmids: pColV-H247, pColV-B188, pColV-K311, pColV-K328, and pColV-P72. (In Fig. 2d, lanes C and M, the hybridization signal seen is due to vector cross reaction with a smaller plasmid present in the B188 strain.) REPII-homologous sequences could only be seen upon prolonged exposure and within a single 4.0 kb HindIII-BamHI fragment of pColV-292, pColV-F70, and pColV-F54, (Fig. 2d, lanes N, R, and S). This weak signal suggests that REPII was not present intact, or that considerable divergence had occurred. The plasmid pColV-K229 is the only ColV plasmid in addition to pColV-K30 which appears to have an intact REPII region. This region is also genetically linked to its REPI-IS1-aerobactin system-IS1 region, but it is more closely linked than is the REPII region of pColV-K30.

Functionality of the REPI region. The functionality of the replication region REPI was assayed by using a cloning vector which cannot replicate in a bacterial host lacking polymerase I, and transforming these polA bacteria with clones carrying the REPI region from some of the ColV plasmids. In clones of the pColV-B188, pColV-292, and pColV-H247 plasmids, as well as of pColV-K30 (26), this region was found to be a functional replication unit (Table 3). Thus, there has been a conservation of function as well of genetic linkage of the REPI region in these ColV plasmids. We have determined that REPI is unstable unless accompanied by a maintenance region that maps outside REPI but adjacent to the aerobactin system genes, and that this region is also conserved in the ColV plasmids examined to date (J. Perez-Casal and J.H. Crosa, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986,

11-103, p. 144). Therefore, those plasmids without REPII sequences are understandably capable of stable replication.

# DISCUSSION

The results presented here demonstrate not only the conservation of the genes encoding the aerobactin iron uptake system among ColV plasmids, but also the conservation of a region which extends beyond the upstream IS1 to include the replication region REPI, as originally found on the prototypic pColV-K30 plasmid (26). The functionality of this highly conserved REPI region was established in clones of some of the ColV plasmids, and would be expected in the other plasmids based on their identical REPI restriction enzyme maps. The published map of certain Salmonella aerobactin-encoding R plasmids (11) similarly shows the restriction environment of REPI. Note that in the ColV plasmids (Fig. 4) the conservation of sequences begins upstream relative to REPI, and the variability begins downstream from the aerobactin system but prior to the downstream IS1 sequence as indicated by dotted lines in Figure 4 and derived from fine analysis. In the Salmonella R plasmids the conservation appears to begin at REPI but then follows the pattern obtained with the ColV plasmids. It therefore appears that the REPI-aerobactin system unit stands out against the two (Salmonella plasmid and E. coli plasmid) varied backgrounds. We have also found this pattern of conservation in an IncFI, aerobactin-encoding, non-ColV, R-plasmid of a clinical E. coli strain isolated from a neonate with sepsis and pyelonephritis (unpublished results). The conservation of upstream sequences appears therefore to be the general rule among IncFI plasmids coding for aerobactin biosynthesis.

The fact that a replication region is part of the large conserved unit suggests that mechanisms other than simple transposition are at work in

fostering the ubiquity of the aerobactin genes on the plasmids of enteric bacteria. This proposal is supported by three observations: 1) the size of the IS1-bound region is large (16 kb), giving transposition low probability (9); 2) the orientation of the boundary IS1 sequences is not the orientation most often associated with IS1-mediated transposition, such as the transposition of E. coli ST toxin by Tn1681 (29); and, 3) transposition of the IS1-bound aerobactin system genes has not been achieved in the laboratory (unpublished results and S. Payne, personal communication). In support of the transposability of aerobactin genes is the fact that the IS1-bound region was found to be in two different orientations among the Salmonella R plasmids (11). However, even in these plasmids, flanking sequences beyond the IS1-bound region have been conserved, including the restriction enzyme environment of REPI (11 and B. Colonna, personal communication). If the IS1-bound aerobactin genes were spread by transposition, the flanking genetic environments would be expected to vary. Since such variation is not observed, the inversion event may be less consequential than the preservation of a larger unit containing the aerobactin system genes and REPI sequences. Also, there are other copies of IS1 in most of the aerobactin-producing ColV plasmids which may function in the recombination of a larger and possibly more stable aerobactin-replication unit. This formulates what we call a virulence factor-replication unit. A large replicon could undergo deletions: a deleted segment which includes its own replication region could thereby be perpetuated either momentarily or for a longer term. Replicon fusion between independently replicating units could similarly result in the passage of the aerobactin system genes from one replicon to another.

The extrachromosomal aerobactin system genes have thus far only been associated with IncFI plasmids (11,12,33), although in the case of the pSMN1 plasmid of E. aerogenes (22), the incompatibility group is unknown. One important characteristic of most members of the IncFI incompatibility group is the possession of sequences homologous to the secondary replicon of F present in the EcoRI fragment f7 (2,16). These sequences, termed incE, are involved in FI-type incompatibility reactions. Detailed mapping and analysis of the REPI region have shown that it carries, in addition to replication-related genes, sequences homologous to incE (3; J. Perez-Casal and J.H. Crosa, manuscript in preparation). The distribution of extrachromosomal aerobactin system genes among IncFI-type plasmids may then simply reflect the fact that these genes are linked to the REPI region.

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Table 1. Properties and sources of E. coli strains and plasmids.

| Strain | Genotype | Relevant phenotype conferred by plasmid[g] | Source |
|---|---|---|---|
| HB101 | F- hsdS20 recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl5 mtl1 supE44 | na | H. Boyer (5) |
| LE392 | F- hsdR514 supE44 supF58 lacY1 galK2 galT22 metB2 trpR55 | na | Bethesda Res. Lab. |
| C2110 | polA rha his | na | Laboratory stock |
| 3478 | F- polA thy | na | Laboratory stock (13) |
| RW193 | entA proC leu trp tsx thi lacY galK ara mil xyl azi supE44 | na | J.B. Neilands (31) |
| LG1315 (pCo1V-K30) | F- ara endA lac leu mtl proC rpsL supE tonA tri trpE xyl | $Iu^+$ $C^s$ | P.H. Williams (32) |
| LG1522 (pCo1V-K30, iuc) | ara azi fepA lac leu mtl proC rpsL supE tonA tsx thi | $Iu^-$ $C^s$ | P.H. Williams (32) |
| HB101 (pHC79) | | $Ap^rTc^r$ | Bethesda Res. Lab. |
| HB101 (pVK102) | | $Km^rTc^r$ | E. Nester (19) |
| HB101(pJHC-V12) | | $Iu^+$ $C^s$ | Laboratory stock (30) |
| C600 (pBRG29) | F- thi-1 thr-1 leuB6 lacY1 tonA21 supE44 | $Cm^r$ $Tc^r$ $Ap^r$ | Laboratory stock (1,30) |
| 3478(pJHC-P1) | | $Iu^+$ $C^s$ $Ap^r$ | Laboratory stock (30) |
| 3478(pJHC-P2) | | $Iu^-$ $C^r$ $Ap^r$ | Laboratory stock (30) |
| 3478(pJHC-P36)[d] | | $Tc^r$ $C^r$ | Laboratory stock |

| Strain | Genotype | Relevant phenotype conferred by plasmid[g] | Source |
|---|---|---|---|
| 3478(pJHC-P3)[e] | | $Cm^r$ $C^r$ | Laboratory stock (30) |
| P72(pCo1V-P72) | | $Iu^+$ $C^s$ | Esther Lederberg (Plasmid Reference Center[a])[b] |
| B188(pCo1V-B188) | | $Iu^+$ $C^s$ | Dwayne Savage (23)[b] |
| 292(pCo1V-292) | | $Iu^+$ $C^s$ | Esther Lederberg (Plasmid Reference Center)[b] |
| H247(pCo1V-H247) | | $Iu^+$ $C^s$ | Dwayne Savage (23)[b] |
| CA7V(pCo1V-CA7V) | | $Iu^-$ $C^r$ | Esther Lederberg (Plasmid Reference Center)[c] |
| F70(pCo1V-F70) | | $Iu^+$ $C^s$ | Esther Lederberg (Plasmid Reference Center)[b] |
| 355(pCo1V-F70) | | $Iu^+$ $C^s$ | Dwayne Savage (23) |
| F54(pCo1V-F54) | | $Iu^+$ $C^s$ | Esther Lederberg (Plasmid Reference Center)[b] |
| 291(pCo1V-F54) | | $Iu^+$ $C^s$ | Dwayne Savage (23) |
| K229(pCo1V-K229) | | $Iu^+$ $C^s$ | Volkmar Braun (7) |
| K311(pCo1V-K311) | | $Iu^+$ $C^s$ | Volkmar Braun (7) |
| K328(pCo1V-K328) | | $Iu^+$ $C^s$ | Volkmar Braun (7) |
| KB2443(pCo1V3-K30) | | $Iu^+$ $C^s$ | Esther Lederberg (Plasmid Reference Center, via Peter Bergquist)[f] |

[a] Plasmids were obtained from the former Plasmid Reference Center.

[b] Originally from the collection of H.W. Smith.

[c] Originally from the collection of A. Gratia.

[d] Strain containing the cloned deleted version of REPI (see text).

Table 1 (Continued)

[e] Strain containing the cloned replication region REPII.

[f] Although originally (21) the plasmid "factor" from E. coli K30 was called ColV3-K30, the name was apparently shortened to ColV-K30 in many collections.

[g] Iu, iron uptake status; $C^s$ or $C^r$, cloacin sensitive or resistant.

Table 2. Distinguishing characteristics of ColV plasmids.

| Plasmid | MW (kb) | No. of copies of IS1 element | Size (kb) of HindIII fragment carrying: IS1 sequence upstream from aerobactin genes | Size (kb) of HindIII fragment carrying: downstream IS1 and aerobactin genes |
|---|---|---|---|---|
| pColV-P72 | 137 | 3 | 8.6 | 15.5 |
| pColV-B188 | 80 | 2 | 8.6 | 45 |
| pColV-292 | 150 | 4 | 8.6 | 14.5 |
| pColV-H247 | 137 | 3 | 8.6 | 15.5 |
| pColV-CA7V | 98 | 3 | 15.8[a] | na[b] |
| pColV-K30 | 144 | 5 | 8.6 | 16.3 |
| pColV-F70 | 150 | 4 | 8.6 | 14.5 |
| pColV-F54 | 150 | 4 | 8.6 | 14.5 |
| pColV-K229 | 180 | 7 | 8.6 | 14.5 |
| pColV-K311 | 130 | 2 | 8.6 | 45 |
| pColV-K328 | 140 | 2 | 8.6 | 45 |
| pColV3-K30 | 144 | 5 | 8.6 | 16.3 |

[a] In pColV-CA7V all three copies of IS1 are contained within this 15.8 kb HindIII fragment, but the copy which is linked to REPI sequences is partially deleted.

[b] na - Not applicable since pColV-CA7V is genotypically and phenotypically negative for the aerobactin iron uptake system.

Table 3. Functionality of clones carrying REPI

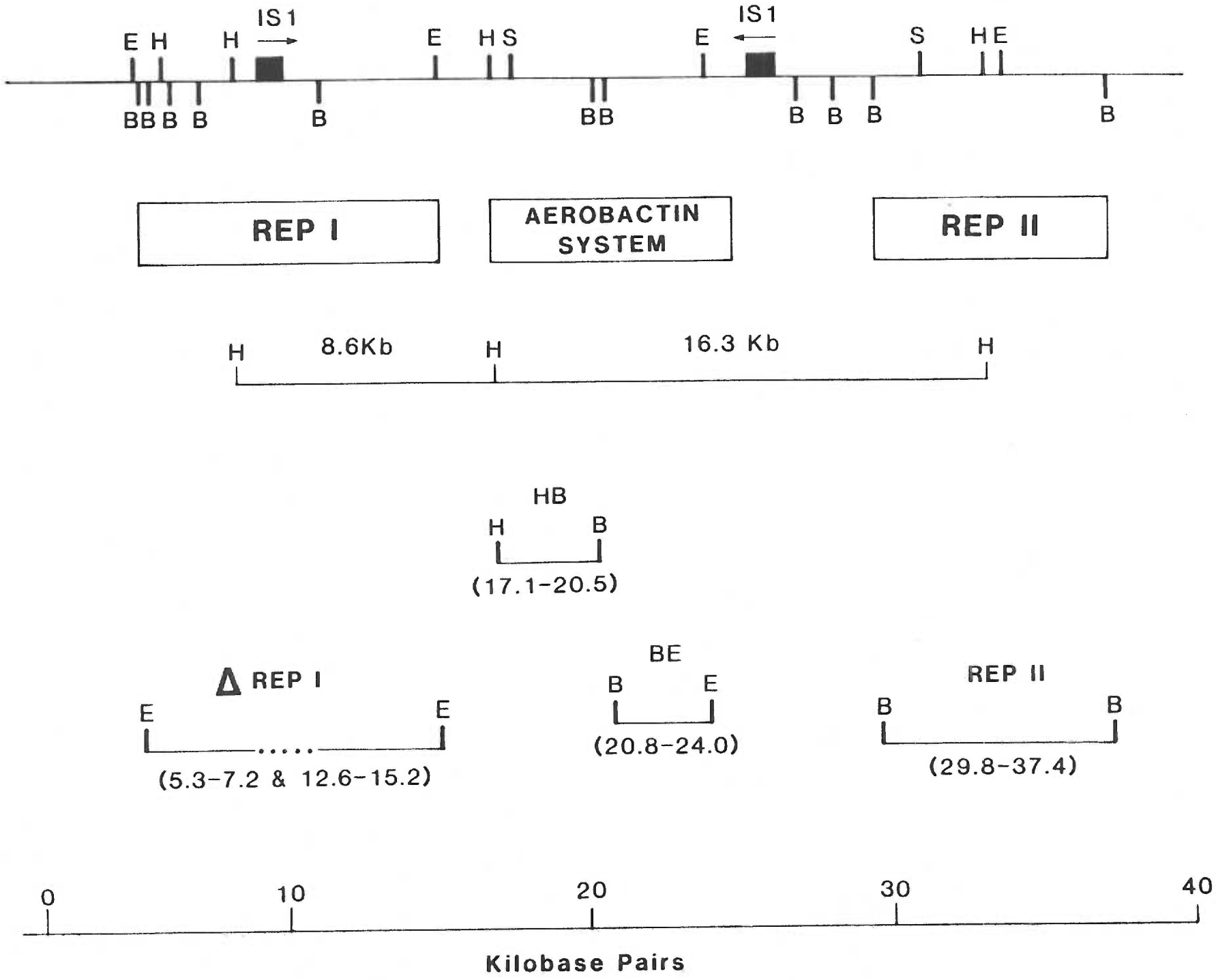
| Clone | ColV plasmid from which clone was made | Size (kb) of Cloned EcoRI Fragments | Replication in E. coli strain | |
|---|---|---|---|---|
| | | | HB101 (polA+) | C2110 or 3478 (polA-) |
| pJHC-VWB4 | pCo1V-B188 | 10[a]; 8.6[b]; 10[c] | + | + |
| pJHC-VW2922 | pCo1V-292 | 10[a]; 8.6[b], 13[c] | + | + |
| pJHC-VWH5 | pCo1V-H247 | 3.7[c]; 10[a] | + | + |
| pJHC-P2 | pCo1V-K30 | 10[a] | + | + |
| pHC79(vector) | | | + | - |

a EcoRI fragment of 10 kb which hybridized with the replication region (cloned from pCo1V-K30) called REPI.

b EcoRI fragment of 8.6 kb which hybridized to the aerobactin-specific probe HB (Fig. 3).

c EcoRI fragments which flank either the left or the right of the REPI-aerobactin system unit in the native plasmid.

