A GENETIC STUDY OF *Erwinia carotovora*

BY

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"And here I may be able to make an observation or two of my own, which may be of use hereafter to those into whose hands these may come..." Daniel Defoe, 'A Journal of the Plague Year'.

CONTENTS

ABSTRACT

Gene transfer systems have been developed in Erwinia carotovora, a phytopathogenic bacterium which is a member of the Enterobacteriaceae. The species is subdivided into two subspecies: E.carotovora subsp. carotovora (Ecc) and E.carotovora subsp. atroseptica (Eca).

Using plasmid isolation methods able to resolve plasmids up to at least 100Md in Ecc, and up to 300Md in other bacterial species, it was observed that 11 of 39 wild Ecc strains and 9 of 28 wild Eca strains tested carried plasmids. The molecular weights of some of these plasmids were determined and all were below 100M. The production of bacteriocins and resistance to high levels of antibiotics by some of these wild strains was also tested.

Spontaneous and induced mutations in Ecc and Eca strains were isolated, characterised, and compared to mutations reported in other members of the Enterobacteriaceae.

Various F-prime plasmids were crossed into E.carotovora strains. One strain of Eca could accept, but could not maintain as a plasmid F'Lac$^+$Tc, and it is proposed that this Tc determinant could transpose onto the Eca chromosome. By contrast, F'Lac$^+$Tc, and some but not other F-prime plasmids, could be conjugated into Ecc SCRI193 where they were inherited and could transfer within this strain and to Escherichia coli. The kinetics of F'Lac$^+$Tc transfer within Ecc SCRI193 and to E.coli were studied under different mating conditions. The chromosome of Ecc SCRI193 was mobilised by F'Lac$^+$Tc and by F'His$^+$.

The behaviour in Ecc SCRI193 of the transposable phage Mu, on the broad host-range plasmid R68, was studied. Although both zygotic and temperature induction of such lysogens were observed, the transposition of Mu onto the chromosome could not be detected by any of several different methods. The mobilisation of the Ecc SCRI193 chromosome from donor strains carrying a chromosomal Mu prophage (thyA1::Mu; constructed elsewhere) and an R68::Mu plasmid, was studied using both the frequency of inheritance and the frequency of coinheritance of chromosomal mutations. The two mechanisms of chromosomal mobilisation observed, are discussed.

Using linkage data obtained from chromosome mobilisation studies in Ecc SCRI193 with donors carrying thyA1::Mu / R68::Mu or the F'Lac^{+}Tc plasmid, a linkage map of Ecc SCRI193 has been constructed which consists of seventeen ordered mutations and one approximately located mutation. This linkage map and the chromosomal mutations isolated in Ecc SCRI193 are compared to those of other species of the Enterobacteriaceae.

SECTION 1

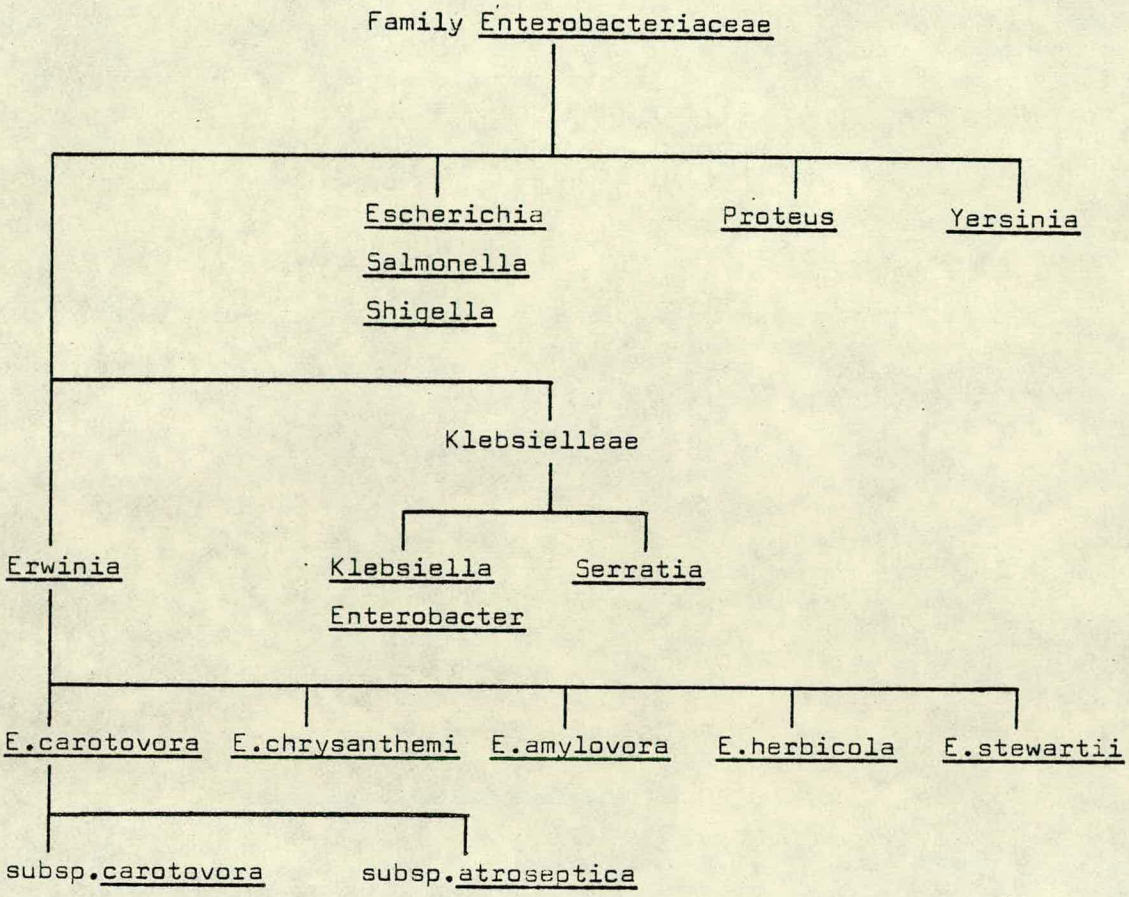
GENERAL INTRODUCTION

The genus Erwinia is a member of the Enterobacteriaceae and includes the soft-rot Erwinia which can enzymatically macerate the parenchymatous tissues of many plants. The soft-rot Erwinia are E.carotovora subsp. carotovora (Ecc), E.carotovora subsp. atroseptica (Eca), E.chrysanthemi, E.crypripedii, and E.rhapontici. This species nomenclature is that recommended by the International Committee on Systematic Bacteriology (Skerman et al.,1980); however other workers have suggested that all of the above species are in fact subspecies of E.carotovora (Dye,1969). Other members of the genus Erwinia include E.amylovora, E.herbicola, E.stewartii and E.uredovora and these are the causal agents of diseases in both plants and animals.

At present the taxonomic position of the Erwinia genus is uncertain and while sharing many features with the Klebsiella and Serratia genera it most probably should belong to a separate tribe rather than to the Klebsielleae tribe. "Bergey's Manual of Determinative Bacteriology" (Buchanan and Gibbons, 1974) classifies Erwinia in the tribe Erwinieae. The phylogeny of these species and genera are shown in Figure 1 together with some of the other genera of the Enterobacteriaceae. Acceptance of Erwinia as a distinct genus is not universal and several workers have suggested that the genus be redistributed amongst other genera of the Enterobacteriaceae.

The relatedness between members of the soft-rot Erwinia was studied at

Figure 1. Taxonomy of the Erwinia and related species.



Adapted from Chatterjee and Starr (1980), Dye (1969), Edwards and Ewing (1972), Skerman et al.(1980) and Starr and Chatterjee (1972).

the DNA level by Brenner et al. (1973) using the kinetics of renaturation of the total DNA of different strains to Ecc ATCC495. They found very high homology between this strain and six other Ecc strains, and only very slightly less DNA binding to DNA from five strains of Eca, supporting the division of E.carotovora into two subspecies. Binding of Ecc ATCC495 DNA to that of six strains of E.chrysanthemi was rather lower, endorsing its classification as a separate species. The degree of binding to E.amylovora DNA and E.herbicola DNA, which was still lower, was comparable with that to the DNA from strains of other members of the Enterobacteriaceae (Escherichia, Salmonella, Serratia). The conclusions of Brenner et al. (1972) and Gardener and Kado (1972) were that "in most instances DNA homologies between Erwinia spp. and E.coli, S.typhimurium, Klebsiella and Shigella species showed about the same amount of relatedness as in Erwinia to Erwinia combinations. The molecular hybridisation data indicate(d) that the genus Erwinia is a loosely composed group of bacteria that often have no greater affinities to each other than to other enteric bacteria" (Gardener and Kado, 1972). The enterobacterial species examined by these workers all contained a fraction of chromosomal DNA which was common to all strains, a fraction which contained a degree of mismatch, and a fraction which was non-homologous. It should be noted however, that the method of preparation of the DNA used in these studies (Berns and Thomas, 1965) involved the extraction of total DNA from the lysed bacteria which was then sheared prior to hybridisation. It is therefore possible that some of the hybridisation observed could be due not to homologous chromosomal DNA sequences, but to sequences carried on plasmids; were these present in the strains. While it is unlikely that the very high

homologies (c.95%) observed within the soft-rot Erwinia would be due to this, the lower homologies observed in the other interspecific comparisons could, in part, be the result of such common plasmid sequences. Such problems can be avoided by using probes of discrete, cloned DNA sequences. Interspecific comparisons of selected DNA sequences by hybridisation using E.coli K12 chromosomal sequences cloned in phage λ (lacZ, thyA, tna and trp) (Riley and Anilionis, 1980) to restricted E.amylovora chromosomal DNA also showed that the extent of homology of different chromosomal sequences between different species varied from sequence to sequence. In view of the low total DNA homologies observed between the soft-rot Erwinia and E.amylovora, the extrapolation of this result from E.amylovora to all of the Erwinia should be made with caution. Thus interspecific comparisons at the DNA level show that while the soft-rot Erwinia (Ecc, Eca and E.chrysanthemi) are closely related, there may be little similarity between this group and the other members of the Erwinia.

The ecology of the soft-rot Erwinia has been reviewed by Pérombelon and Kelman (1980). Of particular interest in the United Kingdom are the two subspecies of E.carotovora: Eca whose host range is mainly restricted to potatoes on which it is the causal agent of blackleg, and Ecc which has a much wider host range. The biochemical basis of the E.carotovora soft-rots has been much studied and has been reviewed by Bateman and Millar (1966), Chatterjee and Starr (1980) and Stack et al. (1980).

This study of E.carotovora was initiated to establish a range of genetic methods in this important, but neglected, member of the

Enterobacteriaceae (Chatterjee and Starr, 1980; Leary and Fulbright, 1982). The genetic study of Ecc, the subspecies used for most of the research, will facilitate future research on the comparative genetics of the genomes of this and other more or less distantly related members of the Enterobacteriaceae, and of genetic elements in this genetic background. Research into the genetic bases of this organism's particular characteristics -its pathogenicity to several plant species (Pérombelon and Kelman, 1980), its catabolism of several different biochemicals and macromolecules, and its pectic enzymes, proteases and phosphatases (Chatterjee and Starr, 1980; Dye, 1969) will also be possible.

Table 1. Bacterial,phage and plasmid strains.

Table 1(a). Bacterial strains.

Strain	Description	Source
Escherichia coli K12		
6895	*argE gyrA metB rpoB* Δ(*lac-proA*)/F'Rep[Ts114]Lac*::Tn10	(Botstein)GMI
AB2847	*aroB mal tsx* λ^{-} λ^{R}	(Pittard)RE
AK5003	*his rpsE* ΔXIII(*lac-proA*)/F^{+}::Tn10	(NK5222/F9505) Kotoujansky
CA7087	*proC thi* HfrH	(Scaife)RE
ED8812	*lacZ leu thi thr* rK^{-} mK^{-} Str^{S}	(Murray)RE
ED8874	*att*λ *his trpR* Δ(*gal-bio*) rK^{-} mK^{+}	(Murray)RE
GMI3230	*leu rpsL thi thr* Mu^{R} rK^{-}mK^{-}	(C600 Mu^{R}) GMI
GMI3246	*thi thyA trp::Mu•-*/R68::Mu c^{+}Δ445-7	(PP54(Wijffelman)/pGMI20)GMI
GMI3247	*thi thyA trp::Mu•-*/R68::Mu c^{+}Δ445-7	(PP54(Wijffelman)/pGMI22)GMI
JC5466	*his trp recA rpsL*	GMI
KF8	*lacZ leu thi thr*/P1*clr*100 Km	P1 into ED8812,this work
KF21	*lacZ leu thi thr*/P1*clr*100 Cm	P1 into ED8812,this work
KF30	*his rpsL trp*/F'His^{+}	spontaneous Str^{R} into RE281 (JC5455/F57),this work
KF39	*leu rpsL thi thr* rK^{-}mK^{-}/F'Rep[Ts114]Lac^{+}::Tn10	plasmid from 6895 into C600, this work
KF53	*thi* Δ(*lac-proA*)/F'*lacI*$^{-}$::*Mucts62* ProA^{+}::Tn10	pKF3,this work, into RE291F^{-}

Table 1. Bacterial,phage and plasmid strains.

Table 1(a) cont.

Strain	Description	Source
E.coli K12 (cont.)		
KF56	*ara argE galK gyrA his lacY leu mtl proA rpoB rpsL thr xyl* /F'His$^+$::Tn10	spontaneous Nal^R Rif^R into AB1157 (KF55;this work) pKF4,this work
KF62	*lacZ leu rpsL thi thr* / RP4	RP4 into ED8812,this work
KF63	*lacZ leu rpsL thi thr* MuΛc$^+\Delta$445-7	Mu from KF1017 into ED8812, this work
KF65	*lacZ leu rpsL thi thr* / RP4::Mu cts62	pKF2 into ED8812,this work
RE26	*his lacY proA trp*	RE
RE254	*gyrA his lacZ rpsL trp*	RE
RE349	*gyrA thi* Δ(*lac-proA*)	RE
RE410	*his lacY pro trp* /F'Lac$^+$Tc	(RE26/F'Lac$^+$Tc) RE
E.coli C		
C6	*lac serB*	RE
Erwinia carotovora		
subsp.*carotovora*	wild strains isolated from:	
SCRI101	tobacco,USA	(NCPPB550)SCRI
SCRI102	avocado,Israel	(NCPPB547)SCRI
SCRI103	potato,UK	SCRI
SCRI106	cucumber,UK	(NCPPB392)SCRI
SCRI109	arum lily,South Africa	(NCPPB929)SCRI

Table 1(a) cont.

Table 1(a) cont.

Strain	Description	Source
E.carotovora subsp. *carotovora* (cont.)		
SCRI110	potato,Brazil	(DAFS- ENA45)SCRI
SCRI112	potato,Japan	(NCPPB1746)SCRI
SCRI113	*Allium cepa*,Japan	(NCPPB1747)SCRI
SCRI114	potato,Denmark	(ATCC15713=NCPPB312)SCRI
SCRI115	potato,UK	SCRI
SCRI117	potato,UK	SCRI
SCRI118	potato,UK	SCRI
SCRI119	potato,UK	(DAFS- G149)SCRI
SCRI120	sunflower,Uganda	(NCPPB1231)SCRI
SCRI121	sugar cane,Jamaica	(NCPPB1640)SCRI
SCRI122	tomato,Tanzania	(NCPPB355)SCRI
SCRI124	potato,UK	(Logan- 14B)SCRI
SCRI125	potato,UK	(Logan- G148)SCRI
SCRI126	potato,UK	(Logan- 362/23)SCRI
SCRI127	wasp,UK	SCRI
SCRI130	potato,UK	SCRI
SCRI132	carrot,Japan	(NCPPB1744)SCRI
SCRI135	potato,USA	(Stanghellini- N'2)SCRI
SCRI139	potato,USA	(Stanghellini- NOB102)SCRI

Table 1(a) cont.

Table 1(a) cont.

Strain	Description	Source
E.carotovora subsp. carotovora (cont.)		
SCRI144	potato,Australia	(Samson- J21A-X)SCRI
SCRI149	potato,Australia	(Samson- T5B-X)SCRI
SCRI152	potato,Australia	(Samson- NI-X)SCRI
SCRI155	potato,UK	SCRI
SCRI166	potato,UK	SCRI
SCRI169	insect,UK	SCRI
SCRI172	potato,UK	(CIP007)SCRI
SCRI174	potato,Peru	(CIP010)SCRI
SCRI178	potato,Peru	(CIP022)SCRI
SCRI191	maize,Israel	(NCPPB552)SCRI
SCRI192	potato,UK	SCRI
SCRI193	potato,USA	(Kelman- SR44)SCRI
SCRI198	potato,USA	(Kelman- SR162L)SCRI
SCRI205	sunflower,Mexico	(Fucikovsky- SR215)SCRI
SCRI211	potato,UK	SCRI
SCRI238	potato,UK	SCRI

Table 1(a) cont.

Table 1(a) cont.

Strain	Description	Source
E.carotovora subsp. *carotovora* SCRI113		
KF1028	*rif-1*	spontaneous Rif^R, this work
KF1042	*nal-1 str-1*	spontaneous Nal^R, Str^R, this work
E.carotovora subsp. *carotovora* SCRI193		
KF1005	*str-1*	spontaneous Str^R (SCRI)
KF1006	*rif-1*	spontaneous Rif^R (SCRI)
KF1007	*his-2 str-1*	NG mutagenesis of KF1005 (SCRI)
KF1010	*his-2 nal-3 str-1* Gal^-	spontaneous Nal^R, NG mutagenesis (Gal^-) of KF1007 (SCRI)
KF1016	*rif-1 thyA1::Mu* $c^+\Delta$*445-7*	Mu induced Tmp^R into KF1006 (SCRI)
KF1017	*deo-1 rif-1 thyA1::Mu* $c^+\Delta$*445-7* /R68 ::Mu $c^+\Delta$445-7	pKF1 (from GMI), and spontaneous Deo^- into KF1016 (SCRI)
KF1019	*his-2 nal-3 str-1* Pec^-	as KF1010 but Pec^- (SCRI, mutant 157)

Table 1(a) cont.

Table 1(a) cont.

Strain	Description	Source
E.carotovora subsp. *carotovora* SCRI193 (cont.)		
KF1033	*nal-1 str-1*	spontaneous Nal^R into KF1005, this work
KF1037	*rif-1* / F'Lac^+Tc	plasmid from RE410 into KF1006, this work
KF1047	*hisD1 nal-1 str-1*	EMS mutagenesis of KF1033, this work
KF1060	*hisD1 leu-2 nal-1 str-1 thr-1*	repeated EMS mutagenesis of KF1047, this work
KF1061	*hisD1 nal-1 pheA1 ser-1 str-1*	as KF1060
KF1062	*nal-1 str-1* / F'Lac^+Tc	plasmid from RE410 into KF1033, this work
KF1063	*nal-1 str-1* F^-	cured isolate of KF1062
KF1064	*aroB1 glyA1 hisD1 nal-1 str-1*	as KF1060
KF1065	*hisD1 nal-1 proA1 str-1 trp-2*	as KF1060
KF1067	*hisD1 nal-1 proA1 str-1 trp-2* / F'His^+	plasmid from KF30 into KF1065, this work
KF1068	*gal-1 hisD1 nal-1 proA1 str-1 trp-2*	EMS mutagenesis of KF1065, this work

Table 1(a) cont.

Table 1(a) cont.

Strain	Description	Source
E.carotovora subsp. *carotovora* SCRI193 (cont.)		
KF1069	*crp-1 hisD1 nal-1 proA1 str-1 trp-2*	as KF1068
KF1070	*galK2 hisD1 nal-1 proA1 str-1 trp-2*	as KF1068
KF1071	*gal-3 hisD1 nal-1 proA1 str-1 trp-2*	as KF1068
KF1072	*aroB1 glyA1 hisD1 manA1 nal-1 str-1*	EMS mutagenesis of KF1064, this work
KF1075	*aroB1 glyA1 hisD1 manA1 nal-1 str-1* / R68::Mu $c^{+}\Delta$445-7	plasmid from KF1017 into KF1072, this work
KF1077	*rif-1* / RP4	plasmid from KF62 into KF1006, this work
KF1078	*chl-1 rif-1*	spontaneous Chl^{R} into KF1006, this work
KF1080	*chl-1 rif-1* / RP4	plasmid from KF62 into KF1078, this work
KF1083	*chl-1 rif-1* / RP4::Mu cts62	plasmid from KF65 into KF1078, this work
KF1084	*chl-1 rif-1 thyA2* / RP4	spontaneous Tmp^{R} into KF1080, this work
KF1085	*rif-1 thyA1*::Mu $c^{+}\Delta$445-7 / RP4::Mu cts62	plasmid from KF65 into KF1016, this work

Table 1(a) cont.

Table 1(a) cont.

Strain	Description	Source
E. carotovora subsp. *carotovora* SCRI193 (cont.)		
KF1086	*chl-4 hisD1 nal-1 pheA1 ser-1 str-1*	spontaneous Chl^R into KF1061, this work
KF1088	*chl-3 rif-1 thyA1::Mu* $c^+\Delta$445-7	spontaneous Chl^R into KF1016, this work
KF1089	*aroB1 glyA1 hisD1 manA1 nal-1 nal-2 str-1*	spontaneous Nal^R into KF1072, this work
KF1090	*aroB1 glyA1 hisD1 manA1 nal-1 str-1 thyA1::Mu* $c^+\Delta$445-7	$ThyA1^-$ conjugated into KF1072 from KF1017, this work
KF1091	*chl-1 rif-1* / R68::Mu $c^+\Delta$445-7	plasmid from KF1017 into KF1078, this work
KF1092	*aroB1 glyA1 hisD1 manA1 nal-1 str-1 thyA1::Mu* $c^+\Delta$445-7 / R68::Mu $c^+\Delta$445-7	plasmid from KF1017 into KF1090, this work
KF1093	*chl-3 rif-1 thyA1::Mu* $c^+\Delta$445-7 / R68::Mu $c^+\Delta$445-7	plasmid from GMI3246 into KF1088, this work
KF1094	*chl-3 rif-1 thyA1::Mu* $c^+\Delta$445-7 / R68::Mu $c^+\Delta$445-7	plasmid from GMI3247 into KF1088, this work

Table 1(a) cont.