Figure 1. Partial map of pColV-K30, indicating fragments used as probes in Southern blot hybridization experiments of the ColV plasmids. Map positions of replications regions REPI and REPII, the aerobactin system genes, and the insertion sequence IS1 are indicated. The cleavage sites for various restriction endonucleases are shown: H, HindIII; B, BamHI; E, EcoRI; and S, SalI.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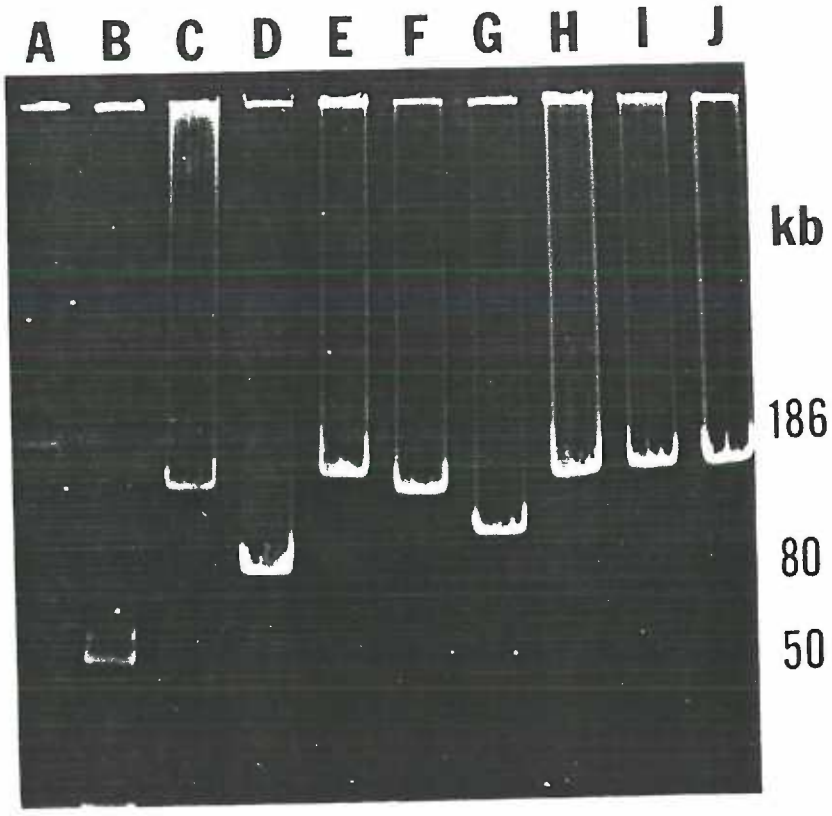
REP I
AEROBACTIN SYSTEM
REP II
H
8.6Kb
H
16.3 Kb
H
HB
H
B
(17.1-20.5)
Δ REP I
E
E
(5.3-7.2 & 12.6-15.2)
BE
B
E
(20.8-24.0)
REP II
B
B
(29.8-37.4)
0
10
20
30
40
Kilobase Pairs

Figure 1a. Electrophoretic profile of undigested covalently closed circular DNA of ColV plasmids used in this study. Panel a. High molecular plasmids in particular are visualized under the conditions used for this gel, so small plasmid species contained in strains pColV-B188 and pColV-P72 are not seen. Lane A, E. coli TP plasmid, molecular weight standard; lane B, pJHC-P1 and E. coli V517 plasmids, molecular weight standards; lane C, pColV-P72; lane D. pColV-B188; lane E, p ColV-292; lane F, pColV-H247; lane G, pColV-CA7V; land H, pColV-K30; lane I, pColV-F70; lane J, pColV-F54. Panel b. Lane A, pColV-B188; lane B, molecular weight standards TP124, V517, and pJHC-P1; lane C, pColV-K299; lane D, pColV-K311; lane E, pColV-K328; and lane F, pColV-3K30; and lane G, pColV-K30.

a




b

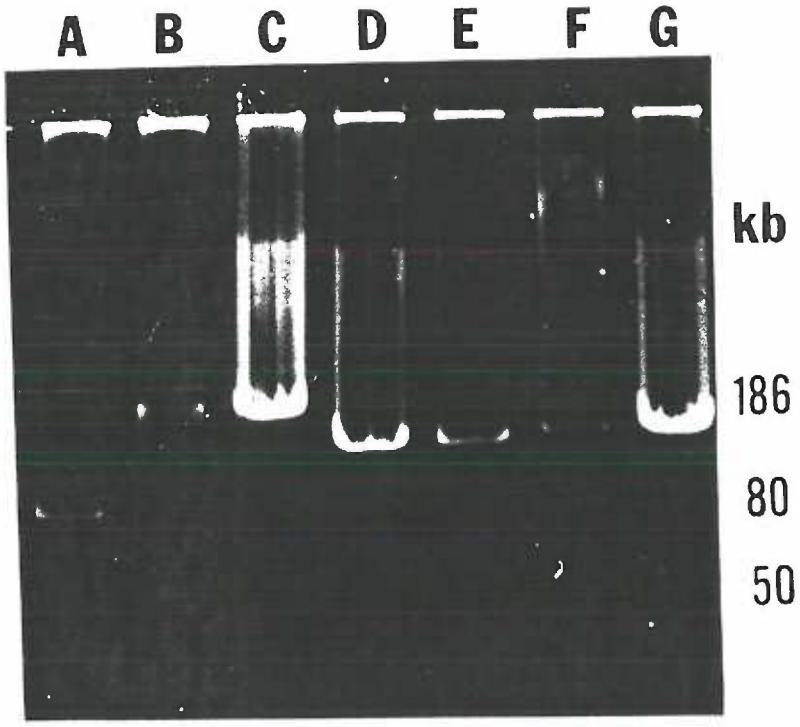


Figure 1b. Electrophoretic profiles of ColV plasmid DNA digested with restriction enzymes HindIII and HindIII-BamHI. Panel a. Lane A, pJHC-P1, a cloned derivative of pColV-K30; lane B, pColV-P72; lane C, pColV-B188; lane D, pColV-292; lane E, pColV-H247; lane F, pColV-CA7V; lane G, pColV-K30; lane H, pColV-F70; lane I, pColV-F54; lane J, pJHC-P1 digested with XhoI and lambda DNA digested with HindIII, molecular weight standards; lane K, pJHC-P1; lane L, pColV-P72; lane M, pColV-B188; lane N, pColV-K292; lane O, pColV-H247; lane P, pColV-CA7V; lane Q, pColV-K30; lane R, pColV-F70, lane S, pColV-F54; lane T, lambda DNA digested with HindIII. Lanes A through I are HindIII digests and lanes K through S are HindIII and BamHI digests. Panel b. Lane A, pJHC-P1; lane B, pColV-K229; lane C, pColV-K311; lane D, pColV-K328; lane E, pColV-3K30; lane F, pColV-P1; lane G, pColV-229; lane H, pColV-K311; lane I, pColV-K328; and lane J, pColV-3K30. Lanes A through E, HindIII; lanes F through J, HindIII and BamHI.

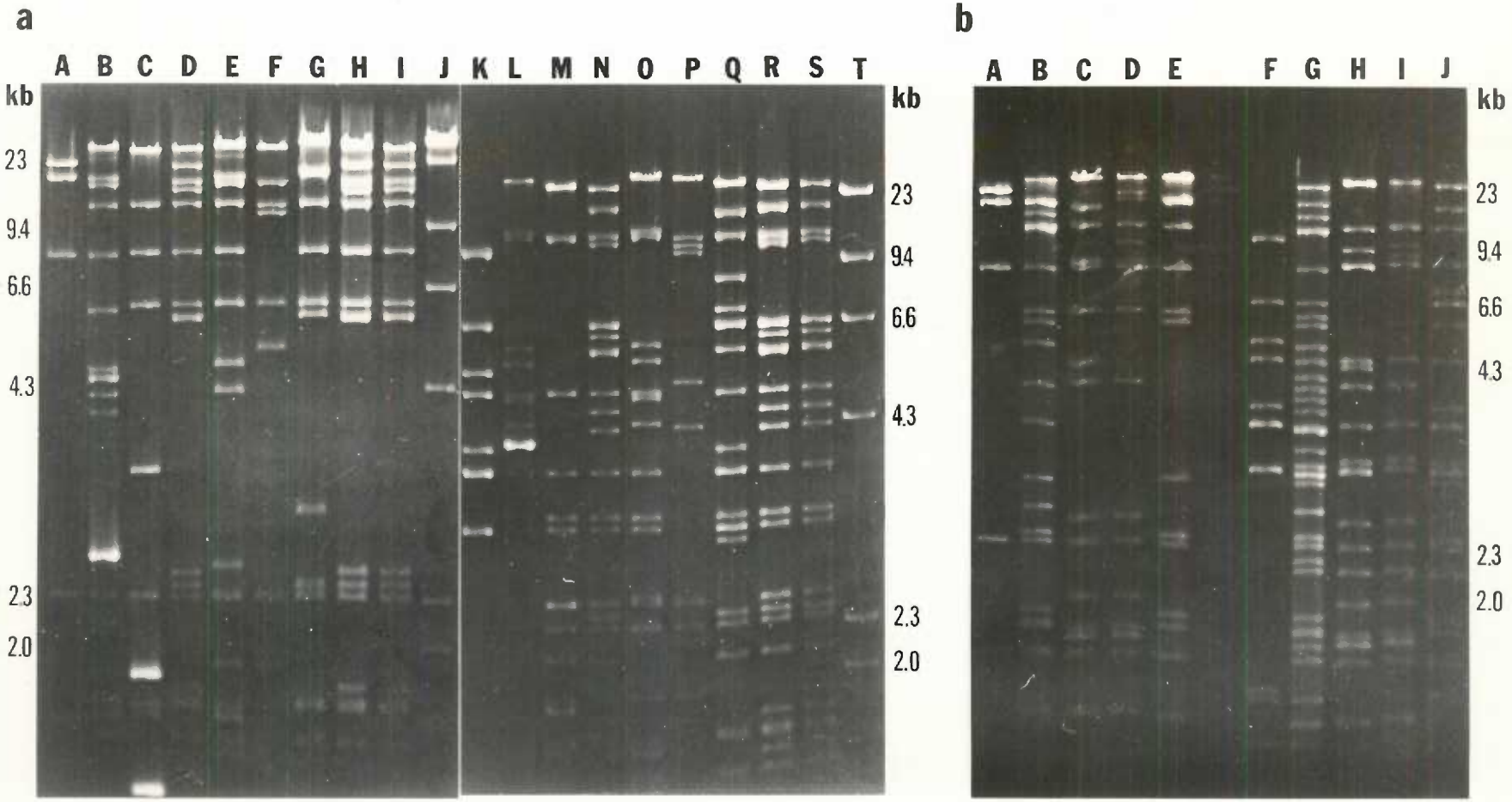

a
A B C D E F G H I J K L M N O P Q R S T
kb
23
9.4
6.6
4.3
2.3
2.0
kb
23
9.4
6.6
4.3
2.3
2.0
b
A B C D E F G H I J
kb
23
9.4
6.6
4.3
2.3
2.0

Figure 3. Hybridization patterns using as probes (described in Fig. 2 legend): HB (panel a); pBRG29 (panel b); pJHC-P36 (panel c); and pJHC-P3 (panel d). In panels a and b, ColV plasmid DNA was digested with HindIII (lanes A through E) and Hind III-BamHI (lane F through J). In panel c, ColV plasmid DNA was digested with HindIII, and in panel d, ColV plasmid DNA was doubly digested with HindIII and BamHI. Lane A, pJHC-P1; lane B, pColV-K229; lane C, pColV-K311; lane D, pColV-K328, and lane E, pColV-3K30 (panels a through d); lane f, pJHC-P1; lane G, pColV-K229; lane H, pColV-K311; lane I, pColV-K328; lane J, pColV-3K30 (panels a and b). In lanes A and F of panels b, c, and d, the vector-containing fragments of the clone pJHC-P1 (26) shows cross reaction with the cloned probes.

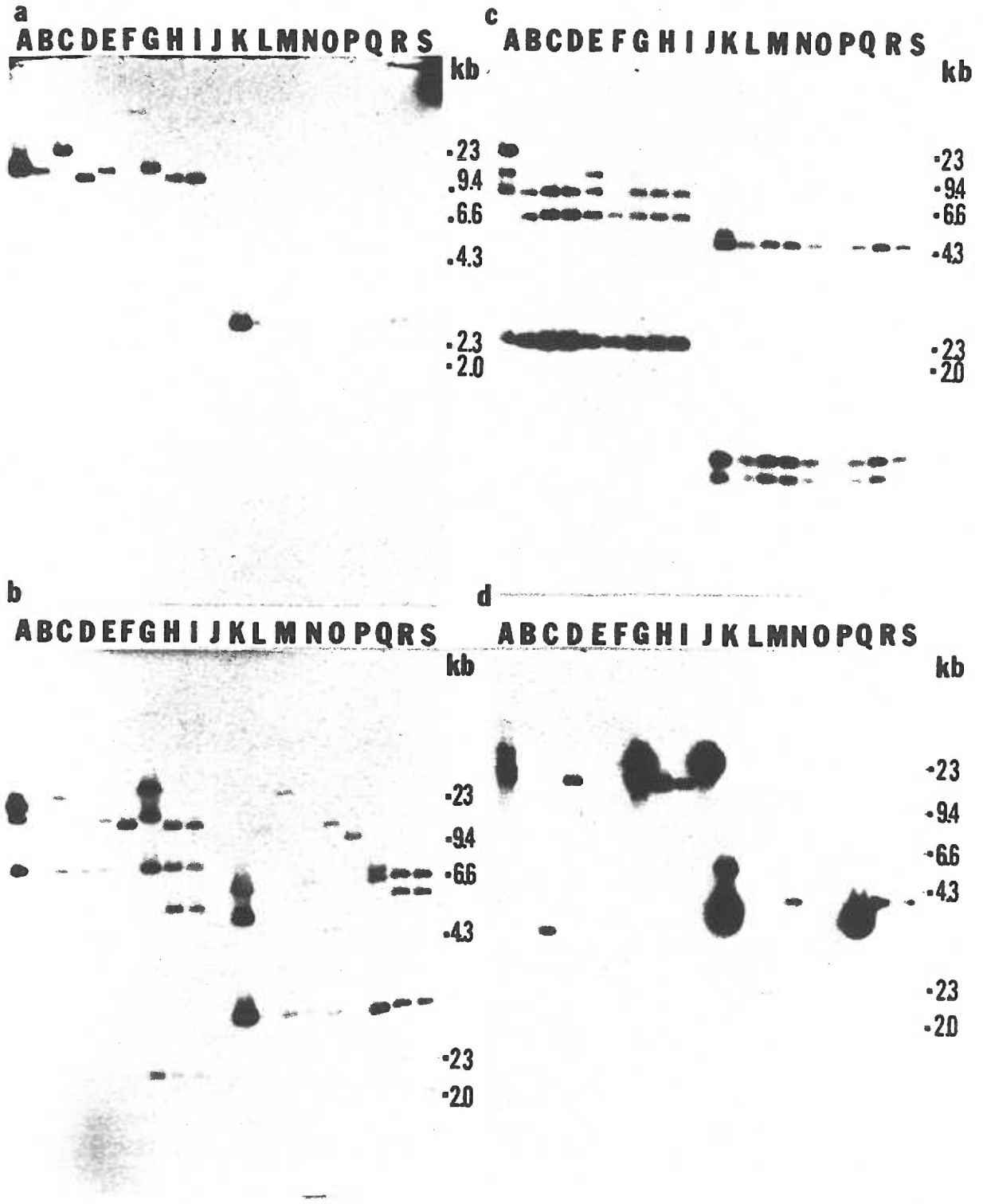

a
A B C D E F G H I J K L M N O P Q R S
kb
·23
·9.4
·6.6
·4.3
·2.3
·2.0
c
A B C D E F G H I J K L M N O P Q R S
kb
·23
·9.4
·6.6
·4.3
·23
·20
b
A B C D E F G H I J K L M N O P Q R S
kb
·23
·9.4
·6.6
·4.3
·23
·20
d
A B C D E F G H I J K L M N O P Q R S
kb
·23
·9.4
·6.6
·4.3
·23
·20

Figure 2. Hybridization patterns using as probes: HB, which is specific for the aerobactin siderophore genes (panel a); pBRG29, which is specific for the insertion sequence IS1 (panel b); pJHC-P36, the ΔREPI probe which is specific for the replication region REPI but has had the IS1 sequence deleted (panel c); and pJHC-P3, which is specific for replication region REPII (panel d). ColV plasmid DNA was digested with HindIII (lanes A through I) and HindIII-BamHI (lanes K through S). Lane A, pJHC-P1; lane B, pColV-P72; lane C, pColV-B188; lane D, pColV-292; lane E, pColV-H247; lane F, pColV-CA7V; lane G, pColV-K30; lane H, pColV-F70; lane I, pColV -F54; lane J, lambda DNA digested with HindIII; lane K, pJHC-P1; lane L, pColV-P72; lane M, pColV-B188; lane N, pColV-292; lane O, pColV-H247; lane P, pColV-CA7V; lane Q, pColV-K30; lane R, pColV-F70; lane S, pColV-F54 (for panels a through d). In order to demonstrate faintly hybridizing fragments, the exposure in Fig. 4d was prolonged, such that the 2 bands (of 3.4 and 3.6 kb) in lanes K and Q are indistinguishable. In lanes A and K of panels b, c, and d, the vector-containing fragments of the clone pJHC-P1 (26) show cross reaction with the cloned probes.

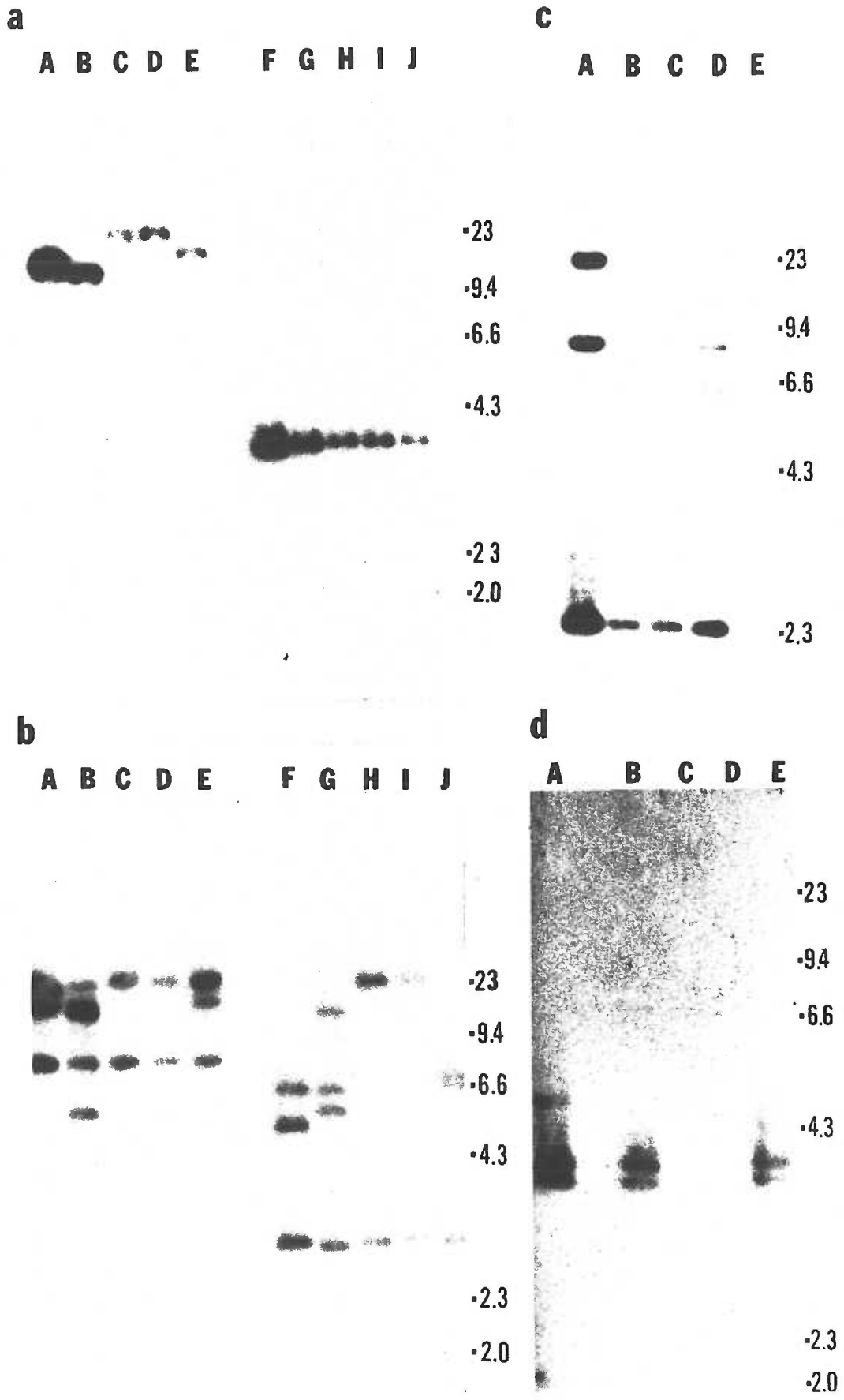
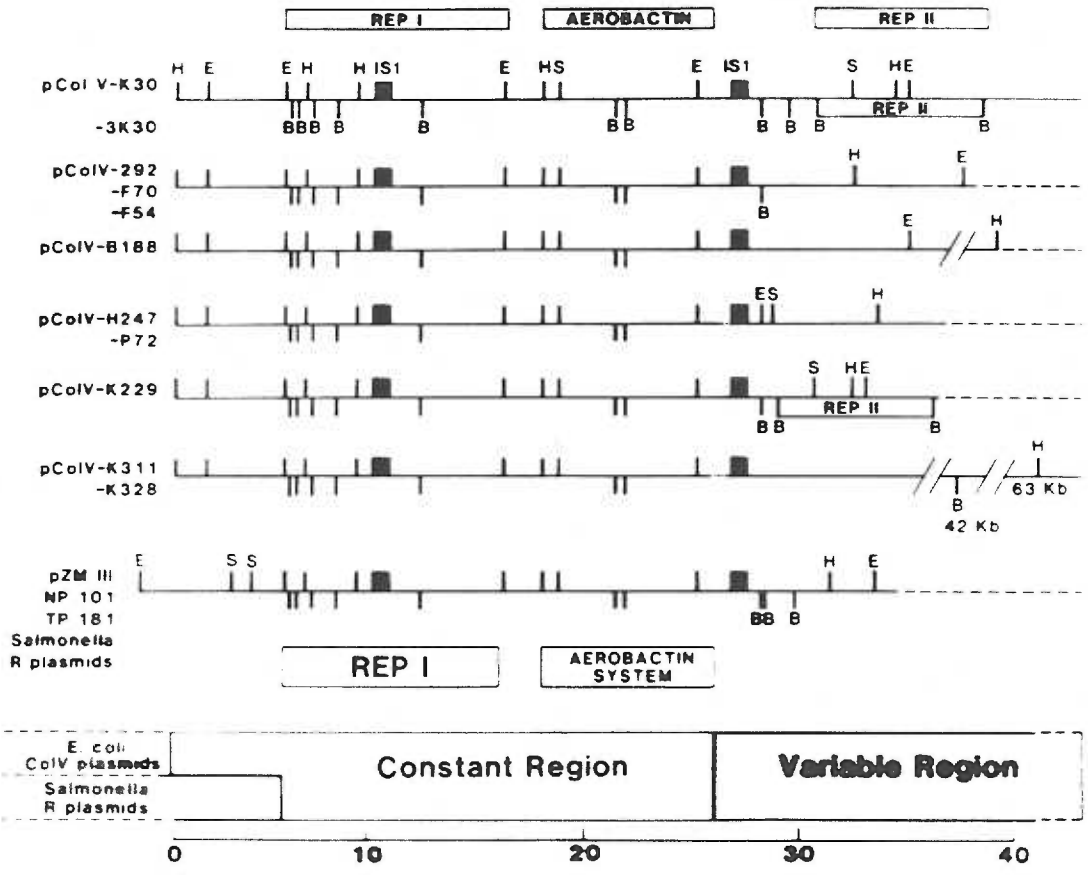

a
A B C D E F G H I J
23
9.4
6.6
4.3
2.3
2.0
c
A B C D E
23
9.4
6.6
4.3
2.3
b
A B C D E F G H I J
23
9.4
6.6
4.3
2.3
2.0
d
A B C D E
23
9.4
6.6
4.3
2.3
2.0

Figure 4. Summary of the mapped regions of ColV plasmids studied herein, and of the Salmonella drug resistance plasmids pZMIII, NTP101, and TP181, included for comparison purposes (see text). The genetic regions held in common by all these plasmids and by only the ColV plasmids are delineated in the Constant Region. The genetic regions which vary among the plasmids are delineated in the Variable Regions. Dashed lines indicate unmapped areas, and dotted lines indicate slight variations which have been mapped to show where the Constant Region ends. The cleavage sites for various restriction endonuclease are shown: H, HindIII; B, BamHI; E, EcoRI; and S, SalI. The enzyme site locations which coincide with those of pColV-K30 are indicated without letter designations.


REP I
AEROBACTIN
REP II
pCol V-K30
-3K30
pColV-292
-F70
-F54
pColV-B188
pColV-H247
-P72
pColV-K229
pColV-K311
-K328
63 Kb
42 Kb
pZM III
NP 101
TP 181
Salmonella
R plasmids
REP I
AEROBACTIN SYSTEM
E. coli
ColV plasmids
Salmonella
R plasmids
Constant Region
Variable Region
0
10
20
30
40

Paper #2:

# ColV Plasmids pColV-B188 and pColV-K30:
# Entire Genetic Maps According to Restriction Enzyme Sites and Landmark Phenotype Characteristics

ABSTRACT

We have obtained the entire genetic maps of the two most studied ColV plasmids, pColV-B188 and pColV-K30, according to restriction enzyme patterns and landmark phenotypic characteristics, such as colicin V, the aerobactin iron uptake system, the transfer region, replication regions, and repeated sequences. These IncFI plmasids are quite large (pColV-B188 is 80 kb and pColV-K30 is 144 kb) and ColV plasmids in general are known to encode a number of virulence properties which have to date neither been studied in detail nor mapped, except for the aerobactin system.

ColV plasmids are IncF1, virulence-related plasmids which have been studied in terms of colicin V production (6,7,8,13,18), the aerobactin iron uptake system (reviewed in 4 and 11), increased serum survival (2,12,22), resistance to phagocytosis (14), adherence to intestinal epithelial cells (3), and, recently, replication (17). There are many questions that remain unanswered in defining the roles that each of these factors might play in contributing to the virulence of bacteria that carry ColV plasmids, although it is known that ColV plasmid carriage is correlated with the invasiveness of such strains (20). Furthermore, it is presently unknown in most cases which of the above phenotypic characteristics are carried by what plasmid, so that the relative virulence potential of each ColV plasmid has not been determined.

We have surveyed a number of prototypic ColV plasmids and found that most of these ColV plasmids do encode for the aerobactin iron uptake system and that this genetic unit is closely linked to the replication region REPI in 100% of the plasmids we studied (24). Plasmids in this category include pColV-K30, pColV-B188, pColV-H247, pColV-P72, pColV-F54, pColV-F70, pColV-292, pColV-K229, pColV-K311 and pColV-K328 ("B" refers to a bovine source of the plasmid-carrying strain, "P" for porcine, "F" for fowl and "H" for human). ColV plasmids which do not encode the aerobactin system include pColV-CA7V and pColV-IK94. It has been our experience that in the recovery of any particularly large plasmid from a clinical (human or animal) isolate, such as an invasive strain of E. coli, the plasmid will commonly resemble in restriction pattern one of the above plasmids, or appear to be the result of DNA insertions into or deletions from one of these plasmids. For example, antibiotic resistance

genes may be inserted, or there may be the absence of common ColV plasmid-associated phenotypes. In fact, we believe that ColV plasmids provide an excellent assembly of replicons to study natural recombinant DNA processes which have survived present-day selective pressures. Such a study could be extended to include all IncF1 virulence-related plasmids, that is, those that may or may not encode for the colicin ColV. The labelling of these plasmids as "ColV" plasmids may then simply be historically the first of many phenotypes that was easily used to unify a large and diverse group of large molecular weight plasmids.

The aerobactin iron-uptake system is widely distributed among enteric bacteria and is encoded both by plasmids and chromosomes (9,15,23,25,28). In the plasmid pColV-K30, the aerobactin operon is surrounded by inverted copies of the insertion sequence IS1, and by two distinct replication regions REPI and REPII (16). In a previous study, we set out to determine if such a DNA environment prevailed in other aerobactin-producing ColV plasmids, to gain insight into the importance of these regions in the preservation and spread of the aerobactin system (24). In the course of such an analysis, we were able to extend the mapping of the DNA which flanked the aerobactin system genes to include the DNA of the entire plasmid. We brought to completion the maps of two of these plasmids, pColV-B188 and pColV-K30, which are presented here. According to their molecular weights and restriction enzyme patterns (24), the plasmids pColV-K30 and pColV3-K30 appear identical, so our map should be applicable to either plasmid.

In our previous investigation of conserved sequences in ColV plasmids, we found that those plasmids which produced aerobactin all shared a ca. 25 kb segment of DNA which contained the replication region REPI and the aerobactin system genes (24). We noticed that these plasmids also shared a cryptic 12 kb HindIII fragment (unpublished observations); this fragment was upstream from the REPI-aerobactin genetic unit but was linked to it via a ca. 22 kb SalI fragment in those plasmids producing aerobactin. The plasmids we investigated ranged in molecular weight from 80 kb to 180 kb. Since the smallest plasmid, pColV-B188, had previously been used to clone the colicin ColV in a 13 kb segment of DNA (6), most of the DNA of this plasmid was thereby charted. We had obtained cosmid clones of the EcoRI fragments of pColV-B188, but we found that mapping was fairly easily accomplished using the intact plasmid pColV-B188 by performing single and double restriction enzyme digests and Southern blot hybridizations (Table 1). Although there are two small molecular weight plasmids of about 3 kb in the strain carrying the 80 kb pColV-B188 plasmid, which had to be kept in mind, the use of the intact plasmid was advantageous because it prevented mismapping as a consequence of using a rearranged cosmid clone.

The plasmid pColV-B188 is defective in transfer (21), and we did not attempt to conjugate it into another strain, but we did find the presence of a region homologous to our "tra" probe (Fig. 1). This probe consists of a self-replicating deletion derivative of plasmid F created by the religation of EcoRI fragments #5 and #3 (19). Since the entire transfer region of F extends over a 30 kb segment of DNA (27), it is clear from

the map that pColV-B188 could not accomodate a transfer region comparable in size to that of F (Fig. 1).

As expected, the radiolabelled colicin V clone pBQ41 (6) detected in pColV-B188 and 4.2 kb EcoRI fragment which encodes the colicin V and colicin V immunity genes (6). Other prototypic ColV plasmids which we have studied have analogous 4.2 kb EcoRI fragments which hybridize to this colicin V probe. We performed similar Southern blot hybridizations with this clone as probe, using HindIII-and HindIII/EcoRI-digested pColV-B188 DNA, and found that this 4.2 kb EcoRI fragment resides within the large 45 kb HindIII fragment of pColV-B188. For this reason, we believe that the original cloning of the colicin V genes via a 13 kb HindIII fragment represents a deletion of this large 45 kb HindIII fragment. These data, as well as the information from previous studies from our lab (16,24), are incorporated into the pColV-B188 map (Fig. 1). The encoding of virulence factors, other than the aerobactin system and intestinal epithelial cell adherence, by the pColV-B188 plasmid has not been explored, to our knowledge, and this plasmid's small molecular weight, relative to other prototypic ColV plasmids, lead us to consider pColV-B188 as a basic (albeit transfer deficient) ColV plasmid.

In the plasmid pColV-K30, the aforementioned 25 kb conserved REPI-aerobactin system unit represents only about 17% of the total plasmid DNA, so we needed more resources in order to map it. Fortunately, we found that there are 5 copies of the insertion sequence IS1 carried by this plasmid (24), although we suspect that in one case (Fig. 1) there may be only a partial copy. We took advantage of this

finding by using our IS1-specific probe (pBRG29, a pBR322 derived plasmid containing the IS1-flanked transposon Tn9) and hybridizing it to pColV-K30 DNA which had been digested by numerous combinations of restriction enzymes (Table 1). Also, we discovered a repeated sequence which we have tentatively called "VRS" and which we similarly used (by cloning, pJHCVW26, and by using it as a probe) to identify overlapping restriction enzyme fragments. As in the plasmid pColV-B188, we localized the colicin V region (within the 4.2 kb EcoRI fragment) and transfer-specific region. We have presumed that, as in the plasmid F, the transfer region genes are contiguous; this may not be the case and awaits confirmation, since our probe (largely traF) did not include all the transfer genes of F. It is also possible that other ColV transfer-specific genes would be nonhomologous with those of F. The functional replication regions REPI and REPII and the maintenance region REPI par were previously mapped in the course of replication studies recently completed in our lab using the plasmid pColV-K30 (16,17). REPI showed high homology to the F plasmid replication region repF1B (in f#7) and REPII showed some homology to the F plasmid replication region repF1A (in f#5), the primary replicon of F. It was also reported that a third replicon, repF1C, functional in IncFII plasmids, showed partial homology with the plasmid pColV3-K30 (1). No functional third replicon has been found for pColV-K30, and the location of this third region showing partial homology with repF1C has not yet been mapped. We have, in light of these different replication regions, distinguished the par region recently discovered in our lab, as REPI par (Fig. 1).

Mapping of a 42 kb segment of pColV-K30 DNA was facilitated by the cosmid clone pJHCP519. This clone carries the EcoRI fragments of 11.0 kb, 5.5 kb, 22.0 kb, and 4.2 kb, inserted into the vector pKY2662 (16). For example, we ascertained the fragments resulting from XhoI-digestion of these EcoRI fragments, and combined this information with the knowledge of those fragments homologous to our IS1- and "VRS"-specific probes. The genetic region spanned by this clone includes the colicin V genes as well as the two repeated sequences, but it also includes a large region devoid of any phenotypic marker (if the tra region is presumed to occupy contiguous DNA). Such a region could be explored for genes encoding plasmid-mediated outer membrane proteins or other proteins perhaps related to virulence. The 12 kb HindIII fragment (of the clone pJHCVW26, Fig. 1), which is highly or absolutely conserved among ColV plasmids, also represents a curiousity, since the region occupied by the "VRS" is probably a minor portion of the total 12 kb.

In comparing the resulting maps of pColV-B188 and pColV-K30, one could easily speculate on the possible natural DNA recombination events that might occur, such as the arrival or departure of the REPII region via IS1-mediated recombination. We suspect that there may be additional repeated sequences on pColV-K30 and on related ColV plasmids which have stimulated recA-dependent recombination events. This type of genetic exchange may be more common in these plasmids than IS1-mediated transposition, since attempts to observe transposition of the aerobactin system operon have been to date unsuccessful (discussed in 24). Indeed, some of these recombination events may be counterproductive, since it has been found for the plasmid pColV2-K94 that a certain inverted repeat can

contribute to mini-plasmid instability which is related to recA-dependent multimerization (26).

It is interesting to note that the region encoding the colicin V genes is surrounded by two distinct sets of repeated sequences (Fig. 1), so it is conceivable that the colicin V genotype is dispensable by means of either of these repeats. The 100% conservation of the 12 kb HindIII fragment (pJHCVW26) among all the ColV plasmids we have studied may mean, however, that there is an essential or favored linkage of this region with the colicin V genes, such that acquisition of the ColV genotype includes this accompanying fragment. The maps presented herein provide a framework for the investigation of these aspects and virulence-related properties of the ColV plasmids.