Table 1(a) cont.

Strain	Description	Source
E.carotovora subsp.		
atroseptica	wild strains isolated from:	
SCRI1	potato,Israel	(?- C1)SCRI
SCRI3	potato,Zimbabwe	(NCPPB435)SCRI
SCRI5	*Schizanthus* sp.,Tanzania	(NCPPB352)SCRI
SCRI6	potato,Romania	(NCPPB1449)SCRI
SCRI8	potato,Netherlands	(DAFS- MG147/43)SCRI
SCRI9	tomato,UK	(DAFS- C403)SCRI
SCRI13	potato,UK	(NCPPB1277)SCRI
SCRI16	potato,USA	(Stanghellini- Pf18)SCRI
SCRI19	potato,USA	(Stanghellini- O'5)SCRI
SCRI22	potato,USA	(Stanghellini- Pf8)SCRI
SCRI26	potato,UK	(NCPPB549)SCRI
SCRI27	*Delphinium ajacis*,USA	(ATCC7403)SCRI
SCRI28	potato,UK	SCRI
SCRI31	potato,USA	(Kelman- SR8)SCRI
SCRI39	? ,USA	(Kelman- SR315)SCRI
SCRI44	potato,Australia	(DAFS- G178)SCRI
SCRI45	sugar beet,USA	(Kelman- SR223)SCRI
SCRI48	? ,USA	(Kelman- SR190)SCRI

Table 1(a) cont.

Table 1(a) cont.

Strain	Description	Source
E.carotovora subsp. *atroseptica* (cont.)		
SCRI49	insects,UK	(DAFS- Fly)SCRI
SCRI52	potato,UK	SCRI
SCRI58	potato,UK	SCRI
SCRI65	potato,UK	SCRI
SCRI71	potato,UK	SCRI
SCRI82	potato,UK	SCRI
SCRI83	potato,UK	SCRI
SCRI84	potato,Peru	(CIP002)SCRI
SCRI85	potato,Peru	(CIP021)SCRI
SCRI86	potato,Peru	(CIP026)SCRI
E.carotovora subsp. *atroseptica* SCRI8		
KF2006	*rif-1*	spontaneous Rif^R,this work
KF2014	*nal-1* *str-1*	spontaneous Nal^R,Str^R, this work
KF2017	*arg-1* *nal-1* *str-1*	EMS mutagenesis of KF2014, this work

Table 1(a) cont.

Table 1(a) cont.

Strain	Description	Source
E.carotovora subsp. atroseptica SCRI8 (cont.)		
KF2018	ilv-1 nal-1 str-1	as KF2017
KF2019	nal-1 ser-1 str-1	as KF2017
KF2020	nal-1 str-1 trp-1	as KF2017
E.carotovora subsp. atroseptica SCRI13		
KF2007	rif-1	spontaneous Rif^R, this work
KF2013	nal-1 str-1	spontaneous Nal^R, Str^r, this work
KF2015	cys-1 nal-1 str-1	EMS mutagenesis of KF2013, this work
KF2021	his-1 nal-1 str-1 trp-1	repeated EMS mutagenesis of KF2013, this work
KF2022	rif-1 Tc	Tc conjugated from RE410 into KF2007, this work
KF2023	nal-1 str-1 Tc	Tc conjugated from RE410 into KF2013, this work
KF2026	his-1 gly-1 nal-1 str-1	as KF2021

Table 1(a) cont.

Table 1(a) cont.

Strain	Description	Source
E.carotovora subsp. *atroseptica* SCRI13 (cont.)		
KF2027	*his-1 leu-1 nal-1 str-1*	as KF2021
KF2029	*nal-1 str-1* Tc^S	Tc^R lost from KF2023, this work
KF2031	*rif-1* / RP4	plasmid from KF62 into KF2007, this work
KF2034	*his-1 mel-1 nal-1 ser-1 str-1*	as KF2021
E.chrysanthemi	wild strains isolated from:	
SCRI401	carnation, UK	(NCPPB426)SCRI
SCRI404	potato, Brazil	(DAFS- ENA49)SCRI
SCRI406	sugar cane, Australia	(DAFS- 15/184)SCRI
SCRI408	*Philodendron* sp., USA	(NCPPB533)SCRI
SCRI412	maize, India	(NCPPB708)SCRI
SCRI413	maize, Egypt	(ATCC27388=NCPPB1065)SCRI
SCRI416	potato, Peru	(CIP001)SCRI
SCRI418	potato, Peru	(CIP030)SCRI

Table 1(a) cont.

Table 1(b). Phage strains

Phage	Description	Source
DNA phage		
P1<u>clr100</u> KM	thermoinducible, Kan^R	RE
P1<u>clr100</u> CM	thermoinducible, Cml^R	RE
PRD1	IncP donor-specific	GMI
RNA phage		
f_2	IncF donor-specific	RE
GU5	IncP donor-specific	GMI
PRR1	IncP donor-specific	GMI
$Q\beta$	IncF donor-specific	GMI
R17	IncF donor-specific	GMI

Table 1(b). Phage strains.

Table 1(c). Plasmids.

Plasmid	Description	Source
F57	F'His$^+$	(Takano)RE
F9505	F$^+$::Tn10	(Kleckner)Kotoujansky
F'lacI$^-$::Mucts62 ProA$^+$		(Van de Putte)RE
F'Lac$^+$Tc		(Harada)RE
FR5	F'Gal$^+$ Ap Cm Sm Su	RE
F$^+$::Tn5		GMI
F'Rep[Ts114]Lac$^+$::Tn10	temperature sensitive for replication	(Botstein)GMI
pAt-C58	Agrobacterium tumefaciens C58 cryptic plasmid	GMI
pBR322	Ap Tc	DS
pGMI20	R68::Mu c$^+\Delta$445-7	GMI
pGMI22	R68::Mu c$^+\Delta$445-7	GMI
pKF1	R68::Mu c$^+\Delta$445-7	GMI(not named by GMI)
pKF2	RP4::Mu cts62	GMI(not named by GMI)
pKF3	F'lacI$^-$::Mu cts62 ProA$^+$::Tn10	F'lacI$^-$::Mucts62 ProA$^+$,F9505 recombinant, this work
pKF4	F'His$^+$::Tn10	F57, F9505 recombinant,this work
pMG1	Gm Sm Su Hg Uv	GMI
pMG5	Ak Bt Km Su Tm Hg Pma	GMI
pTi-C58	A.tumefaciens C58 Ti plasmid	GMI
R6K	Ap Sm	DS
RK2	Ap Km Tc	DS
RP4	Ap Km Tc	GMI

Table 1(c). Plasmids.

Table 1 cont.

Nomenclature is as recommended by Demerec et al.(1966) for bacterial strains, by Novick et al.(1976) for plasmids, by Campbell et al.(1977) for transposable elements and by Howe and Bade (1975) for phage Mu.

Sources of bacterial,phage and plasmid strains,with original sources in parenthesis,were as follows: ATCC-American Type Culture Collection,Rockville,Maryland,USA;Botstein,D.-Massachusetts Institute of Technology, Cambridge,Massachusetts,USA; CIP-International Potato Centre,Apartado 5969,Lima,Peru; DAFS-D.C.Graham and Watson, Department of Agriculture and Fisheries for Scotland,East Craigs,Edinburgh,UK; DS-D.Sherratt,University of Glasgow, UK; Fucikovsky,L.-Colegio de Postgraduados,Fitopatologia,Chapingo,Mexico; GMI-C.Boucher and J.Dénarié,Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes,INRA,Chemin de Borde-Rouge-Auzeville,31320 Castanet Tolosan BP12,France; Goldberg,R.B.-Goldberg et al.(1974); Harada,K.-Harada et al.(1964); Kelman,A.-University of Wisconsin-Madison,Madison,Wisconsin 53706,USA; Kleckner,N.-Massachusetts Institute of Technology,Cambridge, Massachusetts,USA; Kotoujansky,A.-Laboratoire de Pathologie Vegetale,16 Claude Bernard,75231 Paris,France; Logan,C.-Queen's University of Belfast,Belfast,Northern Ireland,UK; Murray,N.-University of Edinburgh,Edinburgh, UK; NCPPB-National Culture of Plant Pathogenic Bacteria,Harpenden,UK; Pittard,J.-Pittard and Wallace (1966); RE-Reeve,E.C.R.,University of Edinburgh,Edinburgh,UK;Rosner,J.L.-Rosner(1972); Samson,P.J.-Department of Agriculture, GPO Box192B,Hobart,Tasmania,Australia; Scaife,J.-University of Edinburgh,Edinburgh,UK; SCRI-M.C.M.Pérombelon, Scottish Crop Research Institute,Invergowrie,Dundee,UK; Stanghellini,M.E.-University of Arizona,Tucson 85721,USA; Takano,T.-referenced in Low(1972); Wijffelman,C.-State University of Leiden,Leiden,Netherlands; Van de Putte,P.-State University of Leiden,Leiden,Netherlands.

Table 1 cont.

SECTION 2

MATERIALS AND METHODS

2)a) BACTERIAL, PHAGE AND PLASMID STRAINS

The bacteria, phage and plasmids used in this study are listed in Table 1.

2)b) MEDIA

Nutrient broth	8g nutrient broth powder (Difco), 5g sodium chloride per litre of distilled water. 10ml 20% sugar, as required, added per litre after autoclaving.
Nutrient agar	as above solidified with 1.4% Difco-Bacto agar.
LB broth	10g tryptone (Difco), 5g yeast extract (Difco), 5g sodium chloride, 1g glucose, 0.1g thymine per litre of distilled water.
LB agar	as above solidified with 1.4% Difco-Bacto agar.
Top agar	15.5g tryptone (Difco), 8.75g yeast extract (Difco), 0.5g NaCl, 4.25ml 1M sodium hydroxide, 5g Difco-Bacto agar per litre of distilled water.
DST agar	40g DST agar (Oxoid) per litre of distilled water.
MacConkey (lactose) agar	51.5g MacConkey agar No.3 (Oxoid) per litre of distilled water.

MacConkey (sugar free) agar	20g peptone (Difco), 1.5g bile salts No.3 (Oxoid), 5g sodium hydroxide, 30mg neutral red, 1mg crystal violet, 15g Difco-Bacto agar per litre distilled water; sugars added to 1%.
Tetrazolium agar	As nutrient agar plus 50mg 2,3,5-triphenyltetrazolium per litre (added prior to autoclaving); sugars added to 1%.
Medium A	10.5g dipotassium hydrogen orthophosphate, 4.5g potassium dihydrogen orthophosphate, 1g ammonium sulphate, 500mg sodium citrate.$2H_2O$ per litre of distilled water.
Stewart's agar-modified	Base: MacConkey (sugar free) agar, 10mM calcium chloride. Overlay (c.10ml): 18g sodium polypectate suspended in 60ml ethanol, to which is added, very slowly, a solution of 1g EDTA and 6ml 1M sodium hydroxide in 1l of distilled water. This is steamed for 40min and autoclaved for 10min at 10p.p.s.i., then adjusted to pH7.4-7.6.
GP agar	10g peptone (Oxoid), 3g yeast extract (Difco), 2g glucose, 10g sodium glycerophosphate.$5l/2H_2O$, 1g Tris (Sigma 7-9), 250mg anhydrous calcium chloride, 15g Difco-Bacto agar per litre of distilled water; adjust to pH7.8.

M9 salts (10x)	60g anhydrous disodium hydrogen orthophosphate, 30g potassium dihydrogen orthophosphate, 10g ammonium chloride, 5g sodium chloride per litre of distilled water.
MM	M9 medium: 1ml 10mM calcium chloride, 1ml 100mM magnesium sulphate, 1ml 20% sugar and 10ml M9 salts (10x) added to 87ml autoclaved distilled water. Calcium chloride, magnesium sulphate and sugar were autoclaved separately.
MM agar	As above but solidified with 1.4% Difco-Bacto agar.
Amino acids and vitamins	Added at a final concentration of 50µg/ml as required. Casamino acids (Difco) used at 0.15% final concentration.
thymine	thymine was added at a final concentration of 80µg/ml as required.

All media (except Stewart's overlay, tryptophan, vitamins, antibiotics and antimetabolites) were sterilised at 15 p.p.s.i. for 15 min; Stewart's overlay was sterilised at 10 p.p.s.i. for 10 min, the remainder were filter sterilised.

2)c) PLASMID ISOLATIONS

Plasmid DNA was isolated by the methods of Casse et al. (1979), Eckhardt (1978), and Schwinghamer (1980). The method of Hansen and Olsen (1978) was also tried but did not give reproducible results and

so was discarded. Whilst the Schwinghamer (1980) method only gives qualitative results, it and the Eckhardt (1978) method do not incorporate an alkaline denaturation step and so are particularly suitable for plasmids which are unstable at high pH; for example IncP plasmids (Hansen and Olsen, 1978; Guiney and Helinski, 1979).

The molecular weights of plasmids were calculated by comparison with plasmids of known molecular weights: pBR322 (2.9 megadaltons (Md) and 5.8Md for the monomeric and dimeric forms; Sutcliffe, 1979), R6K (26Md; Kontomichalou et al., 1970), RK2 (40Md; Burkardt et al., 1979), pGMI20 and pKF1 (65Md; calculated from the molecular weights of R68 (40Md; Burkardt et al., 1979) and Muc$^{+}\Delta$445-7 (25Md; Chow et al., 1977)), pTi-C58 (120Md; Watson et al., 1975), pAt-C58 (273Md; Villaroe, R. and M. Van Montague pers. comm. in Rosenberg et al., 1982), pMG5 (280Md; Hansen and Olsen, 1978), pMG1 (312Md; Hansen and Olsen, 1978).

The Casse et al. (1979) method was unmodified. Isolated DNA was electrophoresed in 0.7% agarose (Sigma, Type 1, Low EEO) in Tris-borate buffer at 5-15 volts/cm.

The Eckhardt technique was modified as follows. The 'lysozyme mixture for gram-negative bacteria' contained one-fifth the bromphenol blue (that is 0.01% was used) which greatly enhanced the clarity of the gels when visualised. The procedure used here was as follows. Plasmid DNA was isolated from c.5x10^{7} bacteria (taken from a static overnight culture grown in 5ml LB in 25mm x 150mm boiling tubes). The bacteria were pelleted by centrifugation in 1.5ml Eppendorf centrifuge

tubes, and washed in 1ml TE buffer (50mM Tris, 20mM EDTA, pH8). To the washed pellets was added 40µl of the 'lysozyme mixture', and these were gently vortexed for 1-2s to give a homogeneous suspension. The suspensions were loaded immediately onto the gel (0.7% agarose). After all of the samples were loaded, 40µl of the 'SDS mixture' was loaded and partially mixed in, 100µl of the 'overlay mixture' was loaded and the wells sealed with agarose, all as described by Eckhardt (1978). Constant current electrophoresis was at 8mamps for 1h, and 40mamps for the remaining 2-3h. It was found that it was not possible to reproducibly reduce the amount of linear DNA which occurred in many of the gels. Reducing the vortexing of the bacteria in the 'lysozyme mixture', adding the 'SDS mixture' immediately, and not overloading the quantity of cells treated did help to reduce the linear DNA. Washing the bacteria in TE buffer prior to lysis slightly reduced both the linear DNA band, and the plasmid bands (Figure 2,a). Increasing the SDS concentration of the 'SDS mixture' from 0.2% to 2% significantly increased the linear DNA without increasing the plasmid DNA yield (Figure 2,a). On occasion strains showed additional plasmid bands with the Eckhardt method which were not observed on other Eckhardt gels or with other methods. These additional bands were open circular (OC) forms of the more usual covalently-closed circular (CCC), supercoiled plasmid DNA. Such OC plasmids have a lower mobility in the gel, and will only enter the gel matrix if their greater size permits. These additional bands were less frequent under conditions which reduced the quantities of linear DNA. The OC forms were not observed with the Casse et al. (1979) method as they were removed along with linear DNA, by the alkaline denaturation. The correlation between the OC plasmid bands and greater quantities of

Figure 2. Eckhardt electrophoretic gels.

Figure 2(a). Eckhardt electrophoretic gel of DNA from Eca SCRI9: method modifications.

The method as described in Section 2)c) was modified as follows: tracks A - D: 10^8 bacteria loaded per track, tracks E - H: 2×10^7 bacteria loaded per track, tracks A,B,E,H: bacterial pellet washed in 1ml TE buffer, tracks C,D,G,H: bacterial pellet not washed, tracks A,C,E,G: 'SDS mixture' containing 2% SDS, tracks B,D,F,H: 'SDS mixture' containing 0.2% SDS.

Figure 2(b). Eckhardt electrophoretic gel of plasmid transconjugants isolated from a cross between the plasmid pKF1 carrying strain KF1017 and the Crp-1$^-$ strain KF1069, either by selection for tetracycline resistance (tracks A - F) or for ampicillin resistance (tracks G - K). Track L: KF1017.

p: plasmid DNA

x: linear (chromosomal) DNA

Figure 2,a

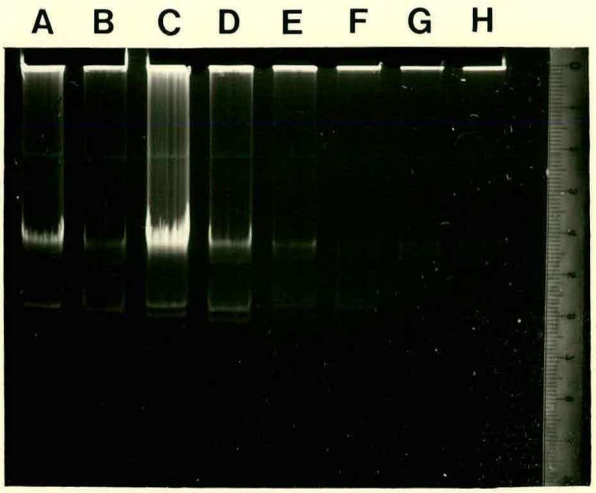
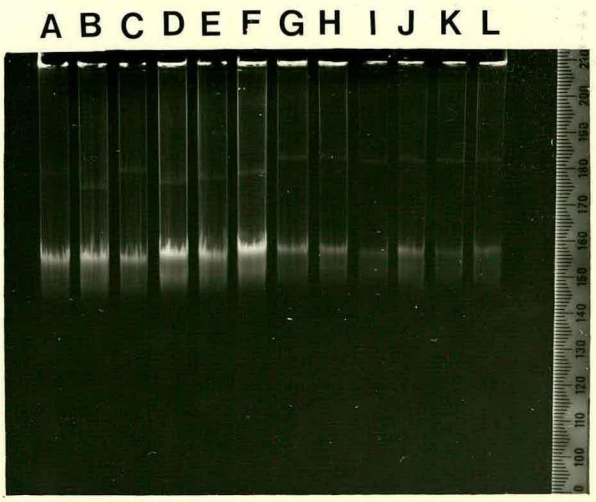


Figure 2,b



linear DNA, along with their thicker, more diffuse appearance on the gel compared to the CCC plasmid bands, reduced misscoring such bands as high molecular weight plasmids.

In the Schwinghamer (1980) technique c.10^{10} cells were lysed by lysozyme which had been driven into the cell walls by osmotic shock; after treatment with sarkosyl, the suspension was gently vortexed to disrupt the chromosomal DNA, and caesium chloride and ethidium bromide added prior to ultracentrifugation. The presence of a satellite band below that of the linear DNA was indicative of the presence of plasmid(s) in the strain. The method was modified from that of Schwinghamer (1980) by the omission of the prewash in sarkosyl, and a reduction of the lysozyme treatment time to 2.5min (from C. Boucher).

2)d) BACTERIOCINS

Glass petri-dishes were used throughout. Strains to be tested were patched onto GP agar plates (12 patches per plate) and incubated overnight at 30°C. These patches were replicated onto GP agar plates with a twelve-spiked stencil, incubated for two days at 30°C, and the bacteria killed by inverting the plates over 2ml of chloroform for 30min. The production of bacteriocins by these strains was tested by overlaying with top agar (2.5ml) containing the indicator strain (1ml of a static overnight LB culture). The plates were scored after incubation at 30°C overnight for zones of lysis around the patches. This method cannot detect the presence of phage in the strains as the patches would have obscured the very narrow zones of inhibition typically produced by phage under these conditions.

2)e) ANTIBIOTIC RESISTANCES

Wild strains were tested for resistance to various antibiotics using Oxoid Multodiscs and Sensitivity Discs by looking for strains showing narrower zones of inhibition for any given antibiotic. This test is dependent on the assumption that all of the strains of a particular species have a common basal level of sensitivity to a particular antibiotic; resistant strains are thus those which show a greater tolerance to an antibiotic. The nature of the test is such that only large differences in the degree of resistance will be detected. 100µl of a one-fiftieth dilution of an overnight LB culture of the strain to be tested was spread onto Oxoid D.S.T. agar plates, overlayed with the discs, and scored after overnight incubation at 28°C. Control E.carotovora strains carrying chromosomal and plasmid borne antibiotic resistances were used throughout.

2)f) ISOLATION OF MUTANTS RESISTANT TO ANTIBACTERIAL AGENTS

MINIMUM INHIBITORY CONCENTRATION

The minimum inhibitory concentrations (MIC) of antibiotics and antimetabolites of non-mutant parents, resistant mutants and plasmid-borne antibiotic resistances were determined by inoculating c.10^3 cells from an overnight LB culture into 1ml serial doubling dilutions of the antibiotic in LB and scoring after 1-2d incubation. The MIC was the lowest concentration of the antibiotic which prevented growth.

MUTANT ISOLATION

Spontaneous mutations in Ecc and Eca to resistance to various antibacterial agents were isolated as below. In each instance the bacteria from 10ml of an overnight LB culture incubated at 28°C were washed in 10ml saline, recentrifuged, and resuspended in 1ml saline. Samples were spread onto appropriate selection media.

Spontaneous Chlorate (CHL) resistant mutants were isolated in Ecc after 3-4 days anaerobic growth (in a GasPak jar) on glucose nutrient agar containing 2mg/ml potassium chlorate. On MacConkey lactose agar these mutants are a deep red colour with a rough colony morphology in comparison to the pink, smooth appearance of the wild type strains.