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TABLE 1

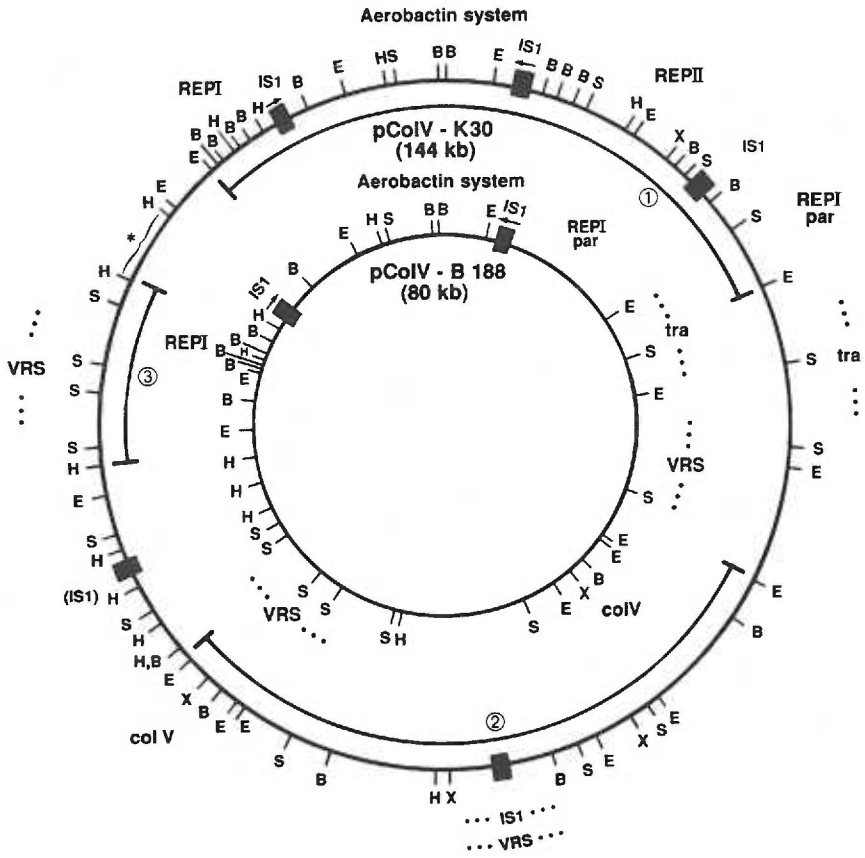
Restriction Enzyme Fragments

(according to size, in kb)

| HindIII | | BamHI | | EcoRI | | SalI | | XhoI | |
|---|---|---|---|---|---|---|---|---|---|
| pColV- | | pColV- | | pColV- | | pColV- | | pColV- | |
| B188 | K30 | B188 | K30 | B188 | K30 | B188 | K30 | B188 | K30 |
| 45 | 61 | 31 | 33 | 27 | 22 | 22 | 23 | 80 | 69 |
| 12 | 17.5 | 30 | 30 | 10 | 20 | 21 | 22 | | 46 |
| 8.6 | 16.5 | 8.6 | 15.5 | 10 | 14 | 10 | 18 | | 17 |
| 6.2 | 12.5 | 4.1 | 13.5 | 10 | 13.5 | 9.5 | 13 | | 12 |
| 2.3 | 8.6 | 2.3 | 8.6 | 8.6 | 13.0 | 8.7 | 10 | | |
| 1.8 | 6.2 | 1.1 | 8.2 | 5.5 | 11.0 | 3.7 | 9.5 | | |
| 1.7 | 5.9 | 1.0 | 7.8 | 4.2 | 10.0 | 3.2 | 8.6 | | |
| | 2.9 | 0.9 | 6.6 | 3.7 | 9.8 | 1.8 | 6.0 | | |
| | 2.35 | 0.5 | 4.1 | 0.8 | 8.6 | 0.8 | 5.7 | | |
| | 2.3 | 0.3 | 4.1 | | 8.6 | | 5.4 | | |
| | 1.8 | | 2.9 | | 5.5 | | 5.0 | | |
| | 1.7 | | 2.3 | | 4.2 | | 5.0 | | |
| | 1.6 | | 1.6 | | 3.7 | | 3.9 | | |
| | 1.2 | | 1.5 | | 1.0 | | 3.7 | | |
| | 0.9 | | 1.1 | | | | 2.5 | | |
| | 0.8 | | 1.0 | | | | 1.8 | | |
| | 0.6 | | 1.0 | | | | | | |
| | | | 0.8 | | | | | | |
| | | | 0.3 | | | | | | |

## LEGEND

Figure 1. Maps of pColV-B188 and pColV-K30, drawn to scale, such that the circumference of the pColV-K30 map is 1.8 times that of pColV-B188. Indicated on the inside border of the pColV-K30 map are the boundaries of the clones critical in mapping: 1) pJHCP1 (16); 2) pJHCP519; and 3) pJHCVW26. The location of copies of the insertion sequence IS1 is shown in cartoon fashion by black boxes which are not representative of the small size of IS1. Where known, the arrows indicate the orientation of the particular copy of IS1. The IS1 copy which we believe may be present only partially (according to the weak Southern hybridization signal) is labelled in parentheses. Dotted lines indicate that a particular phenotype location is approximate, within the limits of available restriction enzyme sites. The asterisk (*) means that there are several (5 or 6) small HindIII fragments in the area indicated by the accompanying bracket.


Aerobactin system
pCoIV - K30
(144 kb)
Aerobactin system
pCoIV - B 188
(80 kb)
REPI
REPII
REPI par
IS1
tra
VRS
colV
col V
(IS1)
H,B
... IS1 ...
... VRS ...

Appendix to paper #2:

# Restriction Mapping of Large Plasmids

ColV plasmid DNA molecules are very large and yield numerous restricion fragments when digested with most restriction enzymes that recognize six base pairs. Therefore, cosmid cloning regions of 35 to 45 kb from these plasmids makes the project much simpler. With the 144 kb plasmid called pColV-K30, this was essential: each portion had to be mapped individually and pieced together to achieve the entire restriction map. With the 80 kb pColV-B188 plasmid, the single aerobactin system-containing cosmid clone was adequate since the plasmid is relatively small (for a ColV plasmid).

The practice of restriction mapping requires the use of single enzyme DNA digestions, double enzyme digestions, and triple or quadruple enzyme digestions. Mapping two restriction enzyme sites with respect to each other entails three sets of digestions: one with each enzyme alone, and one with both enzymes together. For example, if each enzyme cuts a 13.5 kb DNA clone twice, the sizes of each of the resulting fragments should first be determined by gel electrophoresis. The sum of each set of fragments obtained by any enzyme digestion should total 13.5 kb (Fig. 1a). The double digestion with these two enzymes of the same 13.5 kb clone of DNA could yield several possibilities, but the sum of the four resulting fragments should also be 13.5 kb. Two of these possiblities are illustrated (Fig. 1b) and the maps derived for each possiblity are deduced. By continuing this process with different pairs of enzymes, a detailed map can be constructed.

Another method should be applied when the plasmid is large and yields numerous restriction enzyme fragments, that is, the method of

Southern blot hybridization. The probe can be one of these fragments, hybridized to the cloned DNA as digested with several sets of enzymes. Those fragments which overlap with the probe fragment will give a hybridization signal. This procedure is more informative if the probe contains a sequence that is repeated in the clone, so several sets of overlapping fragments would then give a signal, each set specific for the particular location of the repeat. Southern blot hybridization is a very powerful tool and can eliminate many of the doubts that may linger if the map is derived only by the method of performing restriction enzyme digests. In the mapping of pColV-K30, Southern blot hybridization was very useful because there are five copies of the insertion sequence IS1, one copy of which is only a partial copy and therefore gives a weaker signal, and another insertion sequence called here VRS. Each repeat hybridized to a family of restriction enzyme fragments that overlap.

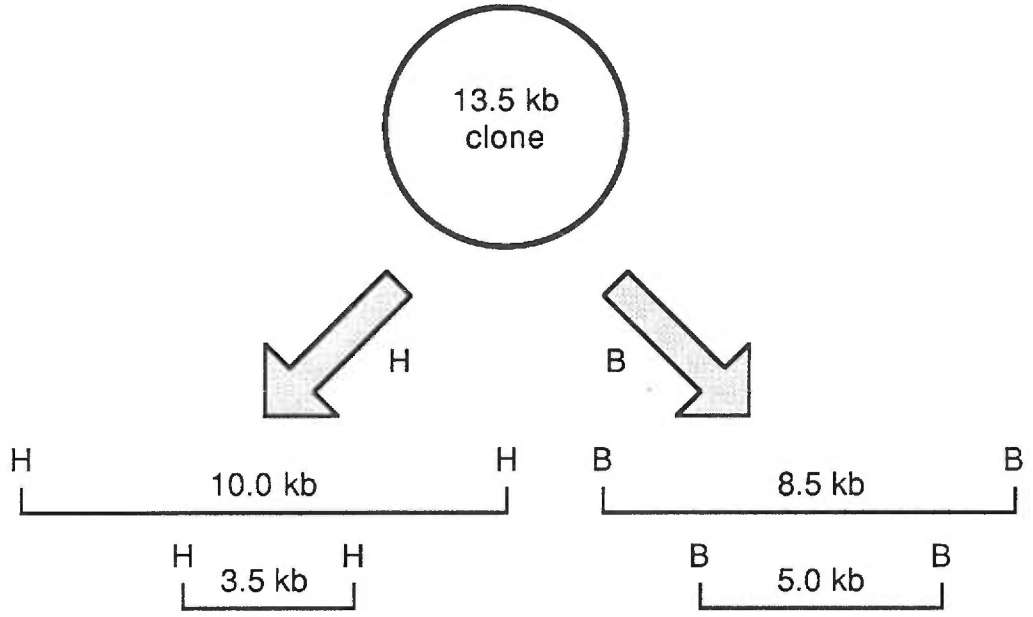
The data obtained from Southern blot hybridizations using as probes sequences specific for important phenotypic markers, and likewise using the repeated sequences, in experiments in which either the intact plasmid or a cloned derivative of the intact plasmid was hybridized to these probes, was thus added to the information obtained by restriction analysis. The maps were then deduced with a degree of certainty that can only await confirmation or correction by DNA sequencing.

Legend

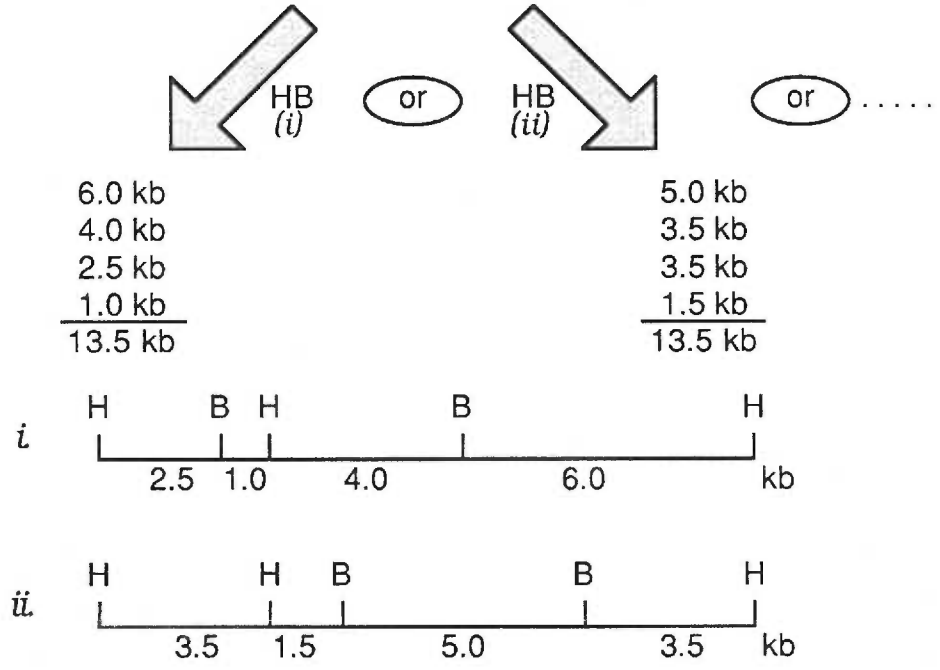
Figure 1a. The digestion of a 13.5 kb DNA clone with two restriction enzymes, H or B, as two single digests.

Figure 1b. The double digestion of the same 13.5 kb DNA clone with both enzymes H and B, given as two possible results that would arise from two possible maps, i. or ii.

1a


13.5 kb
clone
H
B
H
10.0 kb
H
H
3.5 kb
H
B
8.5 kb
B
B
5.0 kb
B

1b


HB
(i)
or
HB
(ii)
or . . . . .
6.0 kb
4.0 kb
2.5 kb
1.0 kb
13.5 kb
5.0 kb
3.5 kb
3.5 kb
1.5 kb
13.5 kb
i
H
B
H
B
H
2.5
1.0
4.0
6.0
kb
ii.
H
H
B
B
H
3.5
1.5
5.0
3.5
kb

Paper #3:

# The Aerobactin Iron Uptake System of *Aerobacter aerogenes* 62-1

# ABSTRACT

Although the aerobactin-mediated iron-uptake system has been characterized genetically in E. coli, the siderophore aerobactin was chemically characterized as purified from culture supernatants of Aerobacter aerogenes 62-1, a member of the Klebsielleae. We have cloned, mapped, and begun characterization of the relevant proteins of this plasmid-mediated aerobactin system of A. aerogenes 62-1. Published chemical data indicate that the siderophore aerobactin of E. coli is the same molecule as the aerobactin of Aerobacter aerogenes 62-1, but we have found that both the genes and the complement of proteins making up the biosynthetic enzymes have diverged in the two systems. In contrast, the outer membrane receptors for ferric aerobactin of the two systems show immunologic cross reactivity, are of the same molecular weight (74 kd) and are encoded by homologous DNA sequences. The fact that the genes encoding the biosynthetic enzymes differ greatly by restriction site analysis, while the essentials of the systems have been preserved, i.e., the siderophore itself and its outer membrane receptor, attests to the importance of this virulence factor across bacterial species.

The significance of iron-uptake proficiency in the growth and virulence of invasive bacteria has been established in several bacterial species (9). Iron-uptake systems typically involve the secretion of an iron-chelating siderophore which competes for iron as bound by host proteins. Aerobactin is such a siderophore and is produced by strains of Enterobacter (19,29), E. coli (33), Shigella (23), Salmonella (19), Yersinia (27) and Klebsiella (21). Due to its chemical and biological character, aerobactin is particularly suited for iron sequestration from blood transferrin (5,20). The biosynthetic genes for aerobactin and for the bacterial outer membrane receptor for ferric-aerobactin have been found both on bacterial plasmids and chromosomes (17,28,33).

The siderophore aerobactin was first discovered in 1969 in culture supernatants of Aerobacter aerogenes 62-1 (13). This hydroxamate siderophore was characterized as purified from A. aerogenes 62-1 supernatants, but, when it was established that ColV plasmid-containing E. coli also secreted the identical aerobactin molecule (31), the genetics of aerobactin synthesis was pursued using E. coli 1315 pColV-K30 (7,10,11,12,14,15). A. aerogenes 62-1 plasmid genes showing some homology to the cloned E. coli aerobactin biosynthetic genes were reported to be residents of analogous 16.3 kb HindIII fragments (19), so the makeup of the aerobactin systems of the two organisms was presumed to be the same. We report here that the aerobactin biosynthetic genes from E. coli and A. aerogenes 62-1 are distinct: they are rather weakly homologous, they reside on 16.3 and 14.0 kb HindIII fragments, respectively, and they encode a different set of functionally analogous biosynthetic enzymes. This is the first report of an aerobactin

biosynthetic system that is genetically distinct from that of pColV-K30. We also report that, on the other hand, the ferric-aerobactin receptor structural genes and proteins appear very closely related in the two systems.

(Results of this investigation were presented in part at the 16th Annual UCLA Symposium on Molecular and Cellular Biology, February 13-19, 1987, Park City, Utah.)

# MATERIALS AND METHODS

Bacteria, plasmids and media. E. coli HB101, E. coli DH5, E. coli BN660, E. coli LG 1315 pColV-K30 and the vector plasmids (pBR322, pVK102, pACYC184 and pACYC177) used to generate cloned derivatives of the plasmids were of the laboratory stock and discussed previously (24,32). The E. coli pColV-K30-derived plasmids pABN1, pABN5, pABN6 and the strain Aerobacter aerogenes 62-1 pSMN1 were obtained from J.B. Neilands (3). Lawn strains used for the bioassay designed to detect the production of aerobactin were E. coli LG1522 containing a mutated version of the pColV-K30 plasmid unable to synthesize aerobactin (33) and E. coli UT2360 (fepArecA) transformed with pABN6 and pACYC177. E. coli C600 F'$ts_{114}$lac::Tn5Tn10 was obtained from K. Richardson. MacConkey, Luria (L) or M9 minimal media were used for growth and supplemented where necessary with additives such as the appropriate antibiotic (18), 400 uM dipyridyl or 10 uM ferric chloride.

Curing of Aerobacter aerogenes 62-1 pSMN1. Curing the strain A. aerogenes 62-1 of its single plasmid pSMN1 was accomplished using novobiocin as previously described (19), with the modification that selection for plasmidless derivatives was by spreading 100 ul of the novobiocin-treated cultures onto Luria agar plates which had been previously spread with 200 ul of a crude preparation of cloacin (24). Single colonies which grew by virtue of their cloacin resistance were tested by colony hybridization against the radiolabelled purified plasmid pSMN1 and by bioassay. Cured and uncured A. aerogenes 62-1 were then submitted to the clinical microbiology lab at The Oregon Health Sciences

University to assay the integrity of the cured derivative according to a panel of biochemical tests.