Spontaneous nalidixic acid (NAL) resistant mutants were isolated in Ecc and Eca on LB and glucose MM media containing nalidixic acid (see discussion in Section 4)b)). The most suitable method of preparation of the nalidixic acid stock solutions was that of Inoue et al. (1978): 5mg/ml nalidixic acid in 30mM sodium hydroxide. The antibiotic was used at a final concentration of 50µg/ml where single colonies were required. Higher concentrations were used where the bacteria were patched (onto glucose MM): 100µg/ml (with Nal-1^R strains) or 500µg/ml (with Nal-2^R and Nal-3^R strains).

Spontaneous rifampicin (RIF) resistant mutants were isolated on LB containing the antibiotic at 50µg/ml.

Spontaneous streptomycin (STR) resistant mutants were isolated on LB

containing 50µg/ml of streptomycin sulphate and repurified on LB plus 200µg/ml of the antibiotic.

Spontaneous trimethoprim (TMP) resistant mutants were isolated on LB containing 50µg/ml of trimethoprim.

2)g) MUTAGENESIS

Bacteria were treated with ethyl methanesulphonic acid (EMS) using the protocol of Lin et al. (1962) as described in Miller (1972). Bacteria were cultured in Medium A containing 1mM magnesium sulphate, 0.2% glucose and amino acids as required; treatment medium was Medium A containing 1mM magnesium sulphate and 200mM Tris (25.2g Tris HCl and 4.7g Tris base per litre medium) at pH7.5. To 15ml of fresh culture medium was added 1ml of an overnight culture of the strain in the culture medium; this was incubated at 28°C to a concentration of $3x10^8$ cells /ml. After washing the bacteria once in fresh medium, the bacteria were resuspended into half the original volume of the treatment medium. 2ml aliquots of this suspension, to which was added 30µl EMS, were shaken in 30ml 'Corex' centrifuge tubes at 28°C for 2h. Following treatment, the bacteria were washed in fresh culture medium, grown up in duplicate 10ml volumes of LB, glycerol added to 20%, and stored at -12°C.

2)h) ISOLATION OF AUXOTROPHS

The suspensions of EMS treated bacteria were diluted and plated on LB agar to give 200-300 colonies per plate after overnight incubation, and then replicated onto glucose MM to identify potential auxotrophs.

Colonies unable to grow on glucose MM were characterised for their nutrient requirements by replicating from LB agar onto glucose MM with various combinations of amino acids, bases and vitamins (Holliday, 1956).

Specific auxotrophs not obtained by the above method were selected from EMS treated bacteria by the ampicillin enrichment method (Miller, 1972). Bacteria were cultured in complete medium, that is glucose MM containing all required amino acids including the selected amino acid (if an auxotroph was being selected); enrichment medium was MM containing amino acids and carbon source as appropriate for the selection, (glucose was used if an amino acid requirement was being selected). An overnight culture of the strain in complete medium was washed twice in enrichment medium and sufficient of this suspension added to an appropriate volume of enrichment medium to give a 20ml suspension at $1\text{-}2\times10^7$ cells /ml. After culturing at 28°C, with shaking, to a density of no greater than 8×10^7 cells /ml (to eliminate the selected amino acid or carbon source), ampicillin was added at a final concentration of 100μg/ml. At greater cell concentrations the lysis of the growing cells, which usually occurred after 60-90min of treatment, was much reduced. Following lysis, the culture was washed twice in fresh enrichment medium, resuspended in the same volume of complete medium and cultured overnight at 28°C. From the overnight culture the required mutants were selected by replica plating from complete medium master plates onto enrichment medium plates.

2)i) BIOCHEMICAL CHARACTERISATION OF AUXOTROPHS

The nature of the auxotrophic mutations isolated in Ecc were further analysed by syntrophy (cross feeding) and by growth on appropriate media. Syntrophism was tested between the mutant Ecc under study and other Ecc and E.coli mutants using the method described by Clowes and Hayes (1968). Growth on biochemical intermediates was tested on glucose MM, with the intermediate at 50µg/ml, using the method of Clowes and Hayes (1968).

2)j) GROWTH OF Ecc MUTANTS ON POTATO TUBER SLICES AND MODIFIED STEWART'S MEDIUM

Rotting of potato tuber slices by Ecc strains was tested as follows. A loopful of a washed overnight LB culture of the strain in saline was gently rubbed onto the surface of a 5-10mm thick slice of potato tuber (variety, King Edward). This was incubated in a petri-dish containing 10ml of sterile distilled water (care being taken that the inoculated surface was not flooded), in the dark, at room temperature and scored after 1-2d.

The degradation of pectate by Ecc strains was tested by streaking a sample of the strain, for single colonies, onto modified Stewart's medium. It was observed that the pitting of the overlayer around pectolytic colonies was enhanced by the use of MacConkey agar underlayer which did not contain a carbon source.

2)k) PHAGE P1

The isolation of E.carotovora strains sensitive to phage P1 was by the method of Goldberg et al. (1974) whereby lysogens are recovered by selecting for chloramphenicol resistance (with P1clr100 CM) or kanamycin resistance (with P1clr100 KM) (Rosner, 1972; Goldberg et al., 1974).

Phage lysates were prepared by temperature induction of the E.coli lysogens KF8 (ED8812 /P1clr100 KM) and KF21 (ED8812 /P1clr100 CM) by the method of Goldberg et al. (1974). Cultures of the lysogen in LBC (LB, 5mM calcium chloride) were cultured to c.5x10^8 cells /ml at 30°C, heat induced at 40°C for 30min, and then cultured for c.90min at 30°C by which time lysis had usually occurred. After sterilisation with 1% chloroform, cell debris was removed by centrifugation and the lysate stored at 4°C. Titres of lysates prepared for each phage were from 2-4x10^9 plaque forming units /ml. To select P1 lysogens of E.carotovora strains, phage were added at a multiplicity of infection of 2 to overnight cultures of the bacteria grown in LBC and concentrated five-fold by centrifugation. Following adsorption at room temperature for 30min, samples were spread onto plates selective for lysogenic cells (LB with either chloramphenicol or kanamycin at 20µg/ml) and incubated at 28°C. Antibiotic-resistant colonies were purified on the same media and then tested for inability to grow at 37°C (if the parent strains could grow at this temperature), and for ability to produce phage by spotting onto a lawn of E.coli ED8874 and scoring for phage production. Curing of the prophage was by selection

for survivors following incubation at 37°C on antibiotic free LB medium.

2)l) IncF PLASMIDS

MATING AND TRANSCONJUGANT SELECTION

All matings involving IncF plasmids were performed with parental strains cultured overnight in LB; E.coli strains were cultured at 37°C (unless the plasmid was temperature-sensitive), and E.carotovora strains at 30°C. Unless otherwise stated, the ratio of donors to recipients was c.1:2; thus in liquid culture 1-2x10^8 donors /ml were mated with 2-4x10^8 recipients /ml. The matings were either (i) in LB in 100ml flasks, or 25mmx150mm boiling tubes, static, (ii) in LB in 100ml flasks gently shaken in a water bath with a 1cm reciprocal movement at 90-100 cycles /min, (iii) on the surface of LB agar by mixing together 50µl of a culture of each parent, (iv) on a membrane incubated on LB agar - as described in Section 2)m).

The concentrations of parental strains and transconjugants from matings were scored by selective growth on MM with supplements as required, and usually with a glucose carbon source. Where possible the antibiotic resistances of the strains were selected, particularly to distinguish between parentals and to recover transconjugants. The use of nalidixic acid when determining the frequency of plasmid transconjugants prevented continued plasmid transfer on the selection plates.

CURING F'Lac$^+$Tc PLASMID

The frequency of curing of F-factors from bacteria can be enhanced by culture in media containing acridine orange. The MIC of acridine orange of Ecc SCRI193 strain KF1037 and Eca SCRI13 strain KF2022 were determined by the usual method and found to be 250µg/ml for both strains. To cure the F'Lac$^+$Tc plasmid from Ecc the method of Miller (1972) was used with modifications: cultures were spread onto LB agar (pH7.2) containing acridine orange, and following overnight incubation were replicated onto LB plus 20µg/ml tetracycline to detect colonies of cured cells. Two concentrations of acridine orange were used, the 25µg/ml recommended by Miller (1972) and 125µg/ml (the maximum concentration at which these strains can grow). Curing of tetracycline resistance from the Ecc SCRI193 Str^R Nal^R strain KF1062 occurred at 2% of survivors at the lower concentration and at 0.5% at the higher concentration, while curing from the Rif^R strain KF1037 was not observed at either the lower (<0.3%) or the higher (<0.08%) concentrations of acridine orange. Curing of tetracycline resistance was also observed from the Eca Str^R Nal^R strain KF2023 but not from the Rif^R strain KF2022, all at the same frequencies as for the Ecc SCRI193 strains.

2)m) PLASMID::Mu COINTEGRATES AND PHAGE Mu

MATING AND TRANSCONJUGANT SELECTION

The parental strains were cultured overnight at 30^{o}C (for Ecc) or at 37^{o}C (for E.coli) in LB without antibiotic selection. Appropriate volumes of each culture were added to a sufficient volume of LB to give 10^9 cells of each parent in a final volume of 20ml. From this, 4ml samples were filtered down onto membrane filters (25mm, Oxoid

'Nuflow' cellulose acetate membrane filter, 0.45μm pore size). The filter was incubated on LB agar at the required temperature (usually 30°C) for the duration of the mating (usually overnight - 18h) after which the mating was terminated by vigorously vortexing the membrane in 1-3ml of saline for 1min. Appropriate dilutions were spread onto selection plates. The concentrations of each parent and the transconjugants were measured at the start of the mating using a replicate filter. Back mutation frequencies of relevant recipient strain mutations were measured by concentrating the overnight broth culture 5-10-fold, washing twice in saline, and spreading onto appropriate selection plates.

MM media with appropriate supplements were used to select for the parental strains and the transconjugants. The plates were scored after 2-4d incubation at 28°C. The concentrations of donor strains containing plasmids conferring resistance to tetracycline were determined on plates with and without tetracycline, as the concentrations of such strains was observed to be lower on plates with tetracycline; presumably as a result of delayed expression of the tetracycline resistance.

The coinheritance of markers was determined by patching transconjugants (45 patches per plate) onto the original selection medium and, after 2d incubation, replicating these onto fresh plates selective for the coinherited markers. These were scored after 1-2d incubation at 28°C. Preliminary tests showed that purification of the transconjugant colonies to remove contaminating parental bacteria prior patching for the coinheritance test was not necessary. The

coinheritance of Mu was ascertained by replicating the master plate onto LB, overlaying with a lawn of the Mu sensitive E.coli ED8812, and after incubation at 37°C, scoring patches which had lysed the lawn.

MEASUREMENT OF Mu PRODUCTION

The presence of Mu in the lysate of a strain was tested using the Mu sensitive indicator strain E.coli ED8812; strains KF63 (ED8812::Mu) and GMI3230 (C600 Mu resistant) were used as negative controls (Mu does not form plaques on these strains).

A qualitative measurement of the presence of Mu in a strain was made by spotting 10µl of an overnight culture of the strain onto a lawn of an indicator bacterium in top agar, and incubating overnight at 37°C.

A quantitative measurement of the concentration of free Mu particles in a culture was made by treating the culture to be tested with chloroform, removing the cell debris by centrifugation, sparging the culture with sterile air to remove the chloroform, and pouring appropriate serial dilutions of the culture with 0.2ml of an overnight LB culture of the indicator strain ED8812 in top agar onto LB agar plates and scoring after overnight incubation at 37°C.

INDUCTION OF Mu

Induction was by the method of Murooka et al. (1981). The Mucts lysogen was cultured at 30°C overnight in LB 5mM calcium chloride. This was then diluted into fresh LB 5mM calcium chloride, 200mM

magnesium sulphate, to an optical density of 0.7, and temperature induced at 43°C with vigorous aeration (by sparging with air) for 30min, followed by incubation at 37°C with continued vigorous aeration. Samples were taken as required. As evaporation of the culture medium was significant, sterile distilled water was added to compensate for the reduction in volume.

Partial induction of Mucts lysogens was by incubating a 20ml culture of the strain in LB in a large static flask at 36°C overnight.

Mu INSERTION MUTATIONS

The transposition of Mu onto the Ecc chromosome was selected by the following methods after zygotic or temperature induction:

Acquisition of trimethoprim resistance as a result of insertion of Mu into thyA (using the selection procedure in Section 2)f)) following zygotic induction.

Mu insertions into the chromosome, after zygotic induction of His^+ chromosomal transconjugants (in crosses between KF1017 donors and non-lysogenic, multiply auxotrophic recipients) were sought by scoring the His^+ transconjugants for the coinheritance of Mu (and the absence of the mobilising plasmid pKF1), by the replica plate method above.

Enrichment of zygotically induced cells for xylose mutants (xyl) and of temperature induced lysogens for auxotrophy, was by D-cycloserine enrichment (using the ampicillin enrichment method with the

substitution of D-cycloserine at 200μg/ml for the ampicillin. (The MIC of D-cycloserine of Ecc SCRI193 is 62μg/ml).

2)n) DONOR-SPECIFIC PHAGE

Pilus production by strains was tested by determining their sensitivity to the appropriate donor-specific phage. Thus the IncP plasmids RP4 and R68 rendered strains sensitive to the RNA phages GU5 and PRR1 (Hua et al., 1981) and the DNA phage PRD1 (Bradley and Cohen, 1977), while IncF plasmids rendered transconjugants sensitive to the RNA phages ϕf_2, R17, and Qβ. The use of different phages allowed for possible differences in the expression of the phages in the different bacterial strains, and the possibility of false negative results.

Lysates of these phage were prepared on E.coli K12 strains carrying either RP4 or F'Lac$^+$Tc as appropriate, and were stored over chloroform (GU5 was filter sterilised instead as it is chloroform sensitive) at 4°C; the titres of these lysates was from 10^8-10^{10}p.f.u./ml. The test for the presence of pili was by spotting 5μl of the appropriate lysates onto a lawn of the bacterium in soft LB agar; clearing of the lawn after overnight incubation at 30°C was indicative of the susceptibility of the strain to the phage. The RNA phages were used in preference to the DNA phages as this avoided the possibility of the restriction of the phage by the strain and thus an incorrectly scored strain.

SECTION 3

PLASMIDS IN WILD STRAINS OF E.carotovora AND OTHER Erwinia SPECIES

3)a) Introduction

It has long been realised that not all of the characteristics possessed by bacteria are necessarily chromosomally inherited; extrachromosomal elements, either phage or plasmids, can play a role, often contributing secondary or supplementary characteristics which are not strictly required for the survival of the bacterium. It has been suggested that they may play a significant part in the evolution of bacterial species by allowing gene acquisition and maintenance, and transfer systems, which are not dependent on mutational changes to the pre-existing genome of the bacterium (Anderson, 1966).

Plasmids have been found in several Erwinia species. In E.amylovora up to three plasmids have been found in some strains upwards in molecular weight of 1Md (megadaltons) (Panopoulos et al., 1978). Plasmids of diverse sizes were found in a number of isolates of E.chrysanthemi from maize: one strain analysed in some detail contained cryptic plasmids of 5Md and 50Md (Sparks and Lacy, 1980). In several clinical isolates of E.herbicola a 65Md, E-Lac$^+$ plasmid was identified which was compatible with plasmids of twelve other incompatibility groups including IncF (Chatterjee and Starr, 1973,b; Chatterjee et al., 1978). Gibbins et al. (1976) reported that one strain of E.herbicola examined had a 6.4Md cryptic plasmid while Gantotti and Beer (1982) located thiamine prototrophy and yellow pigmentation to a 350Md plasmid in two strains of E.herbicola.

E.stewartii isolates have been reported to "contain at least eight species of plasmid DNA (D.L.Coplin and M.Rudinski, unpublished)" (Coplin, 1978), and some of these may belong to either IncFI or to IncP (D.L.Coplin and R.Rowan, unpublished, in Coplin, 1978). Coplin and Rowan (1978) reported the recovery of conjugative plasmids in two strains of E.stewartii which could mobilise a derivative of the non-conjugative plasmid ColE1. There are no reports of plasmid searches in E.carotovora.

The plasmid content of several strains of Ecc, Eca and E.chrysanthemi were examined using methods which were able to resolve both small and large plasmids. Several of the strains were also tested for bacteriocin production and high-level antibiotic resistances as these are often plasmid borne (Echandi and Moyer, 1979; Gantotti and Beer, 1982). Some plasmid carrying strains were tested for sensitivity to donor-specific phages and for incompatibility of the resident plasmids to the IncP plasmid RP4.

3)b) PLASMIDS IN WILD STRAINS OF E.carotovora AND OTHER Erwinia SPECIES

Those strains of Ecc, Eca and E.chrysanthemi which were tested for the presence of plasmids, the plasmid isolation methods used, and the results, are listed in Table 2.

Using these methods it was possible to resolve plasmids up to at least 100Md in E.carotovora (i.e. F'Lac^{+}Tc) and up to 300Md in other bacterial species (i.e. pMG1 and pMG5 in E.coli and pAt-C58 in A.tumefaciens). The molecular weights of the plasmids in some of these E.carotovora strains are given in Table 3(a), and a summary of the plasmid contents of all of the E.carotovora strains in Table 3(b).

A comparison of the results from the Casse et al. (1979) method with those from the Eckhardt (1978) method shows that there was generally good agreement between the two methods. The Schwinghamer (1980) method was used to analyse five of the Eca strains which had been found to be plasmid free using the other two methods. In all five cases plasmids were not detected.

Several of the small plasmids observed were probably multicopy - as judged from the intensity of the plasmid bands on the gels. That the small plasmids in those strains with two or more small plasmids (e.g. Eca SCRI9) were in fact monomers and dimers of a single plasmid is suggested from the calculated molecular weights of these plasmids, a phenomenon most commonly associated with multicopy plasmids (in recombination proficient genetic backgrounds).

Table 2.Plasmids in wild strains of E.carotovora and E.chrysanthemi.

Strains	Plasmid isolation method[a]							
	Casse et al.(1979)			Eckhardt(1978)			Schwinghamer(1980)	
	Number plasmids[b]		Number expts.[c]	Number plasmids[b]		Number expts.[c]	Plasmids[b]	Number expts.[c]
	<10Md	>20Md		<10Md	>20Md			
Ecc								
SCRI101				0	1			
SCRI102				0	0	*		
SCRI103				0	0	*		
SCRI109				0	0	*		
SCRI110	0	0	*	0	0			
SCRI112				2	0			
SCRI113	0	0	*	0	0	*		
SCRI114	1	1	*	2	1			
SCRI115				0	0			
SCRI117				0	0	*		
SCRI118				0	0	*		
SCRI119	3	0	*	3	0	*		
SCRI120				0	0	*		
SCRI121				0	0	*		
SCRI122				0	0	*		
SCRI124	1	1	*	0	1	*		
SCRI125				1	0			
SCRI126				0	0			
SCRI127				1	0			
SCRI130				0	0	*		
SCRI132				0	0	*		
SCRI135				0	0	*		
SCRI139				0	0	*		
SCRI144				0	0	*		
SCRI149				1/2	0			
SCRI152				2	0			
SCRI155				0	0	*		
SCRI166				0	0	*		

Table 2 cont.

Strains	Plasmid isolation method[a]								
	Casse et al.(1979)			Eckhardt(1978)			Schwinghamer(1980)		
	Number plasmids[b]		Number expts.[c]	Number plasmids[b]		Number expts.[c]	Plasmids[b]		Number expts.[c]
	<10Md	>20Md		<10Md	>20Md				
Ecc									
SCRI169				0	0	*			
SCRI172				3	0				
SCRI174				0	0	*			
SCRI178				3	0				
SCRI191				0	0	*			
SCRI192				0	0				
SCRI193	0	0	*	0	0		0		*
SCRI198				0	0	*			
SCRI205				0	0	*			
SCRI211				0	0	*			
SCRI238	0	0	*	0	0	*			
Eca									
SCRI1	0	0	*	0	0		0		*
SCRI3				0	0				
SCRI5				0	0				
SCRI6	0	0	*						
SCRI8				0	0				
SCRI9	2	1	*	3	1				
SCRI13	0	0	*	0	0		0		*
SCRI16	0	0	*	0	0		0		*
SCRI19	2	1	*						
SCRI22				0	0	*			
SCRI26				0	0	*			
SCRI27				0	0				
SCRI28				0	0	*			
SCRI31	0	0	*	0	0		0		*
SCRI39				1	1				
SCRI44				0	0				

Table 2 cont.

Strains	Plasmid isolation method[a]							
	Casse et al.(1979)			Eckhardt(1978)			Schwinghamer(1980)	
	Number plasmids[b]		Number expts.[c]	Number plasmids[b]		Number expts.[c]	Plasmids[b]	Number expts.[c]
	<10Md	>20Md		<10Md	>20Md			
Eca								
SCRI45				0	1			
SCRI48				0	0			
SCRI49				0	0			
SCRI52	0	0	*	0	0	*	0	*
SCRI58				0	2			
SCRI65				0	2			
SCRI71				0	0	*		
SCRI82				1	1			
SCRI83				3	0/1			
SCRI84				0	0			
SCRI85				2	0			
SCRI86				0	0	*		
E.chrysanthemi								
SCRI401				0	0	*		
SCRI404				0	0	*		
SCRI406				0	0	*		
SCRI408				0	0	*		
SCRI412				0	1			
SCRI413				0	0	*		
SCRI416				0	0	*		
SCRI418				0	0	*		

a:The plasmid isolation methods used are described in Materials and Methods. b:With the Casse et al.(1979) and Eckhardt (1978) methods the number of plasmids in a strain could be determined and these were classified according to molecular weight: plasmids of <10Md which banded below the linear, chromosomal DNA band, and plasmids of >20Md which banded above the linear, chromosomal DNA band. Plasmids banding within the linear, chromosomal DNA were not reproducibly resolved. Plasmids were either present (+) or absent (-) from strains analysed by the Schwinghamer (1980) method. c:Strains were tested from two to five times, those asterisked were screened using a single, well prepared gel (or gradient).

Table 3(a).The molecular weights of plasmids in some wild strains of *E.carotovora*.