Cloning and mapping of the Aerobacter aerogenes iron-uptake system. Total DNA, prepared from A. aerogenes 62-1 using the method of Hull (16), was partially digested with HindIII and ligated to completely HindIII-digested cosmid vector pVK102. In vitro packaging and transduction were performed according to a system developed by Amersham Corp., Boston, MA and perspective clones were screened by bioassay and confirmed by cloacin sensitivity as prevously described (2,24,33). Subcloning of a deleted version of an original cosmid clone into pBR322 and pACYC184 generated smaller DNA derivatives which facilitated mapping by conventional techniques.

Southern- and immuno-blotting. Southern blot hybridizatin experiments were performed as described previously (24). For immunoblotting, outer membranes were prepared from cultures of bacteria grown in iron-rich or iron-poor minimal media (8). After SDS polyacrylamide gel electrophoresis, the samples were transferred to nitrocellulose paper and reacted with partially purified antibody (1) raised against the 74 kd aerobactin outer membrane receptor of E. coli 1315 pColV-K30. Staining was according to the protein A-peroxidase method (1).

Transposon Tn5 mutagenesis. E. coli C600 f'$ts_{114}$lac::Tn5Tn10 was transformed at 30°C with pBR322-derived clones with ampicillin selection. Transformants were subbed onto plates containing both ampicillin and kanamycin and incubated at room temperature. Luria broth cultures

containing ampicillin and kanamycin were then inoculated and incubated at 42°C overnight followed by two serial passages into the same media by diluting a culture 1:250 and maintaining the 42°C incubation temperature. Diluted aliquots of each final culture were plated onto MacConkey plates containing ampicillin and kanamycin to note the lactose phenotype. Plasmid DNA was then prepared in order to transform E. coli HB101, selecting on Luria plates containing ampicillin and kanamycin. Small-scale plasmid DNA preparations were made from the resulting transformants to position each Tn5 insertion by mapping.

Identification of plasmid-encoded proteins. In vitro DNA-directed protein synthesis was accomplished according to a system developed by Amersham Corp., Boston, MA. Maxicells were prepared as described (25,26) by irradiating BN660 cultures which contained high copy cloned derivatives of the original A. aerogenes iron-uptake system cosmid clone. Proteins radiolabelled by either method were visualized by processing by 12.5% SDS polyacrylamide gel electrophoresis, drying the gel under heat and vacuum, and exposing it at 70°C with Kodak XRP or XAR film.

# RESULTS

Identification of the plasmid-encoded genes for the aerobactin iron-uptake system in Aerobacter aerogenes 62-1. In the course of studying the genes and the genetic environment of plasmid-mediated aerobactin iron-uptake systems in E. coli carrying various ColV plasmids (32), we also probed the DNA of the plasmid pSMN1 (which is not a ColV plasmid, unpublished results) prepared from the strain A. aerogenes 62-1 with segments of the aerobactin-specific region from the E. coli plasmid pColV-K30. We could not find analogous internal restriction enzyme patterns when comparing the E. coli and A. aerogenes aerobactin-specific regions. This disparity contrasted with the biological activity of the two systems: both the E. coli strains carrying ColV- and aerobactin-encoding plamsids and the A. aerogenes 62-1 strain produced strong halos of growth when spot-inoculated onto a lawn strain of E. coli 1522, indicative of ferric-aerobactin cross-feeding. On the basis of field desorption mass spectroscopy, the aerobactin molecules produced by cultures of E. coli 1315 pColV-K30 and A. aerogenes 62-1 are identical (31). Since the biosynthetic pathway derived for the E. coli system aerobactin involves a series of reactions using four distinct enzymes, we considered the possibility that a divergent set of enzymes could make up the A. aerogenes system. Alternatively, a chromosomal location for the aerobactin system genes of A. aerogenes 62-1 had not been ruled out. To allow for either plasmid- or chromosome-directed synthesis of aerobactin system enzymes, we prepared total DNA from a culture of A. aerogenes 62-1. A cosmid clone bank was generated by in vitro packaging of this DNA which had been partially digested with HindIII and ligated to cosmid

vector pVK102 (32). Initial bioassay screening of 200 cosmid clones yielded three aerobactin-producers which all shared a 14 kb HindIII fragment. Southern blot hybridization experiments using this 14 kb HindIII fragment as probe established that this 14 kb fragment resided within the large, 200 kb plasmid of A. aerogenes 62-1 called pSMN1. (Data not shown.) A deletion derivative of one of these cosmid clones (pJHCVW1738) was subcloned into pBR322. This clone of the 14 kb HindIII fragment, pJHCVW17 (Fig. 1), contains the entire aerobactin system genetic region, including the genes encoding the enzymes required for aerobactin production and the structural gene for the outer membrane receptor for ferric aerobactin. In light of our previous paper (32) which investigated the genetic environment of aerobactin system genes in various ColV plasmids, it is important to note here that this 14 kb HindIII fragment containing the A. aerogenes aerobactin genes does not possess a complete copy of the insertion sequence IS1, nor is it flanked by the replication region REPI which characterizes the IncFI ColV plasmids (data not shown). A smaller clone, pJHCVW100, generated by EcoRV deletion (Fig. 1) retained biological activity and was used extensively for mapping and as the parent clone for Tn5 mutagenesis.

In regard to the bioassay reaction as indicator of aerobactin production, it should be mentioned that we found that the native strain A. aerogenes 62-1 pSMN1 consistently gave a more intense halo of growth to the lawn E. coli indicator strains than did the cloned derivatives. (Both the native strain and the host backgrounds of clones produced enterobactin, so the difference could not be attributed to nonspecific crossfeeding by the native strain.) We attemped to solve this problem

two ways: by constructing a bioassay indicator strain that was ampicillin resistant (UT2360 pABN6) and by returning the clones of pSMN1 to the A. aerogenes background using a cured derivative. The first method, using UT2360 pABN6 (a strain carrying a pBR322 clone of the structural gene for the ferric-aerobactin receptor, within the E. coli pColv-K30 6.6 kb BamHI fragment, Fig. 2) and using ampicillin plates, did result in stronger bioassay reactions, by maintaining the somewhat unstable clones in the E. coli background. The second method, using the cured derivative of A. aerogenes 62-1 as the background, was complicated by the strain's inherent resistance to ampicillin (at concentrations below 2 mg/ml) and to tetracycline (at concentrations below 75 ug/ml) but increasing the antibiotic concentrations to select for the cured strains containing clones allowed us to perform the bioassay. In the cured derivative carrying pBR322 clones, bioassay reactivity also regained intensity, but frequently reverted to negative, presumably because the plasmid clone carrying the aerobactin system genes was lost from cells as they spontaneously generated mutants able to grow in the presence of very high antibiotic concentrations.

In the course of these experiments, we wanted to confirm the integrity of the novobiocin-treated cured strain, so we had it and its parent strain Aerobacter aerogenes 62-1 pSMN1, which was originally obtained from Gibson and Magrath, respeciated in the clinical lab at the Oregon Health Sciences University. We do not believe that the species of this organism has been checked since the 1960s when the siderophore aerobactin was first described. (It has more recently been referred to as Enterobacter aerogenes 62-1, as well as the prevailing but outdated

Aerobacter aerogenes 62-1.). The result of this overdue respeciation was that the organism gave excellent identification as a Klebsiella ozaenae, a species recognized as of 1980. We are presently confirming this result with further taxonomic tests. From these data we will propose that Aerobacter aerogenes 62-1 be referred to as Klebsiella ozaenae 62-1 (V.L. Waters, J.W. Rourke and J.H. Crosa, manuscript in preparation).

Genetic relatedness of the aerobactin systems of A. aerogenes 62-1. The aerobactin iron-uptake system of A. aerogenes genetically distinguished itself from that of E. coli by Southern blot hybridization analysis and by restriction enzyme pattern comparison. The degree of divergence as seen by the variation in restriction enzyme sites (Fig. 2) is reflected in the degree of homology as seen by probing both systems with an internal region of the E. coli system genes (Fig. 3 and 4). After several Southern blot hybridization experiments, it became apparent that the genetic regions encoding the outer membrane receptor protein for ferric aerobactin for the A. aerogenes 62-1 and E. coli systems shared the most homology. Confirming our initial observations, the genetic regions which are specific for the synthesis of the siderophore aerobactin are clearly divergent in the two systems, both in terms of the restriction maps and according to the strength of the signals obtained in Southern blot hybridizations, using agarose gels loaded with comparable DNA concentrations from the two genetic systems, and using a largely siderophore-specific probe (pABN5's 7 kb HindIII-EcoRI fragment, reference 3 and Fig. 2). For example, BamHI-digestion of the E. coli aerobactin system clone pABN1 (3) yields two strongly hybridizing fragments of 9.4 and 6.6 kb with this probe, while the A. aerogenes

analogous clone pJHCVW17 yields the relatively weakly hybridizing 13.5 kb BamHI fragment (Figs. 3 and 4). The conserved 1.95 kb BglII fragment which shows comparable hybridization signals closely approximates the structural genes for the outer membrane receptor proteins of the two systems (Figs. 2 and 4). This result was confirmed using probes inclusive of the entire receptor gene from pColV-K30 (data not shown).

Immunological cross-reactivity of the ferric aerobactin receptor proteins. Sarkosyl-insoluable outer membrane preparations of the native strains E. coli 1315 pColV-K30 and A. aerogenes 62-1 pSMN1 and of the original low copy cosmid clone HB101 pJHCVW1738 (Fig. 1), grown in comparable M9 cultures of iron-rich and iron-poor media, were run out on an SDS-PAGE gel. The samples were then transferred to nitrocellulose paper and reacted with partially purified antibody raised against the E. coli system's 74 kd outer membrane receptor protein. The low iron-inducible 74 kd protein expressed in all of these cultures appears to be of identical molecular weight and is immunologically cross-reactive (Fig. 5). The intensity of the antibody reaction in the low iron-induced culture of A. aerogenes 62-1 pSMN1, as compared to the control reaction with E. coli 1315 pColV-K30, is analogous to the degree of sensitivity that A. aerogenes 62-1 exhibits toward the colicin cloacin, which uses the same ferric aerobactin 74 kd receptor to access the cell (2). Quantitative cloacin sensitivity assays recently performed in our lab demonstrated that A. aerogenes 62-1 pSMN1 is 250 times more sensitive to cloacin than the prototypic strain E. coli 1315 pColV-K30 (C. Marolda, personal communication). The cloned version of the A. aerogenes receptor protein renders HB101 sensitive to cloacin but less so than the parent

strain, a result that is consistent with the different degrees of antibody reaction (Fig. 5). We suggest from these results that the increased antibody reactivity and cloacin sensitivity in A. aerogenes is probably due to an increase in the number of receptor molecules inserted into the outer membrane.

Cis and Trans Complementation. In the E. coli biosynthetic pathway for aerobactin, a molecule of lysine is first hydroxylated to form $N^6$-hydroxylysine. Then an acetyl group is added to form $N^6$-acetyl $N^6$-hydroxylysine, two molecules of which are condensed with a molecule of citrate to create the final product aerobactin (10,11). The gene order in the operon is given: iucA, iucB, iucC and iucD; however, the order in which their gene product enzymes are used in the synthesis of aerobactin from lysine is: iucD, iucB, iucC and iucA, such that the iucD product is the hydroxylase, the iucB product is the acetylase, and the iucA and iucC products are believed to be subunits of the synthetase (20). The biosynthetic pathway for A. aerogenes 62-1 is also believed to involve the hydroxylation and subsequent acetylation of lysine followed by condensation of two moles of the product with citrate (30). One approach to the analysis of the novel genetic system for A. aerogenes aerobactin biosynthetic pathway is to ask if the genes for the two systems are collinear. Divergence of DNA sequence and of the encoded enzymes could still permit analogous chemical functions by a series of enzymes that are collinear as well. To address the questions of collinearity of genotype and duplication of chemical function, we constructed clones designed to complement in cis or in trans in the process of aerobactin synthesis. The clones used and the complementation results are shown in Fig. 6. The

bioassays obtained using the "hybrid-clone" pJHCVW160 and using the coexisting and compatible clones pJHCVW150 and pJHCVW90 were unequivocally positive for aerobactin production. In fact, there may be an enhancing effect in the case of the *trans* complementation possibly due to the double gene dosage of the acetylase (and of the *iucA* product), because the bioassay halo appeared more intense than that of the native clone control. This observation, as well as the positive results obtained in these *cis* and *trans* complementations, argue that three enzymes (the hydroxylase, the acetylase, and one or both of the putative subunits of the synthetase described for *E. coli*) in each system are: 1) analogous in function, and 2) collinear. The *cis* clone actually predicts that a hybrid protein should be made, but these data cannot resolve between two possibilities: 1) that this potential hybrid molecule is functionally analagous to the *E. coli* synthetase "subunit" or, 2) that it is unessential when the hydroxylation is accomplished via the *A. aerogenes* 62-1 hydroxylase-equivalent. Another interpretation would be that the *A. aerogenes* equivalent of the hydroxylase has a dual enzymatic capacity as hydroxylase and synthetase, which seems unlikely. However, we can conclude that the *A. aerogenes* 62-1 hydroxylase-equivalent can serve the function of the 53 kd hydroxylase in the *E. coli* background and that the function of the *iucC* product (synthetase "subunit B") can be duplicated by one or both of the 62.5 kd proteins of *A. aerogenes* 62-1 (Fig.2).

*Identification of proteins and transposon Tn5 mutagenesis*. Analysis of the cloned derivatives of the *E. coli* and *A. aerogenes* aerobactin system by maxicell and *in vitro* DNA-directed protein synthesis experiments

corroborate the production of the 74 kd outer membrane receptor protein in each system. The two receptor proteins appear identical in molecular weight as well as show immunological cross reactivity (discussed above) and are encoded by homologous DNA of similar or identical restriction enzyme pattern (Fig. 2). Among the proteins involved in the biosynthetic pathways for the siderophore aerobactin, only the 33 kd protein, identified in E. coli as the acetylase, comigrates in SDS-PAGE gels in which clones from both systems are compared (Fig. 7). In the E coli system, the first enzyme in the biosynthetic pathway is the hydroxylase which has been shown to be 53 kd (11,15). We also see a protein of that size for the E. coli system but see instead for analogous clones of the A. aerogenes system a protein of about 60 kd (Fig. 7, and data not shown). We also observe the synthetase proteins in the E. coli system, identified as 62 and 63 kd, but see for the A. aerogenes system a band of apparently 62.5 kd. This may be a double band since in a smaller deleted clone a band of similar size is apparent. The analogous smaller clone from E. coli shows one of the two synthetase proteins (Fig. 7). Our data are summarized in the restriction enzyme pattern maps of Figure 2, which indicates the genetic location of the A. aerogenes proteins which have molecular weights of 62.5 kd, 33 kd and 60 kd.

We obtained Tn5 transposon mutants that mapped either in the 74 kd outer membrane receptor protein structural gene or in the regions we believe encode the "hydroxylase equivalent" 60 kd protein or either of the 62.5 kd "synthetase equivalents" (Fig. 2). The expected phenotypic changes occurred with these mutants, i.e., there was a loss of cloacin sensitivity but retention of bioassay activity with the insert in the 74

kd gene and a loss of bioassay activity with the others. (According to map positions, Fig. 2, and data not shown.) The inserts in the region of the aerobactin biosynthetic genes in general gave slight polar effects in terms of expression of the 74 kd gene as judged by variable decreases in, without abolition of, cloacin sensitivity.

# DISCUSSION

This work represents the initial characterization of the iron-uptake system in the organism from which the siderophore aerobactin was first discovered, Aerobacter aerogenes 62-1. The remarkable feature of this system is that it is genetically distinct from the systems in E. coli that are producing the identical molecule aerobactin either by chromosomal or plasmid determinants. Genetic diversity is not unusual in procaryotic organisms but this is the first report of divergence in the coding region for the aerobactin iron-uptake system. We believe that the divergence seen in the aerobactin system genes is somewhat unique because the divergence occurs over a range of proteins which make up the enzymatic steps in the biosynthetic pathway of aerobactin. Thus, the diversity seen in the two operons encoding mercuric ion resistance in Tn21 and in Tn501, in the family of chloramphenicol acetyl transferases, and in the ADP-ribosylating toxins from Pseudomonas aeruginosa and from Corynebacterium diphtheriae, all represent examples of diversity in single structural genes that allow the preservation of protein function.

The evolution of genes/proteins naturally proceeds and is reflected in the separation of the species. The alternative point of view would assert that the more remarkable finding is the conservation of sequences encoding aerobactin in the ColV plasmids and in certain Salmonella R plasmids and in the chromosomal genes of E. coli and Shigella. In the case of the ColV and Salmonella R plasmids, we found an absolute correlation in the presence of these conserved sequences and their linkage to the IncF1-specific replication region REPI (32). The fact

that the pSMN1 plasmid is not of the IncFI incompatibility group (unpublished results) may therefore strengthen speculation that such a linkage acts to preserve the integrity of the replication region-linked sequences. Also, the point of distinction between the two aerobactin genetic systems is at the level of the tribe, not at the level of the species within one tribe. That is, the Escherichieae tribe, containing E. coli and the Shigella species, and the Salmonellase tribe, are distinguished from the Klebsielleae tribe in this discussion of plasmid and chromosomal aerobactin systems.