Strain	Plasmid MW x 10^{-6} daltons
Ecc	
SCRI125	6.7
SCRI127	4.6
Eca	
SCRI9	2.8,5.0,5.7,75
SCRI45	32
SCRI58	28,87
SCRI65	46,57
SCRI82	3.2,20

Plasmid molecular weights (corrected to two significant figures) were calculated from two,three or four Eckhardt gels by comparison with plasmids of known molecular weights (pAt-C58, pBR322, pGMI20, pKF1, pMG1, pMG5,pTI-C58, R6K, RK2).

Table 3(b).Summary of the plasmid contents of wild strains of *E.carotovora*.

	Ecc	*Eca*
Strains tested	39[a]	28
Without plasmids	28	19
With plasmids	11	9
With plasmids >20Md	3	7 or 8[b]
With plasmids <10Md	10	6

a: Number of strains. b: Gels did not reproducibly resolve a large plasmid in *Eca* SCRI83 (see Table 2).

3)c) BACTERIOCINS, ANTIBIOTIC RESISTANCES AND INCOMPATIBILITY OF PLASMIDS IN E.carotovora STRAINS

BACTERIOCINS

As shown in Table 4 and summarised in Figure 3, bacteriocin production was not detected in any of the Eca or E.chrysanthemi strains tested; bacteriocin production was detected in 37% of the Ecc strains tested. It should be noted that 50% of the bacteriocinogenic strains did not harbour plasmids so that either the bacteriocinogenic genes are chromosomally located in these strains, or plasmids are present in them but were not detected. The latter hypothesis is supported by the inherent uncertainty of knowing whether a strain is truly plasmid free, the possibility of plasmids in the range 10-20Md and >100Md being present in strains but undetected, and also the circumstantial observation that to date there are no reports of naturally occurring chromosomally located bacteriocinogenic genes.

ANTIBIOTIC RESISTANCES

Ecc strains SCRI101 and SCRI114 and Eca strains SCRI9 and SCRI39, all of which harbour plasmids of more than 20Md were tested, along with eight other putatively plasmid-free strains, for high level resistance to various antibiotics (Table 5). None of the plasmid-bearing strains, nor the putatively plasmid-free strains, were resistant to any of the antibiotics.

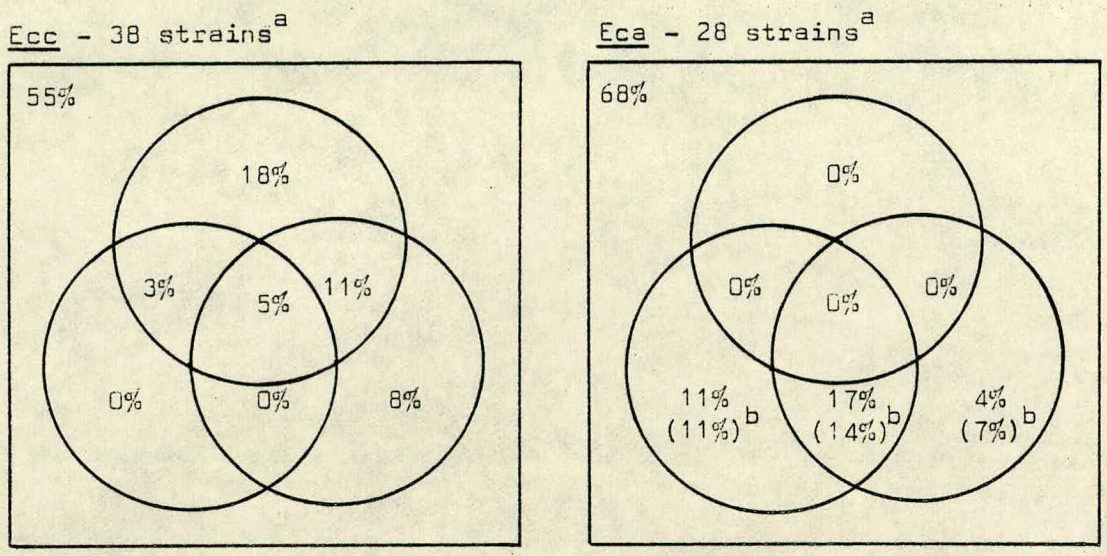
Table 4. Bacteriocin production by wild strains of *E.carotovora* and *E.chrysanthemi*.

Strain	Indicator strain															
	Ecc												*Eca*			
	SCRI106	SCRI112	SCRI113	SCRI114	SCRI119	SCRI121	SCRI124	SCRI126	SCRI192	SCRI193	SCRI205	SCRI211	SCRI8	SCRI13	SCRI31	SCRI408 (*E.chrysanthemi*)
Ecc																
SCRI101	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+
SCRI110	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
SCRI114			-							-	-	-	-	+	-	-
SCRI121			-							-		-	+	+	-	-
SCRI122			-							-		-	-	+	-	-
SCRI124			-							-		-	-	+	-	-
SCRI130			-							+		-	-	-	-	-
SCRI149			-							-	-	-	+	+	+	-
SCRI152	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
SCRI172	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-
SCRI174			-							-		-	-	-	-	+
SCRI178	-	-	+	-	-	-	-	+	-	+	+	-	+	+	+	+
SCRI205	-	+	-	-	-	-	-	+	-	+	-	-	+	+	+	+
SCRI211	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-

Strains were scored for the presence (+) or absence (-) of lytic zones in the overlayer of indicator bacteria around the colony under test.

None of the following strains produced lytic zones: *Ecc* strains SCRI102[c], SCRI103[c], SCRI109[a], SCRI112[b], SCRI113[a], SCRI115[a], SCRI117[c], SCRI118[c], SCRI120[c], SCRI125[b], SCRI126[c], SCRI127[b], SCRI132[c], SCRI135[a], SCRI139[a], SCRI144[a], SCRI155[a], SCRI166[a], SCRI169[c], SCRI191[c], SCRI192[c], SCRI193[c], SCRI198[c], SCRI238[c]; *Eca* strains SCRI1[a], SCRI3[a], SCRI5[a], SCRI8[a], SCRI9[b], SCRI13[a], SCRI16[a], SCRI22[a], SCRI26[a], SCRI27[a], SCRI28[a], SCRI31[a], SCRI39[b], SCRI44[a], SCRI45[b], SCRI48[b], SCRI49[a], SCRI58[b], SCRI65[b], SCRI71[a], SCRI82[b], SCRI83[b], SCRI84[a], SCRI85[c], SCRI86[c]; *E.chrysanthemi* strains SCRI401[b], SCRI404[b], SCRI406[b], SCRI408[b], SCRI412[b], SCRI413[c], SCRI416[c], SCRI418[c] when tested on indicator strains: c=(SCRI113, SCRI193, SCRI211, SCRI8, SCRI13, SCRI31, SCRI408), or b=(c+ SCRI205), or a=(b+ SCRI106, SCRI112, SCRI114, SCRI119, SCRI121, SCRI124, SCRI126, SCRI192).

Figure 3. Distribution of plasmids and bacteriocins in E.carotovora.



Figures show the % frequencies of strains which produced bacteriocins (see Table 4) (upper circle), possessed plasmids of greater than 20Md (see Table 2) (lower left circle), or possessed plasmids of less than 10Md (see Table 2) (lower right circle).

a: Strains tested for both bacteriocin production and plasmid content.

b: Frequencies calculated assuming that Eca SCRI83 does, or does not (in parenthesis) possess a plasmid of greater than 20Md.

Table 5. Resistance to antibiotics of wild strains of E.carotovora.

Strain	Antibiotic (µg/disc)													
	ampicillin (25)	carbenicillin (100)	chloramphenicol (50)	colistin sulphate (30)	gentamicin (30)	kanamycin (30)	nalidixic acid (30)	nitrofurantoin (200)	rifampicin (2)	spectinomycin (10)	streptomycin (25)	sulphafurazole (500)	tetracycline (50)	trimethoprim (5)
Ecc														
SCRI101	-	-	-		-	-	-		+	-	-	-	-	-
SCRI110	-	-	-		-	-	-		+	-	-	-	-	-
SCRI114	-	-	-		-	-	-		+	-	-	-	-	-
SCRI193	-	-	-	-	-	-	-	-	+	-	-	-	-	-
SCRI221	-		-	-		-		-			-	-	-	
SCRI238	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Eca														
SCRI9	-	-	-		-	-	-		+	-	-	-	-	-
SCRI13	-	-	-	-	-	-	-	-	+	-	-	-	-	-
SCRI31	-	-	-	-	-	-	-	-	+	-	-	-	-	-
SCRI39	-	-	-		-	-	-		+	-	-	-	-	-
SCRI52	-		-	-		-		-			-	-	-	

Strains were scored for resistance (+) or susceptibility (-) to the above antibiotics by the method described in Materials and Methods - Section 2)e).

E.carotovora strains were found to be resistant to rifampicin at concentrations upto 7µg/ml; resistant mutants were resistant to 100-200µg/ml of the antibiotic (Section 4)b)).

PLASMID INCOMPATIBILITY GROUPS

Ecc strains SCRI101 and SCRI114 and Eca strains SCRI9 and SCRI39 were not sensitive to the IncF donor-specific phage ϕf_2 or to the IncP donor-specific phages GU5, PRD1 or PRR1, suggesting that these strains do not contain IncF plasmids which are fi$^-$ (F), or IncP plasmids.

The plasmids in Eca strains SCRI9 and SCRI39 were tested for compatibility with the IncP1 plasmid RP4 by transferring this plasmid from E.coli into the Eca strains. Eckhardt gels of these Eca transconjugants showed that they had both their original plasmid complement in addition to RP4, implying that neither of the Eca strains harboured IncP1 plasmids.

3)d) CONCLUSIONS

As might be expected from their common occurrence in many bacterial species, including members of the Erwinia (see above), plasmids up to 100Md were found in both Ecc and Eca. When some of these plasmid carrying strains were tested, they did not carry IncF or IncP group plasmids, nor did they carry resistances to high concentrations of several different antibiotics. Bacteriocin production, which is usually plasmid borne (Hardy, 1975), was only observed from Ecc strains (not Eca strains) and of these bacteriocin producing strains, plasmids were not observed in half of them; the implications of this have already been discussed.

It is interesting to ask why the plasmids observed here are maintained. A survey by Caugant et al. (1981) of the E.coli population of a healthy human host found that of the 64 isolates tested for plasmid content, 58 had plasmids, many having several, which ranged in size from 1Md up to 80Md; all but one of these were cryptic. This proportion of strains carrying plasmids is far greater than was found in the present study. Caugant et al. (1981) discussed why so many of their isolates contained plasmids, and their arguments apply equally well here. Thus, plasmids derepressed for transfer could theoretically pass throughout a population even if they were at a selective disadvantage. In practice, however, most plasmids are found to be repressed, and are only able to transfer to recipients which have compatible resident plasmids, therefore "it is highly unlikely that 'unselected' conjugative plasmids would be maintained in bacterial populations". In the case of non-conjugative plasmids their

maintenance in the absence of positive selection will be even less likely than that of conjugative plasmids. Therefore in general the maintenance of plasmids is unlikely unless there are at least occasional periods when their presence confers a selective advantage.

The phytopathogenicity of many bacterial species is plasmid-borne. The Ti-plasmid of Agrobacterium tumefaciens, which is over 100Md molecular weight, is involved in many of the functions of crown-gall disease. A plasmid in Pseudomonas syringae is required for the production of the syringomycin toxin which has been implicated in the holcus spot disease of maize (Gonzalez and Vidaver, 1977; Staskawicz et al., 1981). In the Enterobacteria plasmids are implicated in pathogenicity of several species of animals, including man. For example in E.coli, toxin production and binding to the intestinal wall are plasmid-borne characteristics (Elwell and Shipley, 1980). Virulence-associated plasmids have also been found in Yersinia enterocolitica and Y.pestis (Portnoy and Falkow, 1981). The rhizobial plasmids are required for many of the functions involved in the formation of effective root-nodules, and contribute to the host-specificity of the bacterium (Johnston et al., 1978; Prakash et al., 1981).

Despite the coincidence of plasmids and the genetic basis of pathogenicity in many bacterial species, there is no report of such being the case in the soft-rot Erwinia, E.chrysanthemi, or in other more distantly related Erwinia species (Chatterjee and Starr, 1980; Leary and Fulbright, 1982). Subject to the qualification that plasmids were not present in several E.carotovora strains, not as a

consequence of inefficient plasmid isolation methods, but rather because they were indeed not present; plasmid determined inheritance of phytopathogenic function(s) can be discounted in E.carotovora also. It is interesting to note however, that Eca SCRI45, which was isolated from sugar-beet, has a plasmid of 31Md; several workers have suggested that the sugar-beet strains of Eca may be either a separate subgroup of Eca or possibly a distinct subspecies of E.carotovora (Stanghellini et al., 1977; Thomson et al., 1981).

SECTION 4

THE ISOLATION OF GENETICALLY MARKED E.carotovora STRAINS

4)a) INTRODUCTION

Spontaneous and induced chromosomal mutations were isolated in Ecc SCRI193 and Eca SCRI8, and SCRI13. The pedigrees of these mutants are shown in Figures 4,5 and 6. Gene and strain nomenclature is as recommended by Demerec et al. (1966), and where possible is the same as that in E.coli.

Figure 4. Pedigrees of Ecc SCRI193 mutants.

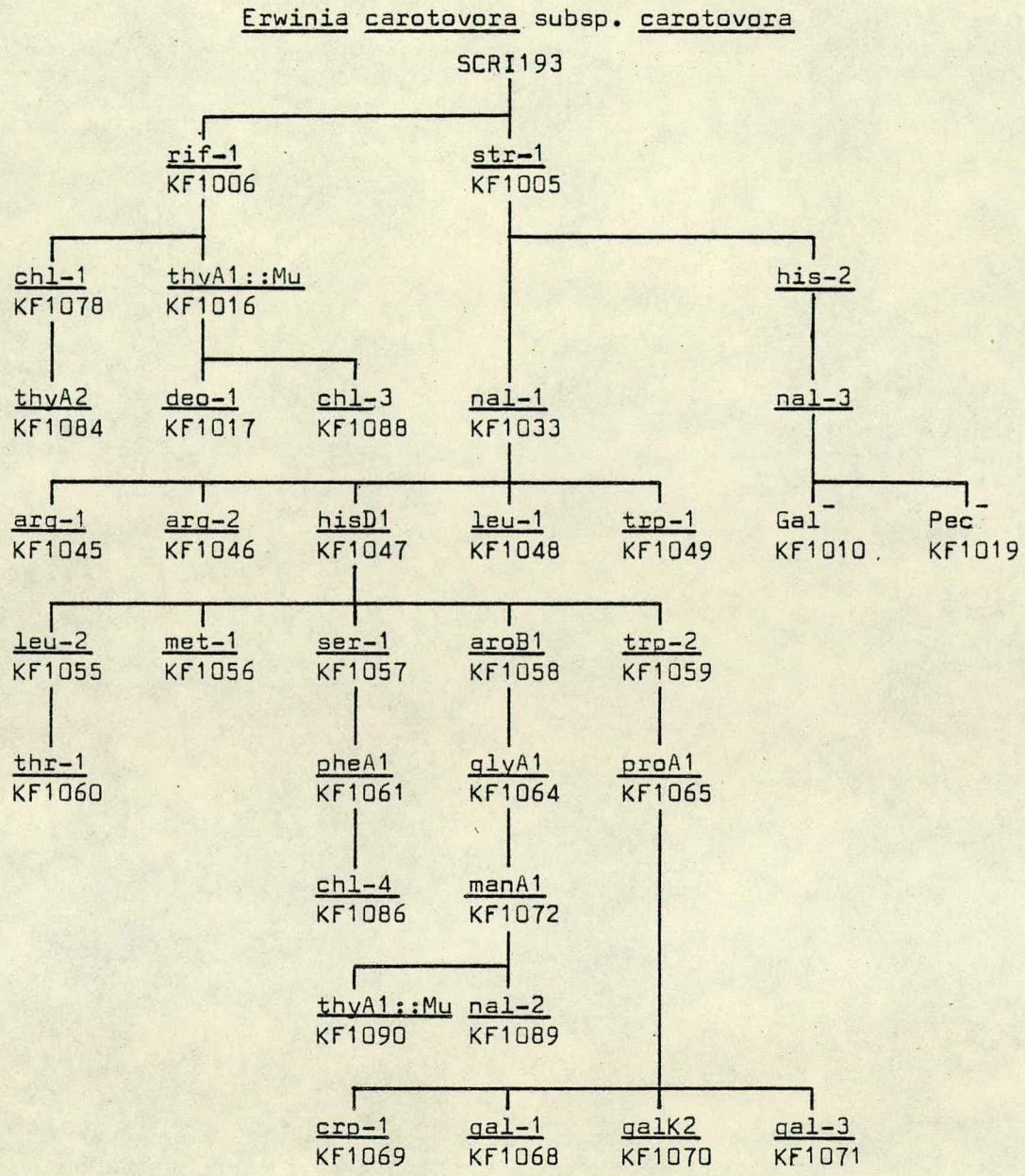


Figure 5. Pedigrees of *Eca* SCRI8 mutants.

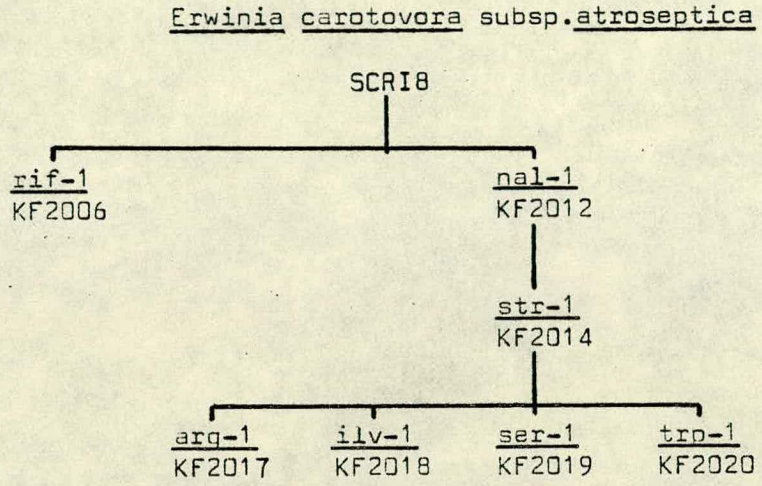
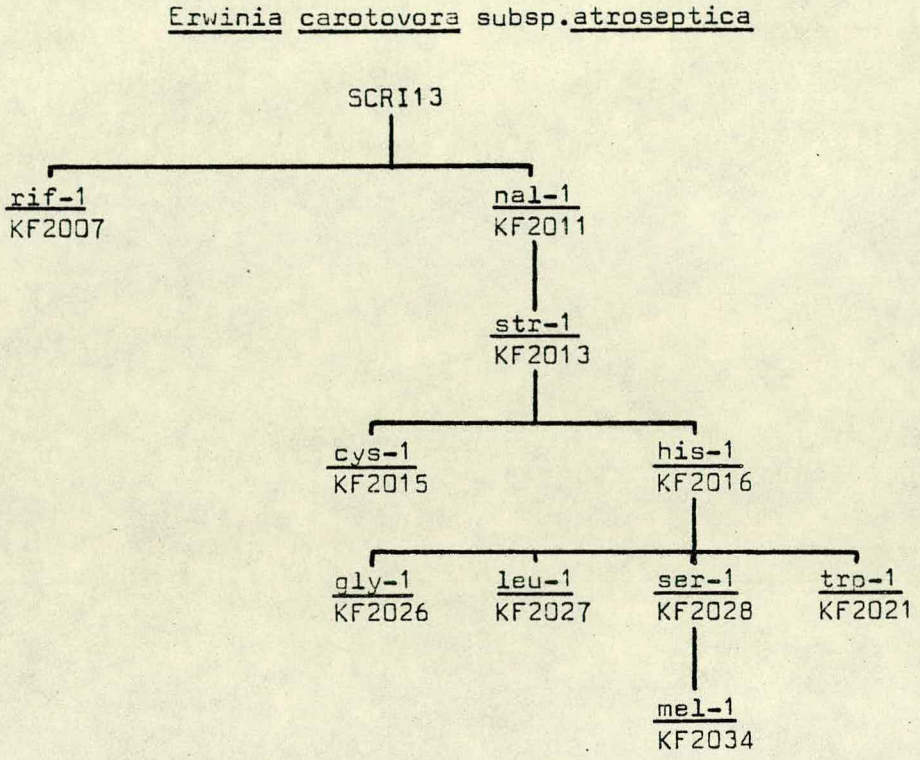


Figure 6. Pedigrees of *Eca* SCRI13 mutants.



4)b) ISOLATION AND CHARACTERISATION OF MUTANTS RESISTANT TO ANTIBACTERIAL AGENTS

CHLORATE: The spontaneous chlorate resistant mutants chl-1,-3 and -4 were isolated in Ecc SCRI193. Mutants of this type in E.coli result from the loss in activity of the formate-nitrate reductases (Bachman, 1983).

NALIDICIC ACID: Spontaneous nalidixic acid (NAL) resistant mutants were isolated in Ecc and Eca. The method of preparation of nalidixic acid solutions was found to be of great importance, particularly the concentration of sodium hydroxide used to neutralise the nalidixic acid. Thus, the preparation of stock solutions of nalidixic acid as recommended by Miller (1972) (2mg nalidixic acid/ml in 140mM sodium hydroxide) results in medium too alkaline for bacterial growth when the antibiotic is used at concentrations greater than c.85µg/ml (KF1033 was able to grow in LB or MM at up to pH11. The method of preparation of Inoue et al. (1978) (5mg nalidixic acid/ml in 30mM sodium hydroxide) did not result in a pH change of either LB or MM when nalidixic acid was used at final concentrations up to at least 500µg/ml.

The Ecc SCRI193 mutations nal-1 and nal-2 were both isolated on LB agar containing 20µg/ml nalidixic acid using the method of Miller (1972), the nal-2 mutation was isolated in the nal-1 carrying strain KF1072, on glucose MM containing 100µg/ml nalidixic acid prepared by the method of Inoue et al. (1978). The MICs (µg/ml) of these strains were:

Mutant	Strain	MIC determined in	
		LB	glucose MM
Nal^S	KF1078	2	2
$Nal\text{-}1^R$	KF1033	1000	125
$Nal\text{-}1^R$ $Nal\text{-}2^R$	KF1089	1000	1000
$Nal\text{-}3^R$	KF1019	1000	1000

In E.coli there are three loci governing resistance to nalidixic acid. NalA mutations are defective in the A-subunit of DNA gyrase (gyrA) (Gellert et al., 1977; Sugino et al., 1977). The resistance mechanism of nalB mutants is unknown (Hane and Wood, 1969). NalC mutations in addition to conferring resistance to nalidixic acid, have variable cross-resistances to pipemidic acid and piromidic acid (structurally related to nalidixic acid), and are the result of mutations in the B-subunit of DNA gyrase (gyrB) (Inoue et al., 1978, 1982). The MICs of these E.coli mutants to nalidixic acid are: Nal^S, 2μg/ml; $NalA^R$, 6 - >100μg/ml; $NalB^R$, <10μg/ml; $NalC^R$, 6 - >100μg/ml (Inoue et al., 1978). The nalA and nalB mutations are also found in S.typhimurium but nalC mutants have not been reported (Sanderson and Hartman, 1978).

Since pipemidic and piromidic acids are not commercially available,

and not all strains carrying nalC show resistance to them anyway (Inoue et al., 1978), the only distinction between nalA and nalC is their chromosomal positions. From the MIC levels obtained for the nalidixic acid resistant Ecc strains (assuming similarity between Ecc and E.coli) it is unlikely that the nal-1, nal-2 or nal-3 mutations map at the nalB locus.

RIFAMPICIN: Spontaneous rifampicin (RIF) resistant mutants were isolated in Ecc, at a frequency of 6×10^{-8}/cell, and also in Eca. The MICs (μg/ml), determined in LB, were:

	Rif^S	Rif^R
Ecc SCRI193:	3.5(KF1033)	230(KF1037)
Eca SCRI8:	3.5(KF2014)	115(KF2006)
Eca SCRI13:	7.0(KF2012)	115(KF2007)

In E.coli and S.typhimurium rifampicin resistant mutants (rpoB) are the result of an alteration to the β-subunit of RNA polymerase (Bachman, 1983; Sanderson and Hartman, 1978)

STREPTOMYCIN: Spontaneous streptomycin (STR) resistant mutants were selected on LB, but were not easily isolated. In Eca preliminary attempts were hampered by the high incidence (all of twelve mutants

isolated) of streptomycin dependent mutants.

In E.coli streptomycin resistant and dependent mutants are both the result of mutations in strA, also called rpsL; however, while streptomycin resistant mutants can grow on medium without streptomycin, the dependent mutants can only grow when the antibiotic is present in the medium. Spotts and Stanier (1961) showed that the dependent mutants could only grow exponentially if the streptomycin concentration was greater than 100μg/ml, while below this concentration growth was arithmetic and in direct proportion to the antibiotic concentration. The initial isolation in Eca of independent mutants was therefore probably unsuccessful because the streptomycin concentration was sufficiently high (100μg/ml) to allow the dependent mutants to grow well and so outnumber the rarer streptomycin-resistant mutants. By halving the concentration of streptomycin in the selection medium fewer colonies appeared, but all proved to be streptomycin resistant (at c.10^{-10}). The str-1 mutation in Ecc SCRI193 was isolated at SCRI as a spontaneous mutations.

The MICs (μg/ml), determined in LB, were:

	Str^S	Str^R
Ecc SCRI193:	14 (KF1037)	>18,000(KF1033)
Eca SCRI8:	2.5 (SCRI8)	3,000(KF2014)
Eca SCRI13:	10 (SCRI13)	3,000(KF2013)

All E.coli mutants resistant to high concentrations of streptomycin map at strA (rpsL) (Bachman, 1983). In S.typhimurium there is an additional mutation conferring resistance to high streptomycin concentrations, strC, which is unmapped (Sanderson and Hartman, 1978).

TRIMETHOPRIM: Ecc SCRI193 trimethoprim (TMP) resistant spontaneous mutations, which were readily isolated, and a mutation resulting from the integration of Mu thyA1::Muc+Δ445-7, (Pérombelon and Boucher (1978) and Section 6) were all found to have a requirement for thymine. The MIC of trimethoprim in the sensitive strain KF1006 and the resistant strain KF1016 were 32 and 4,000 µg/ml respectively.

A thymine requirement is typical of the trimethoprim resistant, thyA mutations, of E.coli (Stacey and Simson, 1965). Trimethoprim resistant mutations also map at folB in E.coli but these are not associated with a requirement for thymine (Breeze et al., 1975). The thyA mutations are of great use since they can be used for both the positive selection (using resistance to trimethoprim) and the negative selection (absence of thymine) of a mutant strain.

4)c) ISOLATION AND CHARACTERISATION OF AUXOTROPHIC MUTANTS

4)c)i) MUTAGENESIS

Auxotrophic mutants were isolated from bacteria following treatment with the mutagen ethyl methanesulphonic acid (EMS) by the method of Lin et al., (1962), who suggest a 2h treatment period. Since it was not known how E.carotovora would respond to this level of treatment, a time course experiment of mutagenesis of E.carotovora KF1033 was

performed using the above procedure, but terminating the treatment at various times up to 4h. The treated samples started to loose viability rapidly after about 2h of treatment. The frequency of auxotrophs in these mutagenised cultures, after culturing them in LB overnight, was $1\text{-}4\times10^{-2}$ auxotrophs /survivor after 2-3h of treatment. That this procedure was mutagenic for Ecc can be inferred from the reduced viability of cultures and the increasing frequency of auxotrophs with increasing treatment times; also auxotrophs were not recovered as frequently from untreated controls ($<10^{-3}$) as from treated cultures.

These results suggest that the treatment time given by Lin et al. (1962) is also applicable to Ecc. The same treatment time and procedure was used for the Eca strains.

4)c)ii) ISOLATION OF AUXOTROPHS

Some of the auxotrophs isolated (in Ecc SCRI193: arg-1,-2, aroB1, hisD1, leu-1,-2, met-1, ser-1, trp-1,-2; in Eca SCRI8: arg-1, ilv-1, ser-1, trp-1; in Eca SCRI13: cys-1, his-1) were simply isolated from the LB cultured, mutagenised cells, as auxotrophs. These auxotrophs were characterised biochemically and those which were not leaky, retained.

The majority of the auxotrophs were recovered following a cycle of enrichment using the ampicillin enrichment method of Lederberg and Zinder (1948) as described in Miller (1972). It was found to be particularly important that the initial concentration of bacteria in

the enrichment step was not too great, as this reduced the proportion of bacteria in the culture which were lysed. A greater concentration of ampicillin (100μg/ml) to that recommended by Miller (20μg/ml) was also found to improve the yield of auxotrophs. By such enrichments it was possible to recover a specific auxotrophic mutant at 1-10% of the bacteria in the enriched culture.

4)c)iii) BIOCHEMICAL CHARACTERISATION OF AUXOTROPHS

Most of the auxotrophs isolated in Ecc SCRI193 were characterised by syntrophy and by growth on appropriate media to ascertain the nature of their biochemical lesions. The results were then compared with mutants of E.coli (Bachman and Low, 1980) and S.typhimurium (Sanderson and Hartman, 1978). The auxotrophs isolated in Eca were not characterised beyond obvious phenotypes.

THE AUXOTROPHS OF Ecc SCRI193

aroB1 Reversion frequency: 2×10^{-9}/cell

This mutant was isolated as a shikimic acid requiring auxotroph. Pittard and Wallace (1966) analysed various E.coli mutants defective in aromatic amino acid biosynthesis and found that they could be grouped by their patterns of growth on different combinations of shikimic acid, phenylalanine, tryptophan, and tyrosine. The pathway in S.typhimurium is very similar to, if not identical with, that in E.coli. The results obtained when the Ecc mutant Aro-1$^-$ was tested on these biochemical combinations are given in Table 6, and it can be

Table 6. Growth requirements of AroB1$^-$ and PheA1$^-$ Ecc SCRI193 mutants.

Strain	Relevant phenotype	Supplements[a]				
		shikimic acid	phenylalanine	tyrosine	phenylalanine + tyrosine	phenylalanine + tyrosine + tryptophan
E.coli mutants[b]	AroB$^-$	++[c]	-	-	-	-
	AroD$^-$	++	-	-	-(+)[d]	++
	AroE$^-$	-	-	-	-	+(-)[e]
	AroC$^-$	-	-(+)[d]	-(+)[d]	++(+)[d]	++
	PheA$^-$	-(+)[d]	++	-(+)[d]	++	++
Ecc						
KF1072	AroB1$^-$	++	-	-	-	-
KF1061	PheA1$^-$	-	++	-	++	++

a: Strains were cultured on glucose MM with amino acids as required in addition to the supplements indicated. b: Taken from Pittard and Wallace (1966). c: Growth of strains was scored after 3d as none (-), weak (+), or strong (++). d:A few mutants grow weakly. e: A few mutants do not grow.

Table 7. Growth of Crp$^-$ and Gal$^-$ Ecc SCRI193 mutants on different carbon sources.

Strain	Relevant phenotype	Carbon source[a]								
		glucose	galactose	arabinose	lactose	raffinose	glycerol	glycerol + galactose	citrate	succinate
KF1033	Crp$^+$Gal$^+$	++[b]	++	++	++	++	++	++	++	+
KF1069	Crp-1$^-$	++	-	-	-	-	-	-	-	+
KF1068	Gal-1$^-$	++	-	++	-	-	++	-	++	+
KF1070	GalK2$^-$	++	-	++	++	++	++	++	++	+
KF1071	Gal-3$^-$	++	-	++	-	-	++	-	++	+

a: Strains were cultured on MM with the indicated carbon sources and amino acids as required. b: Growth of strains was scored after 3d incubation at 28^{o}C as none (-), weak (+), or strong (++).

seen that this is an aroB mutation.

crp-1 Reversion frequency: 1×10^{-8}/cell

Crp-1$^-$ was isolated as a mutant unable to utilise galactose as a carbon source, however as shown in Table 7 it is also unable to utilise many other carbon sources - it was only able to utilise glucose and succinate.

Such a phenotype is also known in other bacterial species where it has been found to result from an alteration in the control of catabolite repression, specifically from a loss of activity of either adenylate synthetase (cya) or of cAMP receptor protein (crp). Mutants of these genes can be differentiated by the suppression by cAMP of Cya$^-$, but not Crp$^-$ mutants. cAMP did not suppress the crp-1 mutation in KF1069.

It is interesting to note that crp-1 also suppresses this strain's ability to metabolise pectate (on modified Stewart's medium) inferring that the enzymes required for pectate degradation are also catabolite repressible in this strain. Catabolite repression of pectolytic enzymes has been reported in Ecc by Mount et al. (1979) who used a Cya$^-$ mutant, and by Hubbard et al. (1978) who varied the extracellular concentration of cAMP, and also in E.chrysanthemi (Chatterjee and Starr, 1977) using a Crp$^-$ mutant.

deo-1

The ThyA$^-$ KF1017 carries a spontaneous mutation which reduces the

strain's requirement for thymine from c.50µg/ml to 1-2µg/ml. In addition this mutation resulted in the strain's inability to utilise deoxyadenosine as a carbon source.

Such phenotypes in thyA E.coli and S.typhimurium are the result of mutations in deoB or deoC (Lomax and Greenberg, 1968; Jørgensen et al., 1977).

gal-1, galK2, gal-3 gal-1 reversion frequency: 7×10^{-9}/cell

Mutations in mutagenised KF1065, resulting in Gal$^-$ phenotypes were isolated.

Two classes of Gal$^-$ mutations are found in E.coli and S.typhimurium. One class, mutations in galK, result in mutants unable to utilise galactose, but which are able to utilise other carbon sources. The other class (gal sensitive), mutations in galE, galT or galU result in mutants unable to utilise any carbon source which has a galactose moiety, such as lactose and raffinose, or to grow in culture medium (such as glycerol MM) which contains galactose at concentrations above 50µg/ml. This second class can be further subdivided into galE mutants which lyse and galT and galU mutants which stop dividing in the presence of galactose (cultured in glycerol MM to avoid catabolite repression (Sundarajan et al., 1962; Yarmolinsky et al., 1959)).

Three Gal$^-$ mutations in KF1065 (gal-1 (KF1068), galK2 (KF1070), gal-3 (KF1071)) were tested for growth on various carbon sources (Table 7). KF1070 was unable to utilise only galactose and so must carry a galK

leu-2 Reversion frequency: 4×10^{-10}/cell

Leu-2 was isolated in KF1047 and was classified as a leucine requiring auxotroph. In both E.coli and S.typhimurium the four structural genes for the leucine biosynthetic enzymes map in a single operon.

manA1 Reversion frequency: $< 2 \times 10^{-9}$/cell

ManA1 was isolated in KF1064 by enriching for mutants unable to utilise mannose as a sole carbon source.

In E.coli and S.typhimurium mannose is phosphorylated to mannose-6-phosphate by the multifunctional hexokinase enzyme, and then isomerised by phosphomannose isomerase (PMI) to fructose-6-phosphate which is catabolised via the glycolytic pathway. Thus the only Man^- mutants that can be isolated result from the loss of PMI activity; these are known as manA in E.coli and pmi in S.typhimurium. In E.coli such mutants are also deficient in their ability to synthesise capsular polysaccharide when grown on glucose MM and are observed to be non-mucoid (Markovitz et al., 1967); there was no obvious alteration of the colony morphology of strain KF1072, which carries manA1, when cultured on glucose MM.

pheA1 Reversion frequency: 2×10^{-7}/cell

This mutation was isolated as a phenylalanine-requiring auxotroph. As shown in Table 6 (from Pittard and Wallace, 1966) such auxotrophs can

arise as a result of mutations in pheA or aroC; however the latter are also able to grow slowly on MM supplemented with tyrosine. KF1061, which carries pheA1, was unable to grow on MM supplemented with tyrosine and so can be classified as a mutation in pheA.

proA1 Reversion frequency: 7×10^{-7}/cell

The proA1 mutation was isolated in KF1059. In E.coli and S.typhimurium there are now thought to be only two genes involved in the biosynthesis of proline from its precursor glutamate: proA and proC. Mutants of these two genes are readily distinguished by the ability of strains carrying proC mutations to cross-feed strains carrying proA. KF1065 was cross-fed by the proC E.coli CA7087 but could not itself cross-feed the proA E.coli RE26 or RE349. This mutation is therefore in proA.

ser-1 Reversion frequency: 4×10^{-9}/cell

This mutant was isolated in KF1047 as a serine-requiring auxotroph. Unlike most serine auxotrophs of E.coli and S.typhimurium, the mutation in KF1061 not only confers serine auxotrophy, but also an inability to utilise glycine or pyridoxine (vitamin B_6). Dempsey and Itoh (1970), who classified several serine and pyridoxine auxotrophs of E.coli also found this class of serine auxotrophs. These E.coli mutants did not grow on MM supplemented with either glycine (unlike serA and serB) or pyridoxine (unlike some serC), and had apparently normal levels of the serine biosynthetic enzymes. This class of mutations has not yet been mapped in E.coli. Cross-feeding tests

showed that the GlyAl$^-$ KF1072 and the SerB$^-$ E.coli C6 could both weakly cross-feed KF1061.

thr-1 Reversion frequency: 4×10^{-10}/cell

The thr-1 mutation was isolated in KF1055. In E.coli and S.typhimurium the three genes (thrA,B,C) coding for the enzyme required for the synthesis of threonine are clustered together in a single operon.

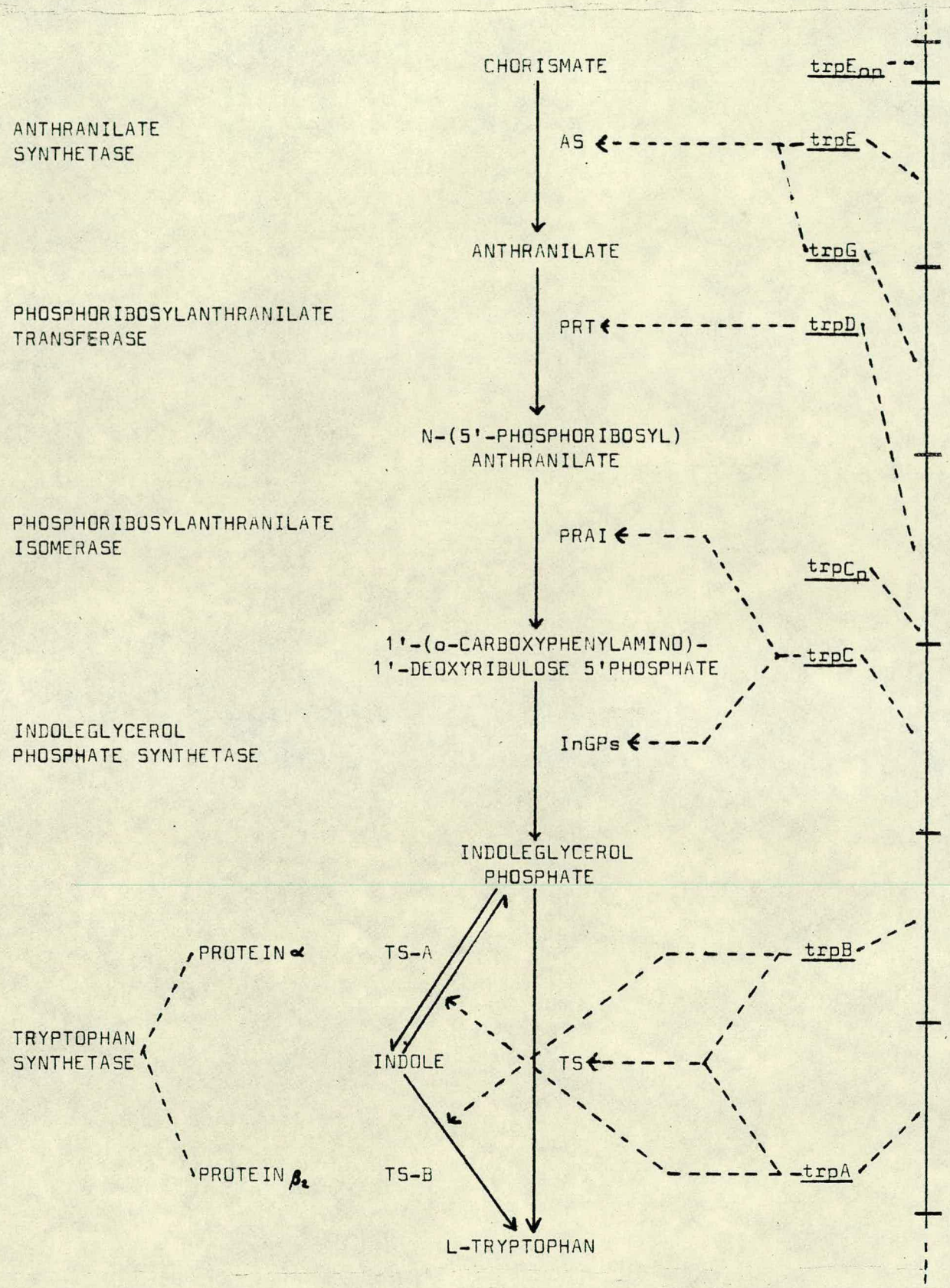
It is unlikely that the thr-1 mutation in KF1060 maps in thrA, as the product of this gene is one of several isoenzymes (with met, lys), all of which synthesis homoserine, and so such mutants would be threonine independent when grown on MM; this was not observed to be the case with KF1060. In addition this mutant was unable to utilise homoserine in place of threonine, further suggesting that it is a mutation in either thrB or thrC.

trp-2 Reversion frequency: 6×10^{-9}/cell

This mutant was isolated in KF1047. From the work of Largen and Belser (1973) (review, Crawford, 1975) on the tryptophan biosynthetic pathway of E.carotovora (ICPB EC153, the subspecies of this isolate was not stated, but is probably subsp. carotovora) several conclusions can be drawn about the organisation of the genes coding for these enzymes in this species; these are summarised in Figure 7.

E.carotovora, like all of the other enteric bacteria which have been

Figure 7.Biosynthesis of tryptophan in E.carotovora and a presumptive genetic map of the E.carotovora trp operon.



Adapted from Crawford (1975), Grabow (1970) and Largen and Belser (1973, 1975).

studied so far, converts chorismate to tryptophan by a pathway with five enzymatic steps. These five enzymes have been found to be co-ordinately repressed and derepressed throughout the enteric bacteria, which would suggest that in all of these species the genes are located in a single operon, as is the case in E.coli and S.typhimurium. In addition, evidence that the second promotor $trpC_p$, which is found in E.coli within the coding region of the trpD structural gene, is also present in E.carotovora and other enteric bacteria, has been obtained. This homology in the control of the trp genes in all of the enteric bacteria so far examined, implies strong conservation of the order of the trp genes in the Enterobacteriaceae. In E.carotovora certain of the tryptophan synthetic enzymes from adjacent genes were observed to be fused together, though in different combinations to that found in E.coli and S.typhimurium; Enterobacter hafniae, and to a lesser extent Proteus and Serratia species were most similar to E.carotovora in this respect (Largen and Belser, 1975; Li et al., 1974). Transductional mapping of four trp genes in Proteus mirabilis obtained a gene order of trpE-D-B-A (Grabow, 1970), while in Serratia marcescens clustering of some trp genes was found (H. Matsumoto, pers. comm. in Largen and Belser, 1975).

4)c)iv) GROWTH OF MUTANT Ecc SCRI193 STRAINS ON POTATO TUBER SLICES AND STEWART'S MEDIUM

Ecc SCRI193 mutant strains KF1006, KF1016, KF1017, KF1033, KF1037, KF1060, KF1061, KF1068, KF1069, KF1070, KF1071, KF1072 and KF1078 were tested for soft-rotting of potato tuber slices and the production of extracellular pectic enzymes on modified Stewart's medium. All of the

mutant strains except the Crp$^-$ KF1069 degraded the pectate on modified Stewart's medium. Rotting of potato tuber slices was found to be dependent on the potato batch - all of the prototrophic strains rotted the tubers in both experiments, whereas rotting by the auxotrophic strains was observed in only one of the two experiments, presumably reflecting a lower concentration of limiting amino acids in the latter batch of potato. It is interesting to note that the Gal$^-$ strains KF1068, KF1070, KF1071 and the Man$^-$ strain KF1072 were able to rot the tubers, suggesting that these mutations in catabolism and cell wall synthesis do not affect rotting under these conditions. The Crp$^-$ strain KF1069 was unable to metabolise pectate on modified Stewart's medium or to rot the tubers, implying that this mutation does affect expression of one, or more, of the enzymes involved in pectate catabolism; this agrees with the results of Hubbard et al. (1978) and Mount et al. (1979) in E.carotovora and of Chatterjee and Starr (1977) in E.chrysanthemi.