There is another form of divergence seen when comparing the iron-uptake system of E. coli and A. aerogenes 62-1. Since the cloned derivatives of the A. aerogenes iron-uptake system exhibited a relatively decreased bioassay reaction, whereas the E. coli clones peformed equally as well as the parent strain, we believe that the two systems have distinct regulators that respond to low iron stress. We have sequenced the promoter/operator for the A. aerogenes system (unpublished results) and found distinctive differences when this region is compared to the analogous sequenced region of E. coli pColV-K30 (4,12). Also, the lack of complete polarity seen in the Tn5 inserts may mean that the receptor protein gene has its own promoter. This would further distinguish the two systems, as it is believed that in E. coli there is a single transcriptional unit for the entire aerobactin system (4). The extreme sensitivity to cloacin exhibited by A. aerogenes 62-1 may indicate differences in the mechanisms of insertion or in the surrounding architecture of the ferric aerobactin receptor protein in the A. aerogenes outer membrane, because the respective receptor proteins from

E. coli and A. aerogenes appear identical by the criteria described herein.

Gibson and Magrath and subsequent investigators found that the addition of iron repressed A. aerogenes 62-1 aerobactin production and that the organism displayed the relationship of iron content to siderophore formation that is typical of many other organisms (13,20,30). The differences in regulation between the E. coli and A. aerogenes systems may therefore be that the various E. coli regulatory factors cannot substitute for the A. aerogenes factors when the A. aerogenes genes are cloned. Viswanatha et al. have found that the first two enzymes, the hydroxylase and the acetylase, are active in association with the inner membrane of A. aerogenes 62-1 and that the synthetase operates in the periplasmic space (30). The constraint in the cloned system may therefore be related to the functionality of these enzymes in the context of a foreign membrane. Likewise the different biochemical environment of the membrane may account for a different level of insertion of the 74 kd outer membrane receptor when the cloned A. aerogenes receptor is expressed in E. coli.

Continuation of the sequencing of the A. aerogenes aerobactin operon may bring out interesting comparisons with the operon of E. coli. That is, from a comparative study one could surmise that the more conserved regions of the divergent enzymes are critical in their enzymatic action. In this regard the most interesting enzyme may be the lysine: $N^6$-hydroxylase. Viswanatha et al., have found that the hydroxylase activity is affected by the concentration of glutamine and it is

postulated that there may be a level of control of lysine hydroxylation by a secondary site on the hydroxylase, i.e., one site for lysine and one site for glutamine (30). There are alternative explanations for such a glutamine effect and comparing the two systems genetically could give insights into where the active sites are and the probability of such auxillary sites.

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Figure 1. Cloning and subcloning strategy for the aerobactin iron uptake system of A. aerogenes 62-1 pSMN1, as described in the text.

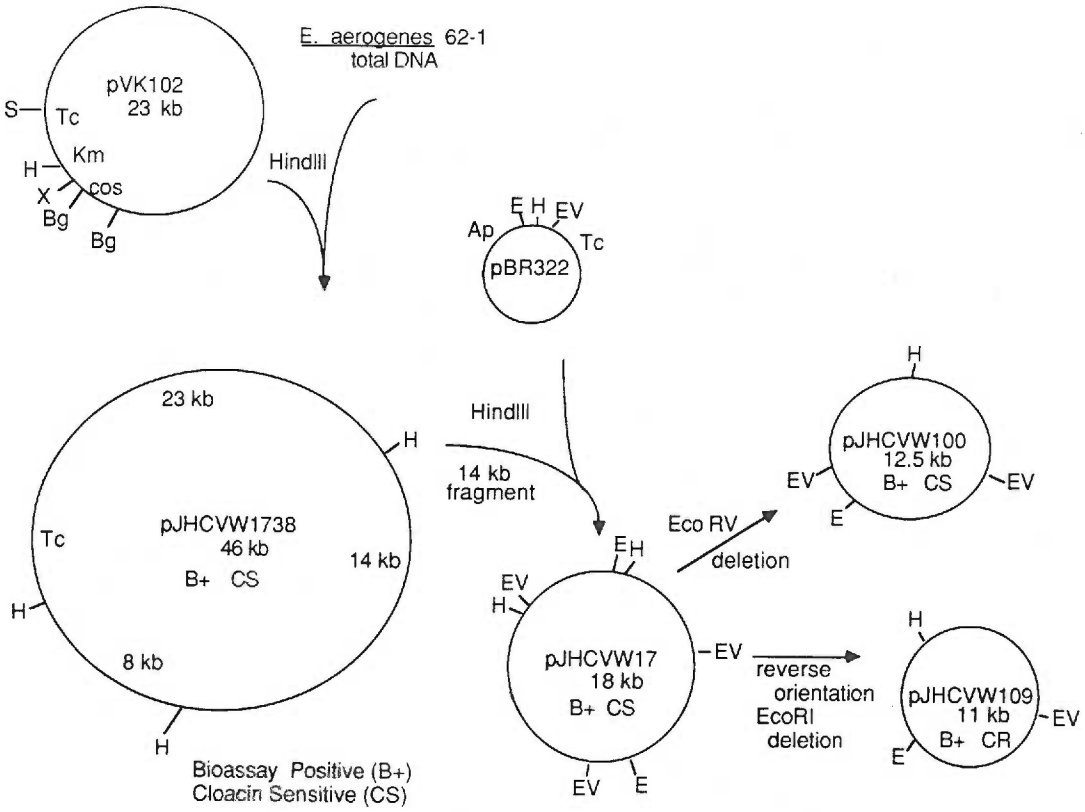
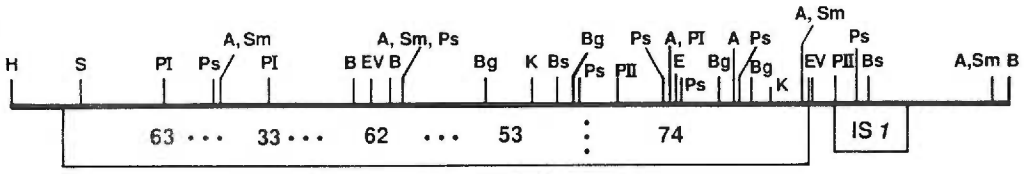

E. aerogenes 62-1
total DNA
pVK102
23 kb
S
Tc
Km
H
X
cos
Bg
Bg
HindIII
E H EV
Ap
Tc
pBR322
23 kb
H
Tc
pJHCVW1738
46 kb
B+ CS
14 kb
H
8 kb
H
Bioassay Positive (B+)
Cloacin Sensitive (CS)
HindIII
14 kb
fragment
E H
EV
H
pJHCVW17
18 kb
B+ CS
EV
EV
E
Eco RV
deletion
H
pJHCVW100
12.5 kb
B+ CS
EV
EV
E
reverse
orientation
EcoRI
deletion
H
pJHCVW109
11 kb
B+ CR
EV
E

Figure 2. Restriction enzyme map derived from the aerobactin system clones of E. coli 1315 pColV-K30 and A. aerogenes 62-1 pSMN1. The numbers beneath the maps indicate the size of the putative encoded proteins, in kilodaltons (kd). The asteriks (*) refers to the putative synthetase subunit molecular weight which we have obtained from data such as in Fig. 7, panel a, lanes C and E. Restriction enzyme sites in the 74 kd protein of E. coli 1315 pColV-K30 were obtained from the published sequence, as well as by mapping. Enzyme abbreviations: H, HindIII, S, SalI, PI, PvuI, PII, PvuII, A, AvaI, Ps, PstI, Sm, SmaI, B, BamHI, EV, EcoRV, Bg, BglII, K, KpnI, Bs, BstEII, E, EcoRI.

## pColV-K30 Aerobactin Region:




## pSMN1 Aerobactin Region:

(no internal sites for Bam HI or Sal I)

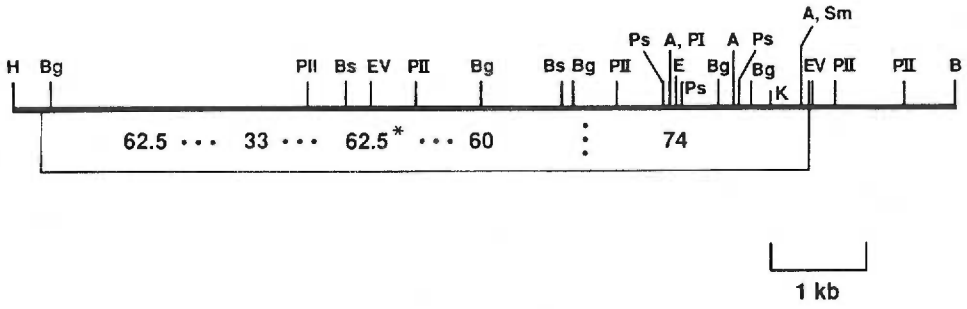


Figure 3. Restriction enzyme digested and electrophoresed DNA prepared from analogous clones of the aerobactin iron-uptake system of E. coli pColV-K30 and A. aerogenes pSMN1. Panel a. Lanes A, C, E, G, I, K, M, O, Q, pABN1, the E. coli system clone of the 16.3 kb HindIII fragment (described in the text). Lanes B, D, F, H, J, L, N, P, R, pJHCVW17 (in Fig. 1), the A. aerogenes system clone of the 14.0 kb HindIII fragment. Lanes A and B, BamHI digest; lanes C and D, BamHI and PstI; lanes E and F, PstI; lanes G and H, PstI and HindIII; lanes I and J, HindIII; lanes K and L, HindIII and BamHI; lanes M and N, HindIII, BamHI and EcoRV; lanes O and P, HindIII and EcoRV; lanes Q and R, EcoRV. Panel b. Lanes A, C, E, G, pABN1; lanes B, D, F, H, pJHCVW6, a pBR322 clone of the 10 kb HindIII to BamHI fragment of A. aerogenes 62-1 pSMN1 (Fig. 2). Lanes A and B, HindIII, BamHI, EcoRV, and a BglII; lanes C and D, BamHI and BglII, lanes E and F, HindIII, BamHI and BglII; lanes G and H, HindIII and BglII. Lane S indicates a molecular weight standard, lambda HindIII.

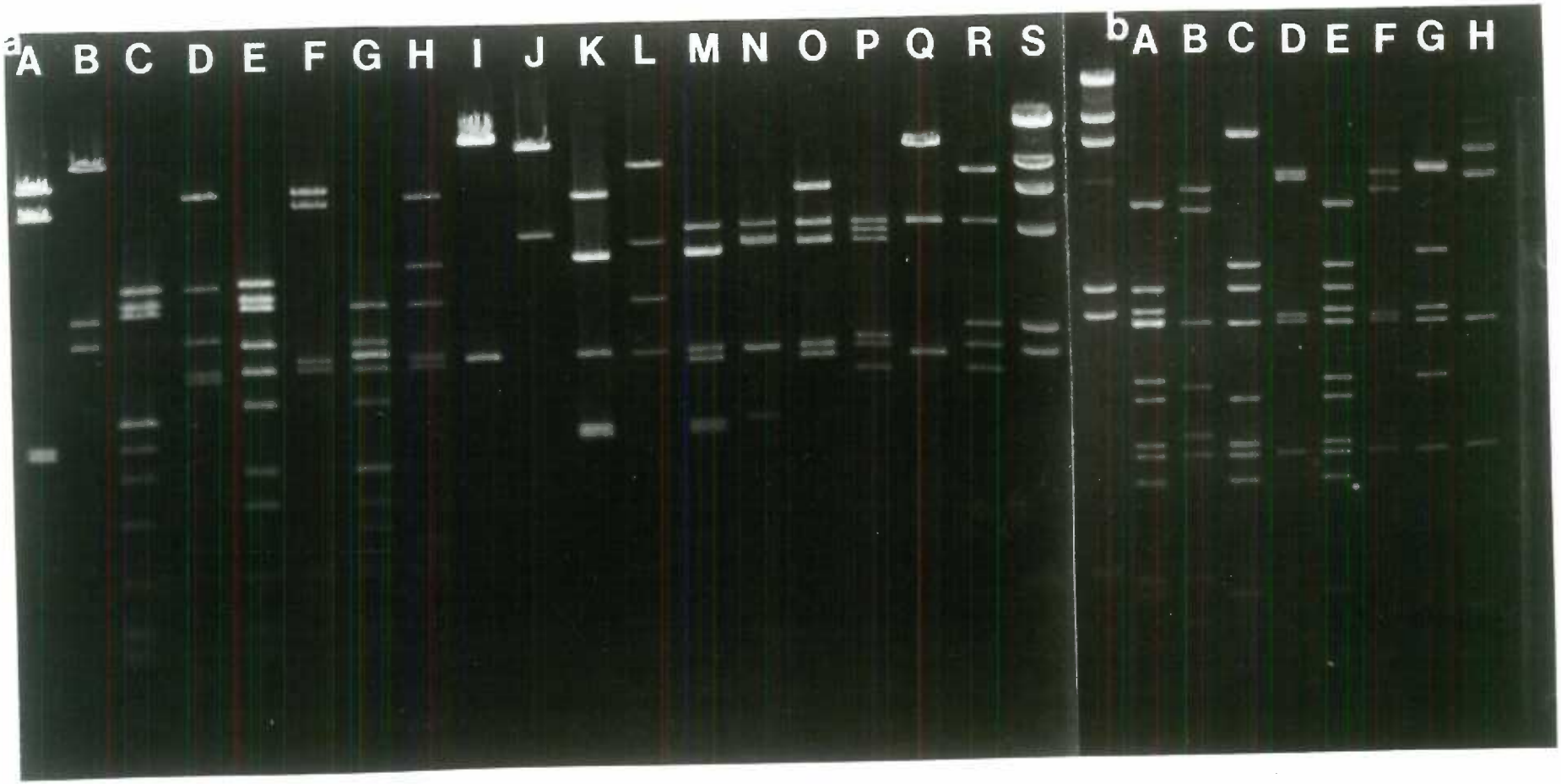

a
A B C D E F G H I J K L M N O P Q R S
b
A B C D E F G H

Figure 4. Southern blot hybridization of the restriction enzyme digested and electrophoresed DNA described in Figure 3. The probe was the radiolablled 7 kb HindIII to EcoRI fragment from the E. coli aerobactin operon, discernable from Figure 2. Panels and lanes are labelled as in Figure 3.


a A B C D E F G H I J K L M N O P Q R
D A B C D E F G H

Figure 5. Anti-74 kd protein immunoblot of SDS-PAGE outer membranes from cultures of E. coli pColV-K30, lanes A and B, and A. aerogenes 62-1 pSMN1, lanes C and D, and the original low copy clone of the aerobactin system of A. aerogenes, HB101 pJHCVW1738, lanes E and F, grown in iron-rich (10 uM FeC13), lanes B, D, and F, and iron poor (no added iron), lanes A, C, and E, minimal media. The arrow indicates the 74 kd proteins. The smaller molecular weight bands in lane C are presumably degradation products.

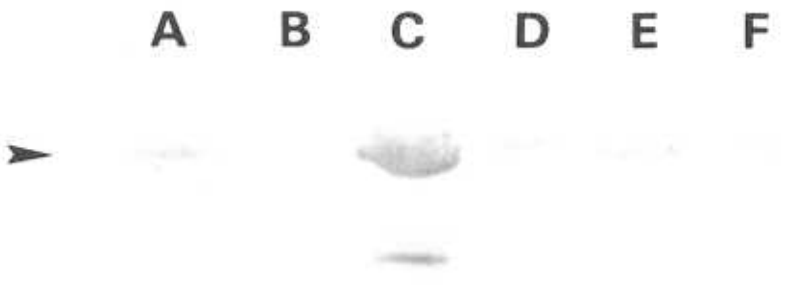
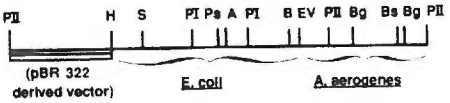

A
B
C
D
E
F

Figure 6. Cis- and Trans-acting clones, from the E. coli and A. aerogenes systems, used to genetically complement the components of the biosynthetic pathway required for aerobactin production, as assayed by bioassay.