4)d) CONCLUSIONS.

When characterised, the antibiotic, antimetabolite and auxotrophic mutations isolated in the E.carotovora strains were all found to have analogous mutations in E.coli and S.typhimurium. The E.carotovora mutations were named on the basis that mutations in different species which have a common phenotype can be given a common name. As to whether the genes in these species are analogous or are in fact homologous, that is having common ancestry, it is not possible to determine using the type of analysis used here; however as most of the mutations isolated are in primary metabolic pathways, it is probable that the genes are indeed homologous rather than simply analogous.

SECTION 5

IncF PLASMIDS IN E.carotovora

5)a) INTRODUCTION

There have been several reports of the transfer of IncF plasmids into Erwinia species: the IncFI plasmids F'Lac$^+$ (Guimaraes et al., 1978; Chatterjee and Starr, 1972,a) and F'His$^+$ (Chatterjee and Starr, 1980), the IncFII plasmids R100drd-56 (Chatterjee and Starr, 1972,b) and R1 (Panopoulos et al., 1978), and the IncFIV plasmid R124 (Panopoulos et al., 1978). From these reports it was clear that while some strains of a species were very good recipients of IncF plasmids, other strains of the same species were not. The maintenance of the plasmids was similarly very variable, with some strains showing very high frequencies of spontaneous curing of the plasmid. Neither were the transconjugants when isolated, invariably sensitive to IncF specific phages, or able to transfer the plasmid to other species.

There are few reports in the literature of the transfer of IncF plasmids to Ecc or Eca, no doubt partly because the most commonly used plasmid, F'Lac$^+$, cannot be directly selected in these naturally lactose-utilising species (the Erwinia strains tested above were all naturally lactose non-utilising). Chatterjee and Starr (1972,b) reported that R100drd56 could transfer to Eca EA153 at a low frequency, but not to an Ecc strain. The Eca transconjugant was resistant to the IncF donor-specific phage M13, but could transfer the plasmid to E.coli at a low frequency (10^{-7}/donor). Panopoulos et al.(1978) did not observe the transfer of plasmids R1 or R124 to three

strains of Ecc or to three strains of Eca. Pérombelon (pers. comm.) transferred F'Lac$^+$Tc (Harada et al., 1964) to two strains of Ecc (SCRI113, SCRI193).

The IncF plasmids, particularly the F-prime plasmids isolated in E.coli, have been used extensively as genetic tools in the Erwinia, primarily by integrating them into the chromosome to form Hfr donors. F'Lac$^+$ was used in E.chrysanthemi (Chatterjee and Starr, 1977; Kotoujansky, 1982), and in E.amylovora (Chatterjee and Starr, 1973,a; Pugashetti and Starr, 1975; Chatterjee and Starr, 1978) by isolating lactose-utilising transconjugants in these lactose-non-utilising species and then selecting for the integration of the plasmid into the chromosome: by selection for stable lactose utilising-transconjugants in strains where there was a high rate of spontaneous curing of the plasmid, or by selection for lactose-utilising transconjugants which were resistant to acridine orange curing, or by using plasmids temperature-sensitive for replication. F'His$^+$ plasmids have been similarly used in E.chrysanthemi (Chatterjee and Starr, 1980) and in E.amylovora (Pugashetti et al., 1978).

These mapping studies have shown that the distribution of genes on the chromosomes of E.amylovora and E.chrysanthemi, discussed in a later Section, are very similar to those on the chromosomes of the well mapped enterobacteria, E.coli and S.typhimurium. There was also homology at the DNA level between E.coli and these species, since the F-prime plasmids were usually integrated into the analogous region of the Erwinia chromosome; however Kotoujansky (1982) isolated an Hfr in E.chrysanthemi using F'Rep[Ts114]Lac$^+$::Tn10 which integrated at a site

far from the integration site of F'Lac$^+$ reported by the workers above.

These successes in linkage map construction using IncF plasmids in E.amylovora and E.chrysanthemi, and the ability of some strains of Ecc and Eca to receive some IncF plasmids suggested that IncF plasmids might also be of use in the genetic mapping of E.carotovora. The plasmids used in this study were all IncFI, were transferable within E.coli, and conferred sensitivity to the IncF donor-specific phages ϕf_2, $Q\beta$ and R17.

5)b) INTRODUCTION OF IncFI PLASMIDS INTO Ecc and Eca.

The transfer of the plasmid F'Lac$^+$Tc from *E.coli* into *E.carotovora* strains was studied.

The transfer kinetics of F'Lac$^+$Tc from *E.coli* RE410 into *Ecc* SCRI193 strain KF1033 was followed during membrane matings at 28°C and 37°C, the results of which are given in Figure 8(a). At 28°C inheritance was barely detectable - c.10^{-6} /donor - and did not increase with time, indicating that plasmid transfer did not continue after the start of the mating. At 37°C the inheritance frequency increased throughout the mating period, showing that transfer continued throughout the mating period. In *E.coli*, F transfers less frequently, and F$^+$ *E.coli* strains are less sensitive to infection by IncF donor-specific phage, when the strains are cultured at temperatures below 37°C (Knolle and Ørskov, 1967; Novotny and Lavin, 1971; Walmsey, 1976), thus the low frequency of transfer of F'Lac$^+$Tc to *Ecc* at the lower temperature is probably a function of the *E.coli* donor rather than the *Ecc* recipient.

Other plasmids which were conjugated in *Ecc* SCRI193 were F$^+$::Tn10, and F'His$^+$; F$^+$::Tn5, F'Rep[Ts114]Lac$^+$::Tn10, F'His$^+$::Tn10, and F'Gal$^+$Ap Cm Sm Su plasmids could not be crossed in (Table 8).

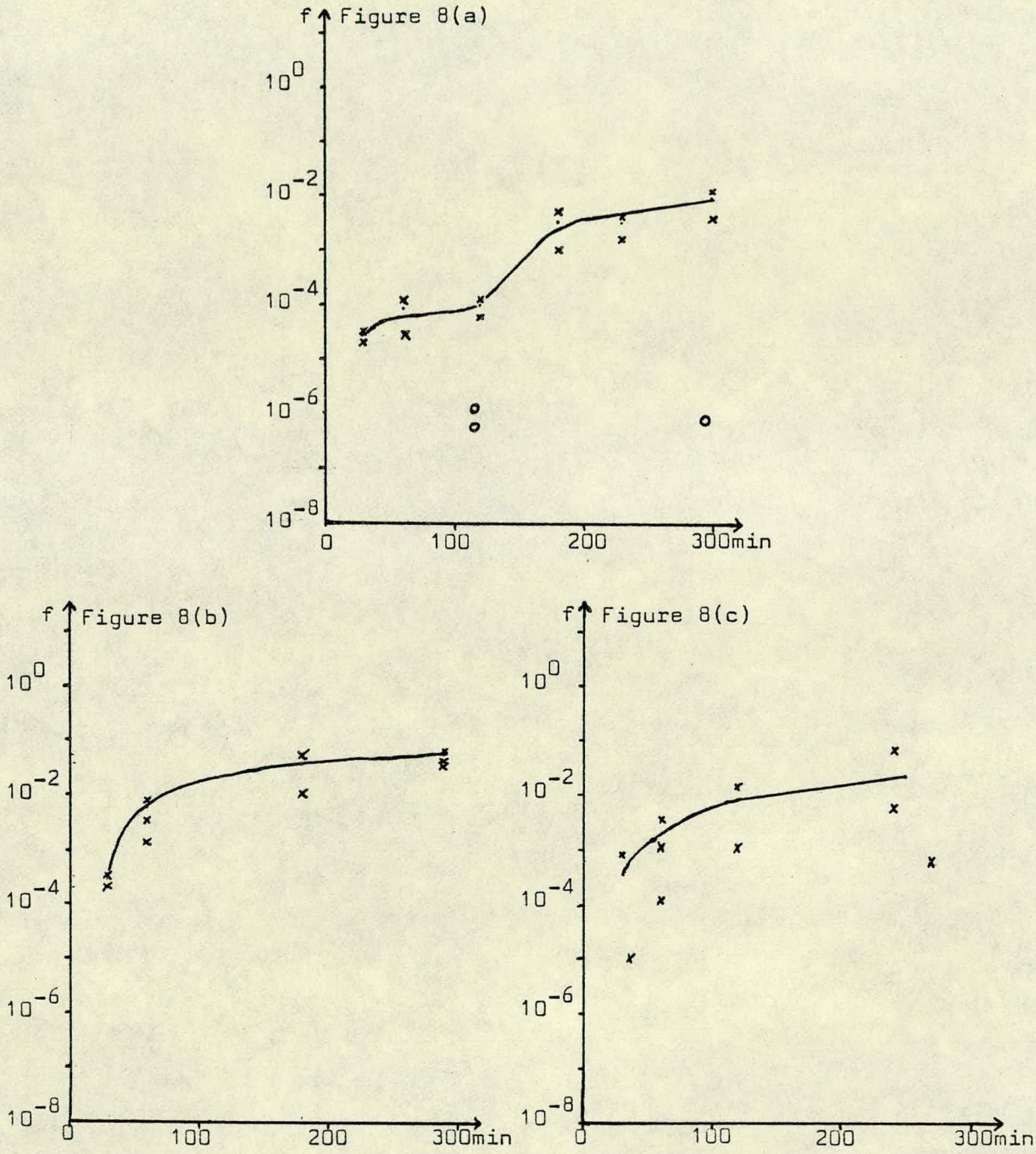
The F'Lac$^+$Tc, F'His$^+$, and F$^+$::Tn10 transconjugants of *Ecc* SCRI193 were sensitive to the IncF donor-specific phages ϕf_2, $Q\beta$ and R17 and harboured plasmids which comigrated on electrophoretic gels with the plasmids in the *E.coli* donor strains. The plasmids could be

Figure 8. F'Lac$^+$Tc transfer into Ecc SCRI193 strain KF1033: different donor strains and mating conditions.

Figure 8(a). Membrane mating between E.coli RE410 and KF1033 at 28°C (o) and 37°C (x).

Figure 8(b). Membrane mating between KF1037 and KF1033 at 28°C; parental strains cultured with gentle shaking.

Figure 8(c). Liquid mating, with gentle shaking, between KF1037 and KF1033 at 28°C.



f: Frequency of F'Lac$^+$Tc transfer per initial donor.

abscissa: Duration of mating.

Table 8. Transfer of IncFI plasmids from *E.coli* into *Ecc*.

Plasmid	Donor strain[a]	Recipient strain[b]	Transconjugants isolated; transfer frequency per donor		Mating[c]	selection for
F^+::Tn5	in JC5466	KF1006	no	$<10^{-8}$ d	a;30°C;5h	Kan^R+Neo^R
F^+::Tn10	AK5003	KF1033	yes	10^{-6}	a;30°C;5h	Tet^R
F'Lac^+Tc	RE410	SCRI193	yes	see text;	a,m;	Tet^R
		KF1006		10^{-2} at 37°C,	30°C,37°C;	
		KF1033		membrane,5h	2-24h	
		KF1028[b]		10^{-7}	a;28°C;1d	
F'Rep[Ts114]Lac^+::Tn10	6895 and KF39	KF1006	no	$<5\times10^{-8}$	a,m; 30°C,34°C; 2-24h;±HS	Tet^R
F'His^+	KF30	KF1065	yes	10^{-6}	m;37°C;4h	His^+
F'His^+::Tn10	KF56	KF1072	no	$<10^{-8}$	m;37°C;6h	His^+;Tet^R
		SCRI193	no	$<10^{-8}$	a;28°C;1d	Cml^R
		KF1010	no	$<10^{-8}$	a;28°C;1d	Gal^+;Cml^R;Amp^R
F'Gal^+Ap Cm Sm Su	in RE26	KF1068	no	$<10^{-7}$	1;37°C;6h	Gal^+;Cml^R
		KF1070				
		KF1071				

a:All donor strains are derivatives of *E.coli* K12. b:All recipients are derivatives of *Ecc* SCRI193 except KF1028 which is a derivative of *Ecc* SCRI113. c:Matings were performed two or more times, on agar surface (a), liquid (l), membrane (m); recipients pre-treated at 50°C for 6min to attempt to inactivate restriction system(s) (+HS). d:The recipient strain mutated to Kan^R+Neo^R at a high frequency (5×10^{-7} mutants /recipient; as two Kan^R+Neo^R recipients were tested for ϕf_2 sensitivity and found to be resistant, F^+::Tn5 transferred at $<10^{-7}$ /recipient (or per donor as equal concentrations of the parents were used).

conjugated from the Ecc strains to other strains of Ecc and E.coli and, at least in the case of F'Lac$^+$Tc, could be cured from Ecc. It is interesting to note that on lactose tetrazolium agar Ecc (and Eca) strains do not give the normal white colony colour of Lac$^+$ strains; however when F'Lac$^+$Tc is present in Ecc a white colony colour is observed. This is presumably due to a low level of lactose catabolism in E.carotovora, as is also the case with the chromosomal lac of the related Klebsiella pneumoniae (Reeve and Braithwaite, 1973).

The transfer of F'Lac$^+$Tc from E.coli RE410 into two strains of Eca was also studied. The inability of Eca strains to grow at 37°C restricted matings at this temperature to short durations (2h); matings of longer duration were at lower temperatures (Table 9). Tetracycline resistant transconjugants were isolated at a low frequency in matings with Eca SCRI13 recipients but not with Eca SCRI8 recipients (Table 9). The tetracycline resistant Eca SCRI13 transconjugants were not sensitive to the IncF donor-specific phages, had not acquired a white colony colour on lactose-tetrazolium agar (see above), did not possess a plasmid detectable by the isolation methods of Casse et al.(1979), Eckhardt (1978), or Schwinghamer (1980), and could not transfer the plasmid to E.coli RE254 or to Eca SCRI13 strain KF2023 ($<4.5\times10^{-8}$ transconjugants/donor; 2h liquid mating at 28°C). Since Eca SCRI13 does not mutate spontaneously to tetracycline resistance ($<3\times10^{-8}$ mutants/cell) the tetracycline resistant transconjugants must have resulted from the transfer of the F'Lac$^+$Tc plasmid into Eca followed by the transposition of the Tc determinant onto the chromosome and the loss of the plasmid. The resistant Eca transconjugants segregated to sensitivity at 1%/cell (on LB acridine

orange). There is no report in the literature on the transposability of this Tc determinant, however Harada et al. (1963) isolated tetracycline resistant transductants of S.typhimurium which however, did not give tetracycline sensitive segregants ($<1.7 \times 10^{-3}$/cell) either spontaneously or after treatment with acridine orange. This Tc determinant was originally isolated from a plasmid carrying Cm Sm Su Tc (R10 of Harada et al., 1963), which is the same phenotype as the Tn10-bearing plasmid R100.

Plasmids F^+::Tn5, F'Rep[Ts114]Lac^+::Tn10, F'Gal^+Ap Cm Sm Su, and F'His^+ could not be conjugated into Eca SCRI13 (Table 9).

Table 9. Transfer of IncFI plasmids from E.coli and Ecc into Eca.

Plasmid	Donor strain	Recipient strain[a]	Transconjugants isolated; transfer frequency per donor		Mating[b]	selection for
	E.coli					
F^+::Tn5	in JC5466	KF2007	no	$<10^{-7}$ c	a;30°C;5h	Kan+Neo^R
F'Lac$^+$Tc	RE410	SCRI13	yes[d]	10^{-6}	a;28°C;1d	Tet^R
		KF2007	yes[d]	10^{-6}	m;37°C;2h	Tet^R
		KF2013	yes[d]	10^{-7}	a;32°C;1d	Tet^R
		KF2006[a]	no	$<10^{-7}$	m;37°C;2h	Tet^R
F'Rep[Ts114]Lac$^+$::Tn10	6895 and KF39	KF2007	no	$<5\times10^{-8}$	a,m;30°C, 34°C;2-24h	Tet^R
F'Gal$^+$Ap Cm Sm Su	in RE26	SCRI13	no	$<10^{-7}$	a;28°C;1d	Cml^R
	Ecc					
F'Lac$^+$Tc	KF1037	KF2027	no	$<8\times10^{-8}$	m;28°C;1d	Tet^R
F'His$^+$	KF1067	KF2027	no	$<3\times10^{-8}$ e	m;28°C;1d	His$^+$

a:All recipients are derivatives of Eca SCRI13 except KF2006 which is a derivative of Eca SCRI8. b:Matings were performed two or more times, on agar surface (a), or on membrane (m). c:The recipient mutated at a high frequency to Kan+Neo^R (5×10^{-7} resistant mutants /cell), and no greater frequency of Kan+Neo^R was observed after the mating, compared to the frequency observed from a culture of the recipient only. d:Transconjugants were ϕf_2^R and plasmid-free -see Section 6)b). e:As the recipient mutated at a high frequency to His$^+$; calculated from the spontaneous mutation frequency to His$^+$ (6×10^{-7}) divided by 20 (the number of His$^+$ recipients tested, and found to be ϕf_2^R).

5)c) MOBILISATION OF IncFI PLASMIDS FROM Ecc

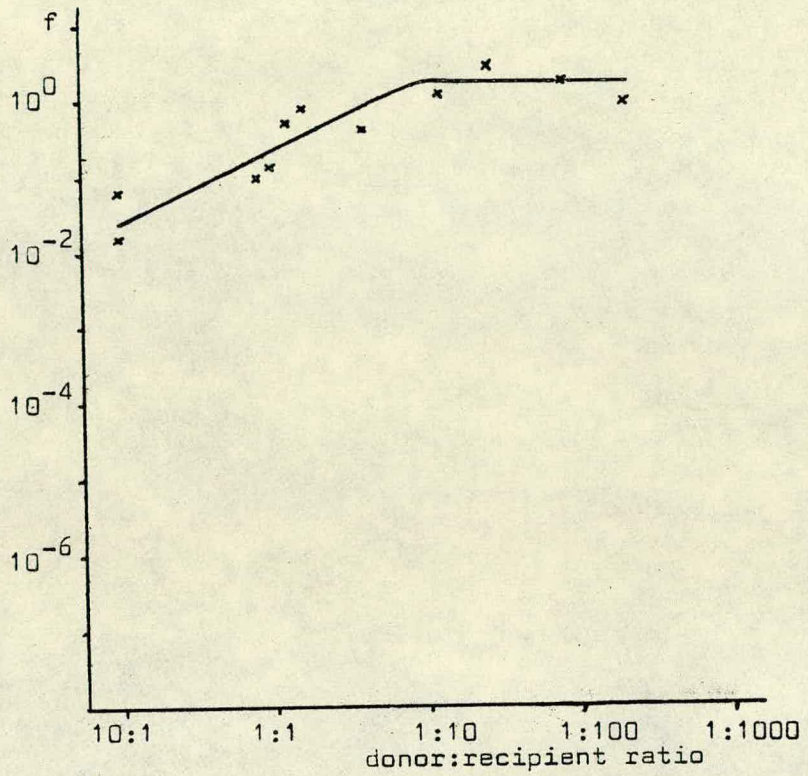
5)c)i) LIQUID AND MEMBRANE MATINGS

When the transfer of F'Lac$^+$Tc in Ecc SCRI193 (KF1037 xKF1033) at 28^{o}C on membrane (Figure 8,b) and in liquid (Figure 8,c) were compared, there was found to be little difference in the frequencies of plasmid inheritance. The dispersion of the inheritance frequencies, particularly from the liquid matings (presumably due to variations in the degree of agitation of the mating cultures), makes more detailed analysis more difficult.

5)c)ii) RATIO OF DONORS TO RECIPIENTS DURING CONJUGATION OF F'Lac$^+$Tc IN Ecc

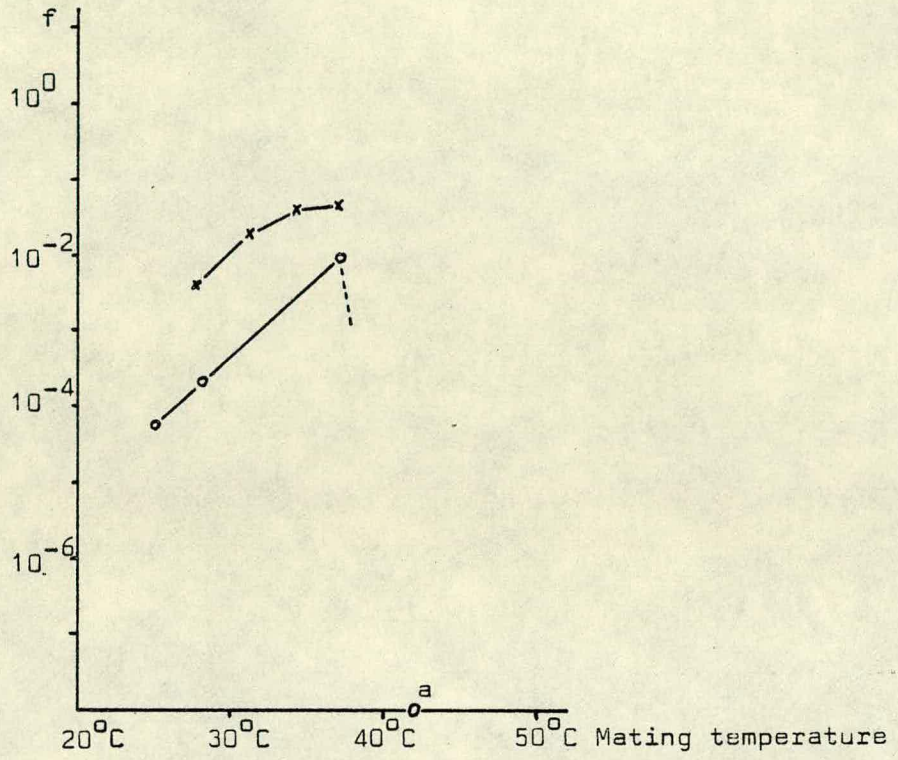
At 37^{o}C the transfer of F'Lac$^+$Tc from KF1037 to KF1033 was found to be much greater than at 28^{o}C (compare Figure 9 to Figure 8,b at 2h mating duration; see also Section 6)c)iii)). The low inheritance frequencies of the plasmid at 28^{o}C, typically up to c.10^{-2}transconjugants /donor after 5h, makes competition for recipients which are still plasmid free, low. At 37^{o}C however, the greater frequency of plasmid transfer, typically up to 100% after 2h makes competition for plasmid free recipients much more severe. This competition was quantified by comparing the frequencies of plasmid inheritance, after matings of 2h duration, from different numbers of donors (c.10^6 - 10^{9}donors /membrane) to recipients of a fixed population size (c.10^{8}recipients /membrane). As shown in Figure 9 there was no detectable competition at donor:recipient ratios of less than c.1:10. As the donor:recipient ratio was increased beyond about 1:10, the plasmid inheritance

Figure 9. F'Lac^{+}Tc transfer in Ecc SCRI193: donor:recipient ratio.



f: Frequency of plasmid transfer per initial donor (KF1037), to KF1033 recipients (2h membrane matings at 37^{o}C).

Figure 10. F'Lac^{+}Tc transfer in Ecc SCRI193: mating temperature.



f: Frequency of plasmid transfer per initial donor (KF1037), to KF1033 recipients during 2h liquid matings, at appropriate temperatures, either in 1ml LB broth in 12x75mm tubes (o), or in 2ml LB broth in 100ml flasks (x). a: Plasmid transfer not detected.

frequencies started to decrease.

5)c)iii) EFFECT OF TEMPERATURE ON F'Lac$^+$Tc TRANSFER IN Ecc

The conjugation of F'Lac$^+$Tc from KF1037 to KF1033 at temperatures between 25°C and 42°C in 2h, static, liquid matings allowed the study of the effect of temperature on the frequency of plasmid transfer (Figure 10). The plasmid was transferred most efficiently at temperatures in the region of 35°C. The reduced aeration during matings in tubes compared to that in flasks reduced the viability of the parental strains at temperatures greater than about 37°C, and reduced the frequency of plasmid transfer at all temperatures.

5)c)iv) F'Lac$^+$Tc PLASMID TRANSFER AND RESTRICTION SYSTEMS IN Ecc

The much increased frequency of inheritance of F'Lac$^+$Tc in KF1033 (under identical mating conditions: membrane mating for 2h at 37°C) from donors of the same Ecc strain (KF1037), $1x10^0$ transconjugants /initial donor (Figure 9), compared with that from E.coli donors (RE410), $c.10^{-4}$ transconjugants /initial donor (Figure 8,c), strongly suggests that Ecc SCRI193 is restricting the incoming plasmids from the E.coli donors. Similarly the transfer of the plasmid from KF1037 donors (under identical mating conditions: liquid mating for 1h at 28°C), to recipients of the same strain (KF1033), $2x10^{-3}$ transconjugants/initial donor (Figure 8,c), was much more frequent than the transfer to E.coli recipients (RE254), 10^{-5} transconjugants/initial donor. It is therefore probable that a

plasmid restriction system(s) is acting in the Ecc SCRI193 strains which is not the same as that in E.coli K12.

That the restriction system in Ecc SCRI193 is different from that in Ecc SCRI113 is suggested from the difference in the frequency of transfer of F'Lac^{+}Tc (under identical mating conditions: membrane mating for 4h at 28^{o}C), from KF1037 donors to the same strain (KF1033) c.10^{-2}transconjugants /initial donor (Figure 8,b) compared to the transfer to the Ecc strain SCRI113 (KF1042), $<10^{-8}$transconjugants/initial donor (in matings of longer duration this plasmid could transfer to Ecc SCRI113 (Table 8) and the transconjugants maintained the plasmid, were sensitive to phage ϕf_2, and could donate the plasmid).

Restriction-modification system(s) in Ecc SCRI193 would account for the variability in the inheritance of different F-primes from E.coli, as it would be expected that some plasmids would be more readily susceptible to restriction by Ecc SCRI193 than others, and would therefore be inherited less frequently in Ecc SCRI193.

The diphasic nature of the inheritance of F'Lac^{+}Tc in Ecc KF1033 from E.coli RE410, if it is not artefactual, is not easily explained. As monophasic transfer was observed from Ecc KF1037 donors (Figure 8,b), this phenomenon is limited to transfer from E.coli donors. If it is the result of the restriction of incoming plasmids on inheritance in Ecc, then one would expect continuous transfer as opposed to the observed diphasic transfer. Rather, the situation must be more complex as initial transfer actually ceases until c.100min into the

mating when it is reinitiated.

5)d) CHROMOSOME MOBILISATION IN Ecc BY F-PRIME PLASMIDS

It has been reported that in *E.coli*, F can mobilise the chromosome in a random, non-polar way by an insertion sequence-dependent mechanism (Willetts and Johnson, 1981) at frequencies of $c.10^{-6}$ recombinants /donor. The *E.coli* F-prime plasmids mobilise the *E.coli* chromosome polarly from a fixed origin, at frequencies of up to $c.10^{-2}$ recombinants /donor. For example, in *E.coli* F'Lac^{+}Tc mobilised *ara* at $c.10^{-3}$ recombinants /donor and *galK* at $c.10^{-6}$ recombinants /donor (30min mating into *E.coli* AB1157 recipients), indicating that this plasmid mobilised the *E.coli* chromosome in a clockwise direction from the *lac* region of the chromosome. When F-prime plasmids from *E.coli* are used to mobilise the chromosomes of other bacterial species the efficiency of chromosomal mobilisation will depend on the extent of genetic homology between the plasmidic and the chromosomal sequences. Thus a gradient in the frequencies of inheritance of chromosomal markers by an F-prime is indicative of genetic homology between *E.coli* and that species.

F'Lac^{+}Tc mobilised the *Ecc* SCRI193 chromosome in matings between KF1037 donors and KF1072 recipients (Table 10). As can be seen from the frequencies of inheritance, *aroB1* and *rif-1* were inherited more frequently than *hisD1* and *manA1*; such a gradient implies that this plasmid recombines preferentially with one region of the *Ecc* chromosome, and that *aroB1* and *rif-1* are proximal to *hisD1* and *manA1*. From the frequencies of coinheritance of the unselected markers the orders *aroB1-rif-1-nal-1* and *manA1-glyA1-hisD1* are obtained; assuming no interference from the counter-selection used - resistance to

Table 10. F'Lac$^+$Tc mediated chromosomal mobilisation:KF1037 to KF1072.

Strains: KF1037: rif-1 / F'Lac$^+$Tc

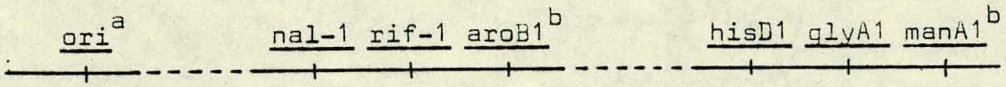
KF1072: aroB1 glyA1 hisD1 manA1 nal-1 str-1

Counter-selection	Plasmid inheritance		Frequency of chromosomal marker inheritance[a]							
			AroB1$^+$		HisD1$^+$		ManA1$^+$		AroB1$^+$Rif-1^R	
	F[b]	n[c]	f[d]	n	f	n	f	n	f	n
Str			300	2	7.3	1	3.5	2	160	1
Nal					3.8	1				
Str Nal	1.0×10^0	2			4.1	1				

Selected marker	Counter-selection	n[c]	T[e]	% coinheritance of unselected marker						
				AroB1$^+$	GlyA1$^+$	HisD1$^+$	ManA1$^+$	Nal-1^S	Rif-1^R	plasmid
AroB1$^+$	Str	1	108	-	<0.9	<0.9	<0.9	35	78	18
								——		34[f]
AroB1$^+$ Rif-1^R	Str	1	83	-	<1.2	<1.2	<1.2	36	-	10
ManA1$^+$	Str	1	11	(<9)[g]	(73)	(18)	-	(<9)	(<9)	
					——					(18)

a: Membrane mating at 37°C for 2.5h. b: Frequency of inheritance per final donor. c: Number of experiments. d: Frequency of inheritance per final donor $\times10^7$. e: Number of transconjugants tested. f: % coinheritance of indicated markers with the selected marker. g: Frequencies in parenthesis are calculated from fewer than 30 transconjugants.

Figure 11. Chromosomal linkage map of Ecc SCRI193: F'Lac$^+$Tc mediated chromosomal transfer from KF1037 to KF1072.



a: The origin is located here since the aroB1 linkage group is inherited more frequently than the manA1 linkage group. b: The orientation of the markers in these two linkage groups cannot be determined from the availiable linkage frequencies.

streptomycin (Figure 11). If both of these linkage groups are indeed mobilised from a single origin, then there should be at least 50% coinheritance of the earlier markers (aroB1,rif-1,nal-1) from the later markers (manA1,glyA1,hisD1); this was not observed to be the case. Two explanations of this are possible. The counter-selection used in these matings, streptomycin resistance, would simultaneously select against markers closely linked to str, in this instance aroB1, rif-1 and nal-1 (see Section 7) thereby reducing their inheritance as unselected markers. Alternatively if the chromosome were mobilised in part by a non-polar mechanism (that is the late markers which are not mobilised efficiently by the polar mechanism), then in this case also there would be no coinheritance of distant markers. In either instance, as the hisD1 linkage group was inherited less frequently than the aroB1 linkage group, the gene order in Figure 11 is obtained.

F'His$^+$ mobilised the Ecc SCRI193 chromosome in matings between KF1067 and KF1072. Using the same mating conditions as with F'Lac$^+$Tc mediated chromosomal transfer (see Table 10), F'His$^+$ mobilised manA1 at 5.5×10^{-7} recombinants /donor (counter-selection for proline and tryptophan prototrophy) (average of two experiments); insufficient chromosomal transconjugants were obtained however, to measure the frequencies of coinheritance of other chromosomal markers.

5)e) CONCLUSIONS

Ecc SCRI193 has been shown to be able to receive and maintain several different F-prime plasmids whilst Ecc SCRI113 also accepted and maintained an F-prime. Other F-prime plasmids could not be conjugated into Ecc SCRI193 and it is proposed that this may be because these plasmids were more susceptible to restriction in this strain. Two prime plasmids, F'Lac$^+$Tc and F'His$^+$, were used to mobilise the Ecc SCRI193 chromosome and the former showed recombinational homology with the chromosome of Ecc.

Since it was not possible to conjugate the temperature-sensitive plasmid F'Rep[Ts114]Lac$^+$::Tn10 into Ecc it was not possible to use this plasmid to isolate Tn10 insertion mutants in Ecc and hence Hfr's with origins where required, as has been done recently in E.chrysanthemi (Kotoujansky et al., 1982). Presumably Hfr strains of Ecc could be isolated using the method of Broda (1967); if prime plasmids were used for this then genetic homology between the plasmid and the chromosome would allow the isolation of Hfr strains with origins as required.

It has been shown that one strain of Eca (SCRI13) was able to receive an F-prime plasmid but was unable to maintain it. This might be developed as a method of transposon mutagenesis in this strain. Eca SCRI8 was unable to receive F'Lac$^+$Tc.

SECTION 6

PHAGE Mu IN Ecc

6)a) INTRODUCTION

The present study set out to utilise the transposability of phage Mu to obtain insertions of the phage at different sites on the Ecc chromosome, thereby creating regions of homology between the chromosomal Mu prophage and another Mu prophage on a conjugative plasmid. Such strains should be able to mobilise the bacterial chromosome in a manner directly analogous to chromosomal mobilisation by prime-plasmids which share homology with chromosomal DNA sequences. In the present study Mu was transferred within Ecc by conjugation, as the Ecc strain used (SCRI193) was not sensitive to Mu infection. M.Pérombelon (pers. comm.) has recently tested several wild strains of Ecc and found all to be Mu-resistant, similarly Faelen et al. (1981,b) found no Mu-sensitive E.carotovora strains.

Phage Mu can integrate into the DNA of bacteria as a transposable genetic element. Taylor (1963) showed that the integration of Mu into the E.coli chromosome resulted in the formation of auxotrophic mutations of many different types at frequencies of 1-3% of all phage integrations; he proposed that this was the result of the integration of the phage into different genes which were then inactivated. Later Bukhari and Taylor (1971) confirmed this when they found complete coinheritance between the mutation and the Mu prophage.

On infection of a non-lysogenic cell the phage can enter either the

lysogenic or the lytic phase, whilst cells lysogenic for Mu can spontaneously enter the lytic phase (in E.coli at c.10^{-4}/lysogen). The switch from the lysogenic to the lytic phase can be induced in phage which carry a temperature-sensitive repressor mutation (cts) by culturing the lysogen at 37-44°C. The multiplication of the phage in the lytic phase has been shown to involve multiple integrations of the phage into the host DNA.

The DNA in Mu phage particles is a linear, double stranded molecule of 25-26Md in length. At each end of the chromosome are regions of host-specific DNA, the so-called split ends, which result from the multiple, random integrations of the phage into the host chromosome during lytic growth, and the headful packaging of DNA in the phage head. The phage DNA itself consists of the α-segment of 21Md, the β-segment of 1.1Md, and between these the G-segment of 2.1Md. All essential genes have so far been mapped to the α-segment. (reviews, see Chow and Bukhari, 1977; Howe and Bade, 1975).

The host range of Mu is limited to a few strains of certain species, and is further limited by the orientation of the G-segment, which can invert by recombination between small homologous regions at each end of the G-segment (Van de Putte et al., 1980,a). Thus in one orientation, G(+), one strain of E.chrysanthemi and E.coli K12 are susceptible, whilst in the other orientation, G(-), E.coli C and one strain each of Erwinia amylovora, E.chrysanthemi, Erwinia uredovora, Shigella sonnei, and Serratia marcescens are phage sensitive (Faelen et al. 1981a, b; Van de Putte et al.,1980,a). Inversion of the prophage G-segment occurs readily such that on induction of the phage

(using Mucts), Mu.G(-) and Mu.G(+) phage particles are produced in equal quantities. Phage lysates produced by infection contain a predominance of the orientation to which the host strain is susceptible.

Since De Graaf et al. (1973) originally suggested mobilising Mu prophage between strains using cointegrate plasmids of Mu and a conjugative plasmid, several such plasmids have been constructed: Mu insertions on the IncFI plasmid F'Lac$^+$Pro$^+$ (Schröder and Van de Putte, 1974; Razzaki and Bukhari, 1975), on the IncP plasmid RP4 (= RP1, RK2, R68) (Dénarié et al., 1977; Van Gijsegem and Toussaint, 1982), and on the IncW plasmid R388 (Leach and Symonds, 1979) are all available.

These cointegrate plasmids can be used to isolate chromosomal Mu insertions following zygotic or temperature induction.

Zygotic induction occurs when a cointegrate plasmid is conjugated into a non-lysogenic recipient, and can be quantified by measuring the frequency of plasmid transfer into lysogenic and non-lysogenic recipients. In this way Schröder and Van de Putte (1974) observed that the transfer of F'Lac$^+$Pro$^+$::Mu was 96% less frequent into E.coli strains which were not lysogenic for Mu. Zygotic induction can also be quantified by the increased frequency of chromosomal mutations in the recipients, resulting from phage insertions. It should be noted however, that zygotic induction cannot be meaningfully quantified from comparisons of the frequencies of inheritance of a plasmid, with and without a phage Mu insertion, as the frequency of transfer of the cointegrate plasmid may well be reduced simply by the greater size of

the plasmid or by insertional inactivation or modification, of a plasmid function involved in conjugation.

By employing cointegrate plasmids with temperature inducible prophage (*cts* mutations), lysogenic strains can be partially heat induced to give chromosomal mutations. Dénarié et al. (1977) reported a seven-fold increase in the frequency of chlorate resistant mutants in *Klebsiella pneumonia* when an RP4::Mu*cts* lysogen was partially induced at 38°C overnight.

Phage Mu, cointegrated with RP4, has been introduced into several *Erwinia* species. The temperature inducible phage, Mu*cts*, was conjugated into *Erwinia amylovora* and *Ecc* by Murooka et al. (1981) where the plasmids were found to be stably inherited and the phage temperature inducible. Coplin (1979) introduced RK2::Mu*cts* into *Erwinia stewartii* where the plasmid was stably inherited and the phage was inducible and able to cause insertional mutations (to non-pathogenesis on maize, auxotrophy, inability to utilise galactose, and resistance to trimethoprim). Coplin (1979) also observed zygotic induction when this plasmid was mobilised into non-lysogenic *E.stewartii* recipients. Pérombelon and Boucher (1978) introduced an R68::Mu $c^{+}\Delta 445$-7 plasmid into *Ecc* SCRI193 where it was transferable and conferred spontaneous Mu production on the strain. The mobilisation of this plasmid within this strain of *Ecc* allowed the isolation of the Mu insertional mutation *thyA1::Mu* $c^{+}\Delta 445$-7 (which was the only lysogenic, trimethoprim-resistant mutant of the 40 trimethoprim-resistant mutants tested) after zygotic induction.

The construction of strains which have a chromosomal Mu insertion and which harbour a conjugative plasmid also with a Mu insertion allows the creation of strains able to mobilise the chromosome in a manner directly analogous to the mobilisation of the chromosome by F-prime plasmids in E.coli. Such Mu dependent chromosomal mobilisation was first used by Zeldis et al. (1973) to orient Mu insertions in E.coli using F'Lac^{+}::Mu plasmids. Dénarié et al. (1977) used RP4::Mu plasmids to mobilise the E.coli chromosome, and found that the transfer was polar and dependent on the orientation of the Mu prophage in both the chromosome and the plasmid. Pérombelon and Boucher (1978) have also reported chromosomal mobilisation with RP4::Mu vectors in Ecc.

Whilst chromosome mobilisation from strains with Mu prophage integrated on both the chromosome and on a conjugative plasmid has been observed to originate from a unique origin on the chromosome it has been found that chromosomal markers on both sides of chromosomal Mu insertions are mobilised, albeit overwhelmingly so in one direction (Zeldis et al., 1973; Dénarié et al., 1977). This is the result of the spontaneous inversion of the G-segment, of either prophage, prior to mobilisation of the chromosome following cross-over between the two prophages in the G-segment. As suggested by Pérombelon and Boucher (1978) this low frequency transfer can be prevented by utilising Mu phage defective in G-segment inversion. Such a phage, which has a deletion across the boundary of the G-segment and the α-segment, was used in the present study. The phage Mu c$^{+}\Delta$445-7 was isolated from the E.coli K12 lysogen DK445 (Chow et al., 1977) in which a spontaneous insertion of 2.6kb had occurred in the β-segment of the

prophage. From this lysogen, progeny phage were obtained which were non-defective for growth on E.coli K12 but which had deletions of the Mu genome. Mu $c^{+}\Delta$445-7 has a 2.15kb deletion which runs from the end of the insertion into the G-segment, leaving the α-segment intact. Mu $c^{+}\Delta$445-7 is unable to invert its G-segment. The nature of the 2.6kb insertion in the DK445 lysogen and in Mu $c^{+}\Delta$445-7 has since been identified (Résibois et al., 1978) as consisting of two adjacent insertion sequences: IS2 and IS5.

6)b) MOBILISATION OF Mu INTO Ecc SCRI193 BY CONJUGATION

Having confirmed that Ecc SCRI193 was resistant to infection by both Mu.G(+) and Mu.G(-) phage, several plasmid::Mu cointegrates were tested for their ability to transfer into this strain. The plasmids were cointegrates with either RP4, R68 or a derivative of F. The Mu moieties were either Mu $c^{+}\Delta 445$-7 or Mucts. Already available in Ecc SCRI193 was the plasmid pKF1, an R68::Mu $c^{+}\Delta 445$-7 plasmid (Pérombelon and Boucher, 1978).

As shown in Table 11 all of the IncP plasmids, carrying either Mu $c^{+}\Delta 445$-7 or Mucts transferred readily into Ecc. The F-prime plasmid pKF3 could not be crossed into lysogenic or non-lysogenic Ecc recipients, even although it transferred within E.coli.

Table 11. Transfer of plasmid::Mu cointegrate plasmids from *E. coli* into *Ecc* SCRI193.

Plasmid	Donor strain[a]	Recipient strain[b]	Transconjugants isolated; transfer frequency per donor		Mating[c]	Selection for
F'*lacI*$^{-}$::*Mucts62* ProA^{+}::Tn10 (pKF3)	KF53	KF1016	no	$<10^{-9}$	m;30°C;1d	Tet^{R}
		KF1065	no	$<10^{-9}$	m;30°C;1d	Tet^{R};Pro^{+}
R68::Mu c$^{+}\Delta$445-7 (pGMI20)	GMI3246	KF1088	yes	10^{-5}	m;30°C;1d	Tet^{R}
R68::Mu c$^{+}\Delta$445-7 (pGMI22)	GMI3247	KF1088	yes	10^{-5}	m;30°C;1d	Tet^{R}
RP4::Mu cts62 (pKF2)	KF65	KF1016	yes	10^{-5}	a;30°C;1d	Tet^{R}
		KF1078	yes	10^{-5}	a;30°C;1d	Tet^{R}

a; All donors are derivatives of *E. coli* K12. b: All recipients are derivatives of *Ecc* SCRI193.

c: Mating on agar surface (a), or membrane (m).

6)c) CONJUGATION AND MUTAGENESIS WITH R68::Mu IN Ecc

6)c)i) CONJUGATION OF R68::Mu PLASMIDS WITHIN Ecc

The frequency of transfer of the R68::Mu $c^{+}\Delta 445\text{-}7$ plasmid, pKF1, within Ecc SCRI193 was studied (Table 12), and even after overnight (18h) membrane matings, the frequency of transfer (using the tetracycline resistance marker) was not high, compared to the frequency of transfer of RP4 (8.7×10^{-1} transconjugants /initial donor, 9h membrane mating at $30^{\circ}C$). Mating temperature ($30^{\circ}C$ or $37^{\circ}C$) had little effect on the efficiency of plasmid transfer.