## Cis-acting Clones

Aerobactin production

### pJHCVW160:

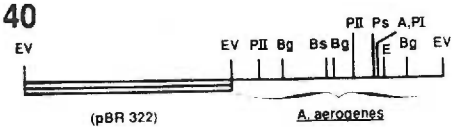



+

### pJHCVW50:


-

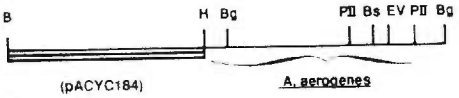
### pJHCVW140




-

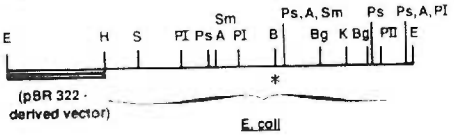
## Trans-acting Clones

### pJHCVW90:




-

### pJHCVW150:

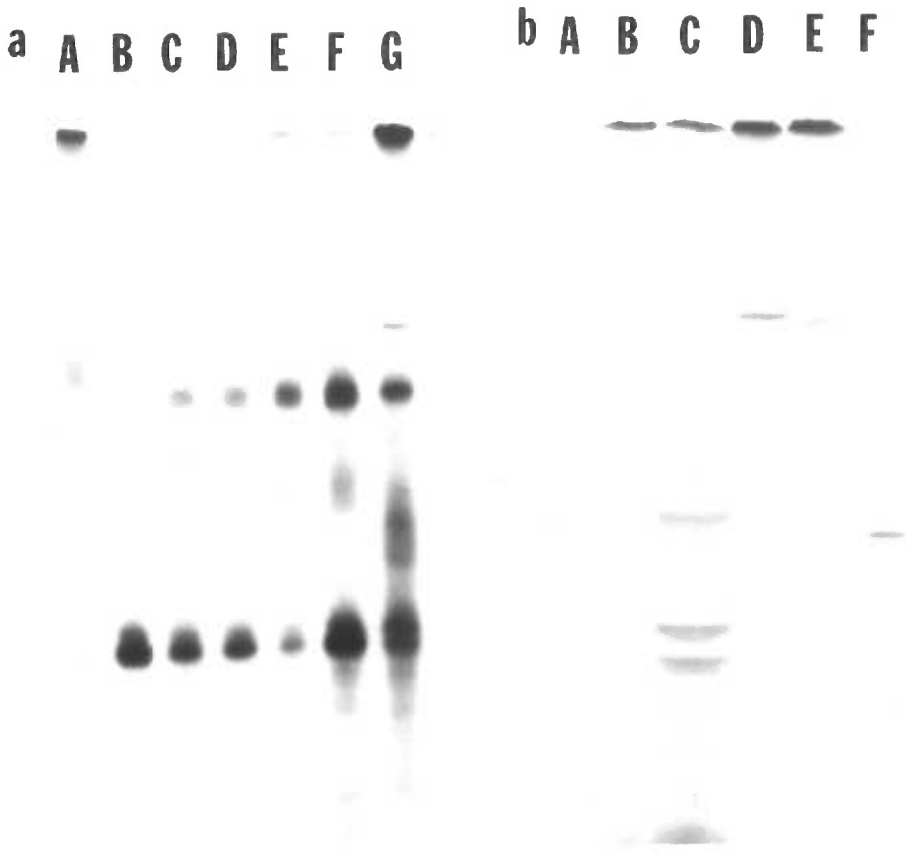



-

Both of the above clones in the same cell

+

Figure 7. Autoradiograph of SDS-PAGE proteins synthesized from clones (panel a) and from DNA fragments (panel b) using an *in vitro* transcription/translation system described in Materials and Methods. Panel a. Lane A, $C^{14}$ molecular weight standards of 94 kd, 68 kd, and 33 kd and 12 kd; Lanes B through G, clones of increasing size originating from the HindIII site of the *A. aerogenes* system: lane B, pBR322 vector; lane C, cloned insert of 3.1 kb from HindIII to PvuII; lane D, cloned 3.8 kb insert from HindIII to EcoRV; lane E, cloned 7 kb insert from HindIII to EcoRI; lane F, cloned 8.5 kb insert from HindIII to an EcoRV site, and Lane G, cloned 10 kb insert from HindIII to BamHI sites. Panel b. Lane A, molecular weight standards as in panel a; Lanes B and C, *E. coli* aerobactin system fragments; lanes D and E, *A. aerogenes* system fragments; lanes B and E, the analogous 3.8 kb HindIII to EcoRV fragments (see Fig. 2); and lanes C and D, the analogous 7.0 kb HindIII to EcoRI fragments. The smaller bands seen below the 33 kd MW standard in lanes C and D are probably truncated products of the 74 kd ferric-aerobactin receptor, since the 7 kb HindIII to EcoRI fragment insert cuts the receptor gene to slightly less than half the native gene.


a A B C D E F G
b A B C D E F

Appendix to paper #3:

# Sequence Analysis of the Promoter Region of the Aerobactin System of *Klebsiella ozaenae* 62-1 (formerly *Aerobacter aerogenes* 62-1)

## ABSTRACT

We have obtained the sequence of the promoter region of the aerobactin system of Klebsiella ozaenae 62-1 (formerly Aerobacter aerogenes 62-1), the organism from which the siderophore aerobactin was discovered in 1969. This sequence is divergent from the analogous region of the E. coli pColV-K30 aerobactin system (the coding regions for the two systems have also been found to be quite divergent). We have compared this sequence to the Fur protein consensus sequence, the specific Fur protein DNaseI-protected region in pColV-K30, and the putative Fur protein binding regions from other promoter regions known to be iron regulated, such as the region for the Shiga-like toxin, for fepA, for fhuA, for fur. We also describe the conservation of an upstream open reading frame which is held in common among the E. coli and Klebsiella ozaenae 62-1 aerobactin systems.

It has become accepted that the invasiveness of enteric bacteria is correlated with the ability to sequester iron from host iron binding proteins such as transferrin and lactoferrin (3). Biologically and chemically, the siderophore is best suited for this role (2,16). The aerobactin iron uptake system has been genetically characterized from clones obtained from the E.coli plasmid pColV-K30, although the siderophore itself was originally discovered in 1969 using the strain Aerobacter aerogenes 62-1 (7). Although this organism has recently been referred to Enterobacter aerogenes 62-1, we have performed extensive biochemical tests indicating that the organism gives excellent identification as a Klebsiella ozaenae, a species recognized as of 1980 (13). We found that the aerobactin system is plasmid-mediated in Klebsiella ozaenae 62-1, encoded by the large 200 kb plasmid called pSMN1 (14). Also, we have recently cloned the aerobactin iron uptake system from this organism, and found that, by restriction analysis, Southern blot hybridization and in vitro protein synthesis, the two aerobactin systems have diverged considerably (14). (Published chemical data show that the two systems produce the identical aerobactin molecule [12]).

To continue the comparison of these two divergent systems, we have begun sequence analysis of the Klebsiella ozaenae 62-1 system, starting at the 5' end of the cloned region that has been shown to contain the entire aerobactin operon. We inferred from an experiment and by comparison with the analogous region of the E. coli pColV-K30 system that the promoter region should be contained within a 400 bp HindIII to BglII fragment. The experiment was by means of an in vitro procaryotic

DNA-directed translation system (Amersham, Corp., Boston, Mass.) in which we used the purified, electroeluted DNA fragment, rather than a clone with accompanying vector and the vector's promoter(s). We used a 7 kb fragment which encoded all the necessary enzymes required to synthesize aerobactin, as determined by a cross-feeding bioassay using cloned versions of this fragment (14). In vitro protein synthesis using this fragment corroborated in vitro protein synthesis using entire clones, so we could conclude that the promoter was included within this 7 kb fragment (14).

We also considered that the first 400 bp, that is, the HindIII-BglII fragment, appeared to be analogous to the promoter region of pColV-K30, which has been S1-mapped and sequenced (1). It is thought that in the E. coli system, transcription results in the synthesis of one large message (1). Although the two aerobactin systems have diverged in terms of the restriction patterns, DNA homology and the complement of proteins making up the enzymes of the biosynthetic pathway, we found that the two systems are collinear and that the enzymes are functionally analogous because we were able to genetically complement both in cis and in trans, using clones that were derived from the two systems (14). For these reasons, we expected the promoters to map in approximately the same genetic location, the E. coli system promoter contained within a 680 bp HindIII to SalI fragment and the Klebsiella ozaenae 62-1 within a 400 bp HindIII to BglII fragment. Our results support this view.

Using the dideoxy chain termination method of Sanger et al. (9),

and cloning the 400 bp HindIII to BglII fragment into the HindIII and BamHI sites of mp18 and mp19, we obtained the DNA sequence (Fig. 1). The sequence was analyzed according to the Pustell Sequence Analysis Program, International Biotechnologies, Inc. We found the characteristics one would expect for a promoter region: the open reading frame beginning at bp #341, presumably the start of the first enzyme of the aerobactin biosynthetic pathway in K. ozaenae 62-1; fairly typical -10 and -35 regions; and an area near the -35 region that could fit the Fur (ferric uptake regulator) protein consensus sequence as found for E. coli iron-regulated promoters. The -10 region we have indicated (CCTAAT, Fig. 2) differs from the corresponding E. coli pColV-K30 -10 region (CATAAT) by one base-pair; alternatively the overlapping sequence TAATAA could be designated as the -10 region of Klebsiella ozaenae 62-1 (Fig. 2). Likewise the -35 region we have indicated for K. ozaenae (AATGAT) perfectly duplicates an alternative -35 region of E. coli (AATGAT). Also, both sets of promoter regions are very AT-rich and are filled with overlapping dyads: in each case one of these dyads represents the Fur binding site (Fig. 2).

The most remarkable observation regarding these dyads is the fact that the longest of these dyads are overlapping and therefore mutually exclusive. Although these dyads are extremely AT-rich and comprise very fluid regions of the DNA molecules, this mutual exclusion of the longest of the dyads might play a role in the transcriptional regulation of the iron uptake system. It is possible that the Fur protein is excluded from its binding site under low iron concentrations by virtue of other DNA-binding proteins which may be involved, and the Fur protein binding

affinity for its dyad probably changes as the iron concentration changes. Although DNaseI footprinting experiments have unequivocally shown that Fur is indeed a DNA-binding protein and that it preferentially binds in high iron concentration as expected (6), its amino acid sequence does not closely resemble that of other DNA-binding proteins (10), so there are probably unique features that make this binding reaction sensitive to iron. In the E. coi system, the "alternative" dyad to the Fur-binding sequence is the more energetically favored, so the high iron situation may leave the Fur protein more passive and therefore allow this "alternative" dyad to prevail, ultimately to favor the functioning of RNA polymerase. In the K. ozaenae system, the dyad which we propose is the alternative dyad is not more energetically favored; however, there is a second alternative dyad also overlapping the Fur sequence so these two alternative dyads may together prevail in the high iron situation, favoring transcription.

In the E. coli system, the proposed -35 region overlaps the Fur-binding region. In the Klebsiella ozaenae system, it appears that there is approximately one turn of the DNA helix between the -35 region and the putative Fur-binding sequence. However, the significant similarity between the two systems is this regard may be that, in both, the Fur-binding region separates the -10 region from the -35 region and the "alternative" dyad.

In addition to the promoter-related aspects of the aerobactin system, this Klebsiella ozaenae 62-1 sequence (Fig. 1) reveals the presence of an open reading frame that appears its origin 5' to this

sequenced DNA fragment. The portion of the open reading frame within this fragment encodes 63 amino acids, and it represents a curiosity, because it is largely conserved when the sequenced aerobactin system promoter areas are compared, that is, the aerobactin promoter region of pColV-K30 (1), of the E. coli K1 chromosome (11), and of K. ozaenae 62-1. The function of this protein is unknown, but its conserved linkage among aerobactin systems, plasmid- and chromosome-encoded, makes it tempting to speculate a role for iron uptake.

Only E. coli iron-regulated promoters have been previously surveyed to generate the Fur protein-binding consensus sequence. Since Klebsiella ozaenae 62-1 is removed from E. coli by species and by tribe, one might expect differences in the way that the iron concentration regulates, even if it is the same siderophore aerobactin that is the final product of this regulation. There are the differences just described, and, although we expect that in Klebsiella ozaenae 62-1 there is a Fur-like protein that serves to down-regulate aerobactin in high iron concentrations, this protein has yet to be found. The Fur-like protein may not be very divergent from the E. coli Fur protein, however, because the sequence that we have chosen as the putative Fur-binding dyad fits the E. coli consensus sequence fairly well, and because the Klebsiella ozaenae 62-1 clone is down-regulated in the E. coli background (14), presumably by Fur protein binding (Fig. 3). This is an interesting expectation, in light of the divergence observed between the aerobactin biosynthetic proteins and the corresponding genes of the two systems (14). In other words, the basic mechanism of iron regulation, using similar repressor proteins, may

be preserved across the species, as well as the integrity of the chemistry of chelation by the siderohpore aerobactin.

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Figure 1. Nucleotide sequence of the putative promoter region, of an upstream open reading frame that is conserved among aerobactin regions of *E. coli* and this organism, and the 5' flanking region of the aerobactin operon of *Klebsiella ozaenae* 62-1, containing the open reading frame of the 5' portion of the first enzyme of the operon. The -10 region, -35 region, and significant dyads are indicated

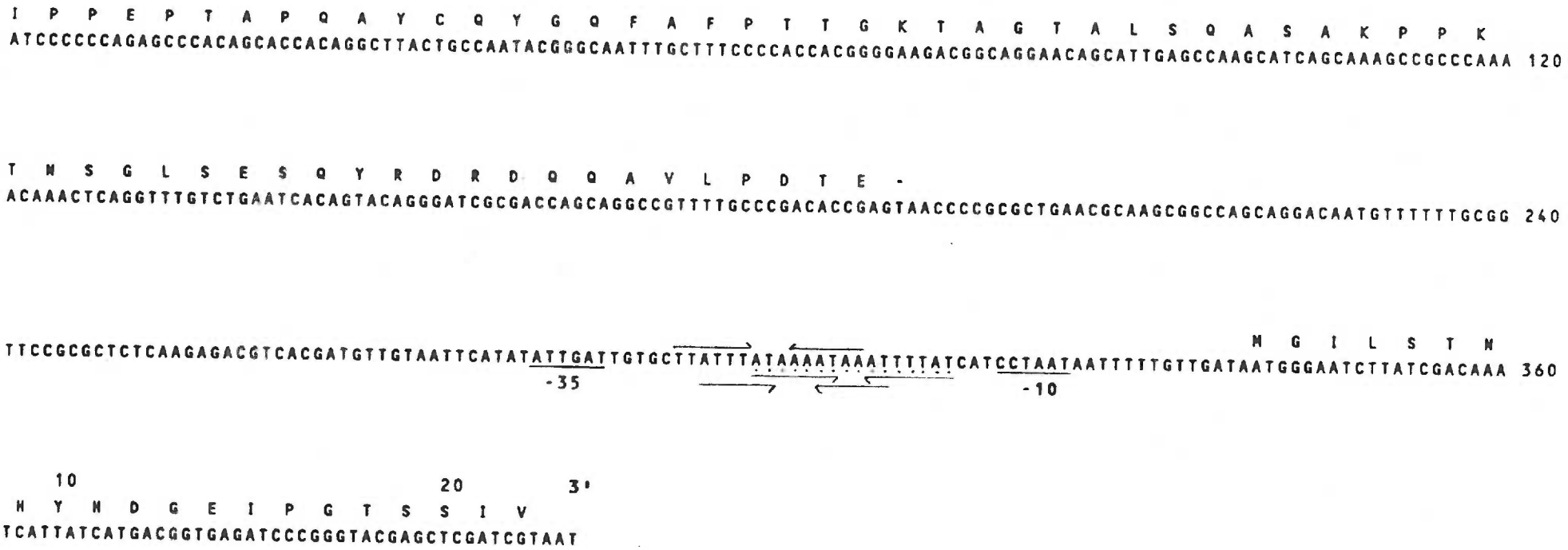
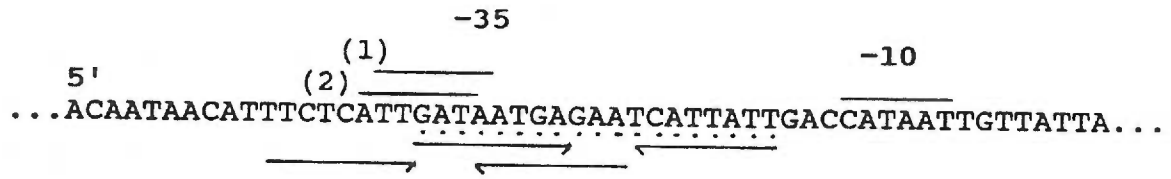

I P P E P T A P Q A Y C Q Y G Q F A F P T T G K T A G T A L S Q A S A K P P K
ATCCCCCCAGAGCCCACAGCACCACAGGCTTACTGCCAATACGGGCAATTTGCTTTCCCCACCACGGGGAAGACGGCAGGAACAGCATTGAGCCAAGCATCAGCAAAGCCGCCCAAA 120
T N S G L S E S Q Y R D R D Q Q A V L P D T E -
ACAAACTCAGGTTTGTCTGAATCACAGTACAGGGATCGCGACCAGCAGGCCGTTTTGCCCGACACCGAGTAACCCCGCGCTGAACGCAAGCGGCCAGCAGGACAATGTTTTTTGCGG 240
M G I L S T M
TTCCGCGCTCTCAAGAGACGTCACGATGTTGTAATTCATATATTGATTGTGCTTATTTATAAAATAAATTTTATCATCCTAATAATTTTTGTTGATAATGGGAATCTTATCGACAAA 360
-35
-10
10
20
3'
H Y H D G E I P G T S S I V
TCATTATCATGACGGTGAGATCCCGGGTACGAGCTCGATCGTAAT

Figure 2. Comparison of the published E. coli pColV-K30 promoter region (2,6) and the analogous region found for Klebsiella ozaenae 62-1. The proposed Fur protein binding sequences are indicated by dots, the dyads by arrows, and the proposed -10 and -35 regions by lines.

E.coli pColV-K30:




Klebsiella ozaenae 62-1 pSMN1:

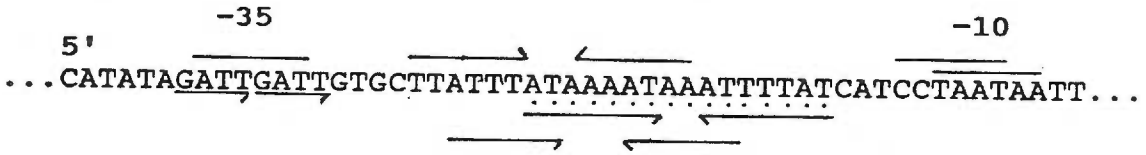


Figure 3. The established Fur-binding region for the primary promoter of the aerobactin system in E. coli pColV-K30, iucA, and the Fur protein-binding consensus sequence (6), and similar promoter regions that are regulated by iron and are thought to bind Fur: fhuA (5), fepA (8), slt (4), fur (10), and iukA, the first gene in the Klebsiella ozaenae 62-1 aerobactin system. Divergence from the consensus is indicated by underlining.

| | |
|---|---|
| fhuA | T C T T T A T A A T A A T C A T T C T C G |
| iuc | T G A T A A T G A G A A T C A T T A T T G |
| fepA | T A T T A T G A T A A C T A T T T G C A |
| sltA | T G A A T A T G A T T A T C A T T T T C A |
| fur | C T A T A A T G A T A C G C A T T A T C T |
| consensus | (T)G A T A A T G A T A A T C A T T A T C(A) |
| K.ozaenae 62-1 | T T A T A A . A A T A A A T T . T T A T C A |

Paper #4:

Respeciation of *Aerobacter aerogenes* 62-1

*Klebsiella ozaenae* 62-1

# ABSTRACT

We present conventionally used criteria to demonstrate that the strain Aerobacter aerogenes 62-1 gives excellent identification as a Klebsiella ozaenae. This strain was originally used in the 1960's to study aromatic amino acid biosynthesis; in 1969 it was the strain from which the iron-uptake siderophore aerobactin was discovered; since then it has been used to chemically and genetically characterize the aerobactin system. We propose that Aerobacter aerogenes 62-1 be henceforth referred to as Klebsiella ozaenae 62-1.

In 1961 a wild-type strain of Aerobacter aerogenes was used to generate a non-encapsulated variant which was named Aerobacter aerogenes T-17 (10). This strain was the parent of Aerobacter aerogenes 61-3, an auxotroph which requires tryptophan and tyrosine, as isolated by the penicillin selection technique originally described by Lederberg (7). By the same technique, Aerobacter aerogenes 61-3 was used to generate Aerobacter aerogenes 62-1, an auxotroph requiring tryptophan, tyrosine, and phenylalanine (5). Such strains, and analogous E. coli auxotrophs, were used by C. Yanofsky, F. Gibson, C. H. Doy, B. Davis and their colleagues in the elucidation of the biosynthetic pathways of aromatic amino acids (2,4,8,9,10). These pathways were deduced by identifying the intermediates that accumulated in each auxotroph. For example, the intermediate chorismic acid was discovered using Aerobacter aerogenes 62-1. Another of these intermediates, 4-hydroxybenzoic acid, is also a precursor in the biosynthetic pathway leading to the iron chelator 2,3-dihydroxybenzoic acid, which, with the addition of a serine moiety, becomes the high affinity catechol siderophore called enterobactin.

In 1969, F. Gibson and D. I. Magrath reported the isolation and purification of another iron chelator, a dihydroxamic acid, which they called aerobactin, in culture supernatants of Aerobacter aerogenes 62-1, grown in iron-deficient medium (6). Aerobactin was ultimately characterized as a virulence factor of invasive enteric bacteria, because it is biologically and chemically suited for the sequestration of iron from serum transferrin (1,11). This ability enables such pathogens to grow in blood, since the essential nutrient iron, as free iron, forms

insoluable and unassimilable compounds under physiological conditions (11).

Since 1969, Aerobacter aerogenes 62-1 has continued to be the prototype strain used for the chemical analysis of the enzymes involved in the biosynthetic pathway of the siderophore aerobactin (12). We recently cloned the aerobactin system genes from this strain (which was originally obtained from Gibson and Magrath), in order to genetically characterize the system. In the course of these studies, we needed to verify the species of this strain, which has occassionally been called Enterobacter aerogenes 62-1, but more often the prevailing but outdated Aerobacter aerogenes 62-1. To our knowledge, the species of this strain has not been checked since the 1960's.

The data presented herein are representative of a conventional but thorough panel of biochemical tests used for speciation in the clinical lab at The Oregon Health Sciences University. In parallel with Aerobacter aerogenes 62-1, we have respeciated three other organisms as controls, obtained from the American Type Culture Collection, Atlanta Georgia. They are: Klebsiella pneumonia ATCC #13906 (formerly an Aerobacter aerogenes), Klebsiella ozaenae ATCC #11296, and Enterobacter aerogenes ATCC #13048 (formerly an Aerobacter aerogenes). The results in total are presented in Table 1.

Most of the biochemical reactions and substrates utilized were fairly typical. The ATCC Klebsiella ozaenae #11296 gave one atypical result, in that it was malonate positive, whereas the typical Klebsiella

ozaenae is malonate negative. In this regard, the "62-1" organism is more typical of the species Klebsiella ozaenae. Distinquishing the two organisms from Enterobacter aerogenes are the following tests: the Vogues-Proskauer, motility, and ornithine decarboxylase. The Enterobacter aerogenes species, and indeed the ATCC Enterobacter aerogenes #13048, are typically positive for all of these tests, whereas the Klebsiella ozaenae are negative (3). Both the Klebsiella ozaenae #11296 and the "62-1" strain are typical and negative for these tests. The species Klebsiella ozaenae is unusual among the Enterobacteriaceae in that 15% are negative for nitrate reduction. All (100%) of the Enterobacter aerogenes are nitrate positive (3). The fact that both of the Klebsiella ozaenae ATCC #11296 and the "62-1 strain tested here are nitrate negative supports the respeciation of Aerobacter aerogenes 62-1 to Klebsiella ozaenae 62-1. Distinguishing this organism from the Klebsiella pneumoniae are the malonate and methyl red tests. The ATCC #13906 Klebsiella pneumoniae was typical and positive for malonate and negative for methyl red, and 62-1 was negative for malonate and positive for methyl red and thus typical of the Klebsiella ozaenae. The 62-1 organism utilizes citrate but not lactose, which is not typical, but the citrate test can be considered variable for the Klebsiella ozaenae species.

The organism called Aerobacter aerogenes 62-1, therefore, is very typical of the Klebsiella ozaenae, and has been distinguished from the Enterobacter aerogenes (especially by motility and nitrate) and the Klebsiella pneumoniae (especially by malonate and methyl red). We

propose that this organism be henceforth referred to as Klebsiella ozaenae 62-1.

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Table 1

Biochemical reactions of simultaneously tested stains

| Test or Substrate | Klebsiella pneumonia[1] ATCC 13906[1] | Enterobacter aerogenes[1] ATCC 13048[1] | Klebsiella ozaenae ATCC 11296 | "62-1" |
|---|---|---|---|---|
| Hydrogen Sulfide (TS1 Agar) | - | - | - | - |
| Urease | - | - | + (Weak) | - |
| Citrate (Simmons') | + | + | - | + |
| Motility | - | + | - | - |
| Ornithine Decarboxylase | - | + | - | - |
| Lysine Decarboxylase | + | + | + | + |
| Arginine Dihydrolase | - | - | - | - |
| Gelatin | - | - | - | - |
| Adonitol | + | + | + | + |
| Arabinose | + | + | + | + |
| Alpha-methylglucoside | + | + | + | + |
| Raffinose | + | + | + | + |
| Rhamnose | + | + | + | + |
| Dextrose Acid | + | + | + | + |
| Dextrose Gas | + | + | + | + |
| Lactose | + | + | + | - |
| Nitrate to Nitrite | + | + | - | - |
| Malonate | + | + | + | - |
| Dextrose Fermentation | + | + | + | + |
| Oxidase | - | - | - | - |

[1] Formerly called Aerobacter aerogenes

# Discussion

The study of iron uptake by means of the aerobactin system demonstrates how bacteria adapt to the hostile environment of the mammaliam host in order to survive, grow, and multiply. The particular hostility that the host is elaborating in this case is the withdrawal of free iron by its binding to host compounds such as transferrin in serum and lactoferrin in secretions. (Another mechanism, besides the aerobactin iron uptake system, that organisms have used in response to the free iron shortage is the production of hemolysin.) Survival and growth is the first step in the bacterial disease process, and this stage includes preventing the host from eliminating the bacteria, at least temporarily. The fact that the host is usually able to eliminate blood-borne bacteria, even those that carry the aerobactin iron uptake system, exemplifies that there are other host mechanisms at work in maintaining health. Nonetheless, the most common Gram-negative organisms found amoung septic neonates are E. coli, and the Klebsiella and Enterobacter species. This fact, in the context of the data presented in this thesis and the data summarized in the introduction, would lead one to believe that a virulence factor such as the aerobactin iron uptake system is one of the more important factors commonly encoded by invasive bacteria.

The advent of the widespread use of antimicrobial agents has selected for the mobility of virulence factors, such that the treatment of one sensitive strain with an antibiotic may in some cases prolong the disease process, as there is a shuffling of plasmid-mediated factors to a resistant strain, or, the transfer of the drug resistance (the latter was

the first and most easily traced example of *in vivo* recombinant DNA technology performed by the bacteria). These types of events may have occurred in a child, a patient here at the OHSU hospital, who experienced three successive episodes of bacteremia caused by three different organisms, an *E. coli*, a *Klebsiella pneumoniae*, and an *Enterobacter cloacae*. I obtained all of these isolates and found that each produced aerobactin. One could envision the transfer of the aerobactin system genes, as they are encoded often on large transferrable plasmids, from the *E. coli* to the *Klebsiella pneumoniae* and ultimately to the *Enterobacter cloacae*. It is known that the child was treated with antibiotics at each episode of bacteremia, and that there were differences in the drug resistant patterns of the successive isolates.

The work described in the first and third papers of this thesis, however, would add a level of complexity to this scenerio. It is known that some antibiotic resistance genotypes are carried by broad host range plasmids, that is, "promiscuous" plasmids that can carry genes across diverse species. In the case of the plasmid-mediated aerobactin iron uptake systems, the plasmids are large and in most cases transferrable, but the host range is limited. Because of the absolute association of the plasmid-mediated *E. coli* aerobactin system genes with the incompatility group IncFI, I would expect this aerobactin system, the "*E. coli* system," to be limited to species that can carry IncFI plasmids, such as *E. coli* and the Salmonelleae. The above patient in fact had come from India as an orphan, and first presented with a bloody diarrhea caused by a *Salmonella* (unfortunately I was unable to obtain

this strain). Salmonella carrying aerobactin genes on IncFI R factors (drug resistance plasmids) have been known to be endemic in this area of the world (see the first paper of this thesis).

In light of the uniqueness of the genotype of the aerobactin system found in the Klebsiella ozaenae, one would expect that there is some constraint that prevents the transfer of the "E. coli system" to the Klebsielleae tribe (see the third manuscript of this thesis). Therefore, in the above case of successive bacteremias, the emergence of aerobactin-producing species probably did not occur by means of aerobactin plasmid mobility across species, at least from the E. coli to the Klebsiella or Enterobacter. We cannot say from the data presented herein whether or not there is plasmid transfer of aerobactin system genes between the latter two species, assuming that these genes would be of the "Klebsiella-aerobactin system type." The lack of any DNA homology between the REPI region of the E. coli pColV-K30 plasmid and the Klebsiella ozaenae aerobactin-producing plasmid DNA implies that the Klebsiella plasmid is not of the IncFI incompatibility group. This difference in incompatibility group in E. coli and Klebsiella plasmids may play a role in the separation of the two aerobactin systems but it does not help in speculation as to the mechanism of spread of the Klebsiella system among the Klebsielleae tribe, if indeed there is such a mechanism.

What might have occurred, then, regarding the child with the four successive infections, is that each organism came prepared with its own

aerobactin system, whether plasmid- or chromosome- encoded. Alternatively, the Salmonella and E. coli could conceivably have shared genetic material in the gut; likewise, the Klebsiella and Enterobacter species might have shared aerobactin genes via plasmid transfer. An in depth genetic investigation could give insight into these speculations but of course could not prove what actually happened in this individual.

Another aspect of the interaction between the host and the invading bacteria is that the bacteria need to be responsive to the changing micro-environment of the niche that they occupy in the host. In other words, bacteria, which are apparently more efficient than higher organisms in their economical use of genetic material, must regulate the expression of gene products according to environmental stimili. This last aspect has been addressed in the sequencing analysis of the promoter region of Klebsiella ozaenae 62-1. The analogous region of the E. coli system, as well as other E. coli iron-regulated promoter regions, were compared to this region. A new concept, that of overlapping and mutually exclusive dyads in the promoter regions, may be used to partially explain how an iron-sensitive repressor may change the aerobactin expression. This concept is invoked for both the Klebsiella ozaenae 62-1 and E. coli systems, so it may be part of a general mechanism of iron regulation. Knowledge of the levels of control of amino acid synthesis, that is, the fine tuning of attenuation vs. the coarse tuning of repression of transcription, leads one to speculate that there may be other levels of control in iron regulation of operons such as the aerobactin system operon. So, the action of the Fur protein may be the "coarse-adjust, or there may be other factors such as the

interaction of proteins with the alternative dyads which could modify the "on-off" nature of the Fur binding to the DNA sequence. The Fur protein is a global regulator, affecting many genetically distant loci which are all responsive to the iron concentration of the environment. Such iron-regulated loci are probably not all equally critical in maintaining the survival of the bacteria, so there are probably sub-regulators that affect individual genes, such as the alternative dyads, positive regulators, or translational factors. The critical nature of iron has also resulted in the possession by many bacteria of several iron uptake systems, each for a different ecological niche or a different level of iron starvation.

Much of the work done in preparation for this thesis has been brought to you by the comparative approach. The power of this approach has become apparent with each set of experiments, and it will continue to be apparent if both the Klebsiella ozaenae and E. coli structural genes for the aerobactin systems are sequenced to completion, a total of about 16 kb. The regions held in common for each enzyme should be critical for enzymatic activity, and the expendability of certain regions may be hypothesized. In the course of preparing the first manuscript of this paper, several ColV plasmids were compared in terms of various genotypes, such as the colicin V genes, the transfer genes, and repeated sequences. By this molecular epidemiology, the evolution of some of these plasmids by means of recA-dependent recombination events using repeated sequences could be surmised. Local regions of conservation and divergence gave insight into the large blocks of DNA that may have recombined into or out of a particular replicon. These observations demonstrate the plasticity

of plasmids. The comparative approach in studying the expression of the aerobactin system by bioassay was also fruitful: differences between the clones and the native strains, and differences between the clones from the two systems, demonstrated that there were other levels of distinction besides the divergence of structural genes and polypeptides between the aerobactin systems of E. coli and Klebsiella ozaenae. The observations made by bioassay were borne out, although not completely explained, by the use of the comparative approach in the study of the sequences of the operator-promoter regions.

The divergence of the E. coli and Klebsiella ozaenae 62-1 aerobactin iron uptake systems includes differences in the sequences encoding the enzymes of the biosynthetic pathway of aerobactin, differences in the enzymes themselves, and differences in expression of both the biosynthetic enzymes and the outer membrane receptor protein. Sequencing the two sets of genes for the biosynthetic pathway enzymes will yield much information regarding the enzymes and perhaps delineate the active sites of some of these enzymes. The differences found between between any two analogous enzymes may give insight into the need for these two aerobactin iron uptake systems. For example, the first two enzymes in the biosynthetic pathway leading to aerobactin, the hydroxylase and the acetylase, are known to operate in association with the inner membrane of Klebsiella ozaenae 62-1, while the synthetase activity is in the periplasmic space of this organism. This localization of enzyme activities is not known for the E. coli system. If the cell localization is found analogous in E. coli, the differences between these enzymes may reflect differences between the inner membranes of E. coli and Klebsiella

ozaenae. Therefore, these differences between these inner membranes need to be analyzed in the context of the functioning of the hydroxylase and the acetylase. Perhaps this analysis would help explain why the Klebsiella ozaenae 62-1 clones do not mediate aerobactin production in the E. coli background as well as in the native strain. Likewise, the expression of the outer membrane receptor protein is not comparable in the two cell backgrounds. This difference in expression may be due to differences in the outer membrane architecture of the two organisms, or it may be due to differences in regulators of expression. Pursuing these aspects will give insight into the significance of the divergent aerobactin systems, and give insight into the differences between the two species. The ultimate goal would be to explain why each particular aerobactin system is suited to its particular genus and to its particular plasmid. The question of why both systems are found encoded only by low copy plasmids (or by chromosomes) and not by plasmids of another copy number is yet to be addressed.

An additional realm that has not been explored is the nature of the pSMN1 plasmid. The incompatiblity group of this plasmid is not known, nor is it known if this plasmid is transferrable. If it can be transferred to the Enterobacter and Serratia species, as well as to other Klebsiella species, knowledge of these other genera can be applied in drawing conclusions about the suitability of this aerobactin iron uptake system for the Klebsielleae tribe. Also, the nature of the replication regions of this plasmid has not been explored, so the relevance of replication regions in the preservation of this aerobactin system is unknown. Answering these kinds of questions could help explain why the

genes for this system are plasmid-encoded, and perhaps why the genes are encoded by low-copy plasmids.

To illustrate and summarize the encounter of an invasive enteric bacteria with the vertebrate host, including the two possibilities of high iron concentration (the unusual) and low iron concentration (the usual), the cartoon on the following page is given. The nomenclature of the E. coli aerobactin system operon is used, but the general mechanism of iron-sensitive repression of transcription should apply to both of the aerobactin iron uptake system genes decribed in this thesis. Thus this cartoon can be called the "Generic Invasive Enteric."

# Generic Invasive Enteric