By contrast, the transfer of pKF1, from KF1017, into the Crp^{-} strain KF1069 had some unusual features (Table 13). From the frequencies of plasmid and chromosome transfer into KF1069 it was apparent that on selection for the transfer of tetracycline resistance, many fewer transconjugants were isolated than would be expected. Analysis of the plasmid transconjugants selected by ampicillin or tetracycline resistance showed that while all of the former were phenotypically normal, those selected using tetracycline resistance commonly had deletions (Figure 2(b)) and were unable to produce viable Mu phage. Presumably the tetracycline resistance of R68 is improperly expressed in the Crp^{-} KF1069 and certain plasmid mutations (usually deletions in the Mu prophage) suppress this defective expression of the tetracycline resistance. Deletions in the phage are not however obligatory for the isolation of such tetracycline resistant transconjugants. The frequency of these plasmid mutations, usually deletions, in pKF1 as detected by selection for tetracycline resistant transconjugants, can be calculated from the quotient of the frequency

Table 12. Temperature and the mobilisation of pKF1 and of the chromosome from Ecc SCRI193 strain KF1017.

	Mating temperature[a]			
	30°C		37°C	
	f[b]	n[c]	f	n
Plasmid inheritance[d]	5.3×10^{-2}	4	3.4×10^{-2}	2
Chromosome inheritance[e]	4.4×10^{-5}	2	6.6×10^{-5}	2

a: KF1017 donors and KF1061 recipients were mated on membranes for 18h at the indicated temperature. b: Frequency of transconjugants per initial donor. c: Number of experiments. d: Inheritance of tetracycline resistance. e: Inheritance of His^+.

Table 13. pKF1 and chromosomal mobilisation from KF1017 to Crp^+ and Crp^- *Ecc* SCRI193 recipients.

Marker transferred	Frequency of inheritance[a]	
	KF1068 (Crp^+) recipients	KF1069 (Crp^-) recipients
Tc^R	3.9×10^{-2}	1.7×10^{-4}
Ap^R		7.0×10^{-2}
His^+	7.0×10^{-5}	8.6×10^{-5}

Unselected phenotype	Phenotype of transconjugants selected in KF1069 recipients			
	Tc^R transconjugants		Ap^R transconjugants	
	f[b]	n[c]	f	n
Tc^R		-	100	248
Ap^R	100	39		-
Sensitivity to IncP donor-specific phages	100	11	100	9
Production of Mu[d]	9	11	100	9
Plasmids[e] - presence	100	11	100	9
size[f]	g	11	h	9

a: Frequency of transconjugants per initial donor (averaged from two experiments) in an 18h membrane mating at 30°C between KF1017 donors and KF1068 (Crp^+) or KF1069 (Crp^-) recipients. b: Proportion of transconjugants tested (%). c: Number of transconjugants tested. d: Transconjugant caused the lysis of the Mu^S indicator strain *E.coli* ED8812. e: Electrophoretic gels by the method of Eckhardt (1978). f: See also Figure 2(b). g: Variable between that of pKF1 and R68. h: Identical to that of pKF1.

of inheritance of tetracycline resistance in the Crp^- recipient KF1069 and in the Crp^+ recipient KF1068 ($1.7x10^{-4}$ tetracycline resistant transconjugants /cell and $3.9x10^{-2}$ tetracycline resistant transconjugants /cell respectively) giving $5x10^{-3}$ mutant plasmids /non-mutant plasmid. The mechanism of this altered tetracycline resistance in KF1069 is unknown, but may be due to poor, or no, expression (transcription ?) of the tetracycline resistance gene in the Crp^- genetic background. Perhaps the plasmids in these rare transconjugants carry mutations which adjoin another promoter to the resistance gene, or alternatively remove an intervening stop codon(s) from between the resistance gene and an upstream promoter. Such a promoter might be in the Mu prophage as all of the deletion mutants observed were unable to produce viable Mu phage. A deletion extending from the Mu replication genes, including any intervening R68 DNA up to the resistance gene, might allow transcription from the early promoter of Mu, $\underline{p}_e$ (Giphart-Gassler et al., 1981,a,b; Priess et al., 1982). Transcriptional readthrough, by the above mechanism, from the repressor promoter, $\underline{p}_i$ (Priess et al., 1982), is unlikely as this would result in the constitutive expression of the remaining Mu replication genes, which would be lethal for the transconjugant.

6)c)ii) ZYGOTIC AND TEMPERATURE INDUCTION OF Mu IN Ecc

By crossing the R68::Mu c^+_A445-7 plasmid, pKF1, from KF1075 into the lysogenic strain KF1016 and the non-lysogenic strain KF1006 it was observed that the plasmid inheritance frequency fell 30-fold from $6.4x10^{-2}$ transconjugants /initial donor to $2.0x10^{-3}$ transconjugants /initial donor. This suggests that the Mu prophage on pKF1 was

zygotically induced on transfer into the non-lysogenic recipient.

The temperature induction of the prophage on the RP4::Mucts plasmid, pKF2, in Ecc KF1083 and in E.coli KF78 (this latter lysogen being isolated from a cross between Ecc KF1083 and E.coli ED8812) were compared. Both lysogens produced phage spontaneously at 10^5 plaque-forming units/ml prior to induction. After induction of the prophage at 37°C, following a prior heat shock at 43°C for 30min, there was a lag (0.5h in E.coli and 1.5h in Ecc) during which phage were still produced at the spontaneous rate. After the lag phase, phage production increased (100-fold in 1h in E.coli and in 2h in Ecc). The longer duration of the lag phase and the slower production of free phage after induction in Ecc probably reflects the slower growth rate of Ecc compared to E.coli.

6)c)iii) Mu INSERTIONAL MUTAGENESIS IN Ecc

The transposition of Mu from cointegrate plasmids onto the bacterial chromosome was studied following zygotic or temperature induction.

pKF1 was conjugated from KF1075 into the non-lysogenic recipient KF1006 during an overnight mating at 30°C, after which the mating culture was cycloserine enriched for xylose non-utilising KF1006 mutants. No such KF1006 mutants were recovered from 1,000 enrichment survivors. From a parallel mating 100 trimethoprim resistant recipients were isolated; none of these carried Mu and so presumably were spontaneous mutants.

A second method for the detection of chromosomal Mu insertions following zygotic induction made use of the observation that the cotransfer, and maintenance, of the pKF1 plasmid in certain chromosomal transconjugants in matings with KF1017 donors to non-lysogenic recipients (e.g. His^+) was a very rare event (see later). Therefore chromosomal transconjugants which have also inherited Mu will have been derived from the transposition of Mu, from the non-autonomously replicable, mobilised chromosomal DNA, onto the chromosome of the transconjugant. This is formally the same as isolating lysogens in E.coli by testing survivors from infection. Following such an infection in E.coli, lysogens are recovered at 5-10% of survivors following a single cycle of infection (Howe and Bade, 1975). Accordingly, His^+ chromosomal transconjugants, from overnight matings at 30°C between KF1017 and KF1060 (183 His^+ transconjugants), KF1061 (125 His^+ transconjugants) and KF1069 (123 His^+ transconjugants), were tested for Mu lysogeny (and the absence of the plasmid); however no lysogenic His^+ transconjugants were found ($<2.3 \times 10^{-3}$ lysogens /His^+ transconjugant).

No Mu insertional mutants ($<4.5 \times 10^{-4}$ auxotrophic mutants/enrichment survivor) were isolated when a liquid, LB culture of the Ecc lysogen KF1083 (an Ecc SCRI193 prototrophic strain carrying RP4::Mucts) was partially heat-induced by incubation at 36°C overnight and then cycloserine-enriched for auxotrophs in glucose MM.

The dichotomy between the zygotic and temperature induction of prophage, on the one hand, and the difficulty of isolating Mu insertional mutations on the other, is interesting. Following

infection of a Mu sensitive strain, Mu can only be maintained if it is integrated into a replicon, typically the host chromosome or a plasmid. This lysogenic pathway requires a short period of expression of the early operon genes which code for the transposition/replication functions, and thereafter the repression of this pathway by the cI repressor. Continued expression of the early operon genes results in the phage entering the lytic pathway through the multiple transposition of the phage. This is followed by the expression of the late operon genes, which code for structural phage proteins, the lysis of the cell, and finally the release of free phage particles. When Mu is introduced into a non-lysogenic bacterium on a plasmid, a short period of expression of the early operon genes is not required for the maintenance of the phage in the bacterium; the immediate expression expression of the cI repressor gene will send the phage into lysogeny. Alternatively the uninterrupted expression of the early operon genes will send the phage into the lytic pathway. Thus there need not be any low-level expression of the early operon, followed by cI repressor synthesis, to allow the establishment of lysogeny when the phage is carried on a plasmid.

In Ecc there seems to be strong selection on phage entering strains either to establish lysogeny immediately or to induce and replicate. The low-level expression of the early operon which allows the transposition of a single copy of the phage seems to be a very rare event. The phage is either switched into lysogeny or into replication (Giphart-Gassler et al.,1981,b; Priess et al., 1982; Van de Putte et al.,1980,b; Van Meeteren and Van de Putte, 1980). The reason for this could be slight differences in the transcription or translation of one

or more of the phage genes in Ecc such that the fine control of expression of the early operon found in E.coli and related species, which allows the transposition of the phage to a new site on the chromosome prior to the establishment of lysogeny, does not occur.

6)d) MOBILISATION OF THE Ecc CHROMOSOME BY R68::Mu

6)d)i) THE CONTRIBUTION OF Mu INSERTIONS ON THE MOBILISING PLASMID R68 OR RP4 AND ON THE CHROMOSOME OF THE DONOR.

In parallel experiments the mobilisation of the Ecc chromosome by the plasmids RP4, R68::Mu $c^{+}\Delta 445$-7 (pKF1), or RP4::Mucts62 (pKF2) was studied (Table 14). Although the plasmid RP4 transferred at a very high frequency, RP4-mediated mobilisation of the chromosome occurred at a very low frequency. By contrast, whilst pKF1 and pKF2 were transferred at less than one-hundredth the frequency of RP4, both these plasmids mobilised the chromosome several hundred-fold more efficiently than RP4. The frequencies of inheritance of hisD1, leu-2 and thr-1, when mobilised by pKF1 or pKF2, were all comparable, suggesting that the chromosome was mobilised randomly by both of these plasmids. These chromosomal transconjugants also carried coinherited chromosomal markers.

When a Mu prophage was present on the chromosome of Ecc, using the thyA1::Mu $c^{+}\Delta 445$-7 insertional mutation, the pattern of chromosomal mobilisation by pKF1 was altered (Tables 16(a), 15(a)). The frequency of inheritance leu-2 and thr-1 was unchanged, but the frequency of inheritance of hisD1 was increased three-fold (from a Student's t-test there is only a 10% probability that this difference was due to chance). Coinheritance of chromosomal markers was still observed.

The coinheritance of the pKF1 plasmid in chromosomal transconjugants was low (1.1×10^{-2} plasmid transconjugants /chromosomal transconjugants; that is 32 tetracycline resistant transconjugants ÷ 2899 chromosomal

Table 14. Chromosomal mobilisation by RP4,pKF1 and pKF2 plasmids in *Ecc* SCRI193.

Strains: KF1084: *chl-1 rif-1 thyA2* / RP4
KF1091: *chl-1 rif-1* / R68::Mu (pKF1)
KF1083: *chl-1 rif-1* / RP4::Mu (pKF2)
KF1060: *hisD1 leu-2 nal-1 str-1 thr-1*

Donor	Counter-selection	Plasmid transfer		Frequency chromosomal marker inheritance[a]					
				$HisD1^+$		$Leu\text{-}2^+$		$Thr\text{-}1^+$	
		f[b]	n[c]	f	n	f	n	f	n
KF1084[f]	Str	8.1×10^{0}	1	5.5×10^{-8}	1	6.8×10^{-8}	1	6.8×10^{-8}	1
KF1091	Str	3.2×10^{-2}	2	3.2×10^{-5}	2	2.5×10^{-5}	2	3.6×10^{-5}	2
KF1083	Str	4.2×10^{-2}	1	9.1×10^{-6}	1	7.5×10^{-6}	1	7.9×10^{-6}	1

Donor	Selected marker	Counter-selection	n[c]	T[d]	%Coinheritance of unselected markers					
					$HisD1^+$	$Leu\text{-}2^+$	$Nal\text{-}1^S$	$Rif\text{-}1^R$	$Str\text{-}1^S$	$Thr\text{-}1^+$
KF1084	$HisD1^+$	Str	1	1	-	(0)[e]	(0)	(0)	-	(0)
	$Leu\text{-}2^+$	Str	1	1	(0)	-	(0)	(0)	-	(0)
	$Thr\text{-}1^+$	Str	1	1	(0)	(0)	(0)	(0)	-	-
KF1091	$HisD1^+$	Str	2	78	-	<1.3	<1.3	<1.3	-	<1.3
	$Leu\text{-}2^+$	Str	2	51	<2.0	-	<2.0	<2.0	-	12
	$Thr\text{-}1^+$	Str	2	109	<0.9	23	<0.9	<0.9	-	<0.9
KF1083	$HisD1^+$	Str	1	34	-	<3.0	<3.0	<3.0	-	<3.0
	$Leu\text{-}2^+$	Str	1	60	<1.7	-	<1.7	<1.7	-	16
	$Thr\text{-}1^+$	Str	1	59	<1.7	19	<1.7	<1.7	-	<1.7

a: Membrane mating at 30°C for 18h. b: Frequency of inheritance per initial donor. c: Number of experiments. d: Number of transconjugants tested. e: Frequencies in parenthesis are calculated from fewer than 30 transconjugants. f: The inheritance of $HisD1^+$ and of $Ser\text{-}1^+$ by RP4 in a cross between KF1084 and KF1061 (membrane mating at 30°C for 9h) was not detected ($<3.0\times10^{-9}$ chromosomal transconjugants / initial donor; counter-selected either with Str or with Nal); RP4 transferred at 5.5×10^{-1} transconjugants / initial donor.

transconjugants) and were presumably the result of the independent transfer of the plasmid from the donors to chromosomal transconjugants (the plasmid was inherited at 3.4×10^{-2} transconjugants /recipient; averaged from eleven matings). It is however, more interesting to compare the frequency of coinheritance of the plasmid in chromosomal transconjugants selected for the inheritance of an 'early' marker (that is one mobilised polarly following the recombination of the plasmid into the Mu prophage in thyA1 on the donor chromosome, e.g. hisD1, glyA1, trp-2, manA1) or of a 'late' marker (that is one mobilised non-polarly following the random integration of the plasmid into the donor chromosome, e.g. pheA1, proA1, leu-2, thr-1); respectively, 3.4×10^{-3} plasmid transconjugants /chromosomal transconjugant (6 tetracycline resistant chromosomal transconjugants ÷ 1752 chromosomal transconjugants) and 2.4×10^{-2} plasmid transconjugants /chromosomal transconjugant (22 tetracycline resistant chromosomal transconjugant ÷ 922 chromosomal transconjugants). Chromosomal transconjugants arising from non-polar transfers were as likely to inherit the plasmid as the recipients in general, implying that such chromosomal mobilisation was not by R-primes as the coinheritance of the plasmid would then be much greater. The proportion of early marker transconjugants which have been mobilised not by the polar, but by the non-polar mechanism, c.10% of hisD1, c.50% of trp-2, in fact would account for all of the observed chromosomal transconjugants which also carried a plasmid. Thus transconjugants derived from non-polar transfers inherited the plasmid at a frequency equal to the recipient population in general. The plasmid was not apparently transferred to chromosomal transconjugants derived from polar chromosomal transfer, as might be expected given the long duration of

the matings. This could be a reflection of a low copy number of episomic plasmids in donors in which the plasmid has integrated into the chromosome.

With respect to the non-polar mobilisation of the chromosome the following points should be noted: (a) It has been reported that there are slight differences between the two IncP plasmids used in this study - RP4 and R68 (Willetts et al., 1981; Krishapillai, 1977); however no differences in the pattern of non-polar chromosome mobilisation by the R68 or RP4 cointegrate plasmids was observed. (b) There was no correlation between the sensitivity of donor strains to donor-specific phages (GU5, PRD1) and non-polar chromosome mobilisation: strains carrying pKF1 (KF1017, KF1092) were sensitive, while strains carrying RP4 (KF1084) and pKF2 (KF1083, KF1085) were all partially resistant. (c) As noted above, the prophage in pKF1 has two internal insertion sequences, IS2 and IS5; however as chromosomal mobilisation by the IS2, IS5 free plasmid pKF2 (in donors without a chromosomal Mu insertion) was as efficient as from donor strains carrying pKF1, these sequences cannot be playing a role. It is apparent then that the presence of Mu on the mobilising plasmid enhances the frequency of mobilisation of the chromosome by these plasmids.

Presumably this involves the spontaneous replication of the prophage on the plasmid such that the plasmid is integrated at random sites into the host chromosome. This intermediate, consisting of the R68 plasmid flanked by Mu prophages can then act as an Hfr for the mobilisation of the host chromosome (Bialy et al., 1980; Toussaint et

al., 1981; Van Guijsegem and Toussaint, 1982). In E.coli such chromosomal mobilisation would be at a lower frequency than that observed here in Ecc (unless the prophage had been induced prior to conjugation). This suggests either that the Ecc genetic background is contributing in some way to the formation of these Hfr-type donors, or that the behaviour of the mobilising vector, R68 in this case, is not identical to that of the F-factors which were used in the studies on E.coli.

If the spontaneous transposition of the phage on the plasmid is involved in the non-polar transfer, as seems probable, then the ready isolation of new Mu insertional mutations might also then be expected. Other studies in E.coli however, have shown that the viability of the donor is not necessary for the donation of chromosomal DNA by this process (Van De Putte and Gruijthuijsen, 1972). Thus the phage in these Ecc donors could be undergoing multiple transpositions, which while integrating the plasmid into the chromosome, will also be lethal for the bacterium. The transposition of single copies of the prophage to give stable, single insertions is not then a prerequisite for non-polar chromosomal mobilisation.

In summary, the mobilisation of the Ecc chromosome by R68 or RP4 plasmids is enhanced when the plasmid carries a Mu insertion, and can be further enhanced for certain regions of the chromosome by the insertion of a Mu prophage onto the chromosome of the donor strain.

6)d)ii) MATING CONDITIONS AND THE MOBILISATION OF THE CHROMOSOME

As the frequency of mobilisation of the chromosome from KF1017 was low (Table 12), 18h matings were used throughout the following analysis (2h matings yielded barely detectable numbers of chromosomal transconjugants). As would be expected from the negligible effect of the mating temperature on the transfer of pKF1 itself, temperature was found to have little effect on the frequency of transfer of the chromosome (Table 12); matings were accordingly performed at 30°C.

6)d)iii) QUANTITATIVE ANALYSIS OF CHROMOSOMAL MOBILISATION FROM KF1017 DONORS INTO MULTIPLY AUXOTROPHIC RECIPIENTS.

The KF1017 donor is a Deo^- derivative of the Ecc SCRI193 rif-1 thyA1::Mu $c^+\Delta$445-7 /R68::Mu $c^+\Delta$445-7 strain isolated by Pérombelon and Boucher (1978). Confirmation that the thyA1 mutation and the Mu insertion in this strain were the result of dependent and not independent events was obtained by determining the frequency of reversion of thyA1 to prototrophy, and by determining the degree of linkage between thyA1 and the Mu insertion. As is characteristic of Mu insertional mutations (Taylor, 1963), and unlike most spontaneous mutations, thyA1 did not revert ($<8.3\times10^{-11}$ revertants/cell). Linkage between thyA1 and Mu was inferred from a mating between KF1017 and KF1072. Trimethoprim resistant recipients were recovered from the mating at 5×10^{-8} mutants/initial donor (twelve were recovered), and of these, 25% were lysogenic (and pKF1 free). Thus coinheritance between thyA1 and Mu is at least 25%; since the nine non-lysogenic, thymine

auxotrophs could have been the result of spontaneous mutations to trimethoprim resistance in the recipient (which occurs at a comparable frequency) the linkage between thyA1 and Mu may be greater than 25%. Linkage between thyA1::Mu in the chromosomal transconjugants and aroB1, deo-1, glyA1, hisD1, nal-1, rif-1, and str-1 was not detected (<33%).

The multiply auxotrophic recipients used in the matings were not lysogenic for Mu as previous results (Section 6)c)ii)) had shown that 3% of transconjugants receiving Mu survived zygotic induction. The thyA1::Mu donor mutation also allowed the counter selection of the donors by a mutation which was not mobilised (at least from polar donors).

Given that KF1017 mobilises the chromosome by both a polar and a non-polar mechanism, the following predictions can be made: (a) All markers should be mobilised. (b) The counter-selection against the KF1017 donors, by the omission of thymine from the selection media, will not select against chromosomal transconjugants which are derived from polar transfers (as thyA1 will be transferred as a late marker). In the case of non-polar transfers this counter-selection will depress the apparent frequency of inheritance of markers which are closely linked to thyA1, and will also reduce the frequency of coinheritance of markers in this region; gene orders will not be altered. (c) The frequency of inheritance of a marker will depend on its location on the chromosome. Markers which are mobilised by the polar mechanism will be inherited more frequently than markers which are mobilised only by the non-polar mechanism. (d) Markers mobilised by the polar

mechanism will show a gradient in the inheritance frequencies such that early markers will be inherited more frequently than late markers. The coinheritance of markers adjacent to the selected marker will also show a polarity of the coinheritance frequencies. Transconjugants selected for a late marker will inherit earlier markers with a high probability which is dependent only on the frequency of the recombination of the markers into the recipient chromosome. These coinheritance frequencies will decrease towards a minimum of 50% with increasing distance between the two markers. The coinheritance of late markers from early markers will also show a decrease in frequency with increasing distance between the markers but which will be additionally dependent on the entry of the later marker into the recipient. These latter coinheritance frequencies will be lower than the reciprocal coinheritance frequencies. (e) Pairs of markers which are both mobilised only by the non-polar mechanism will show no difference in the coinheritance frequencies from reciprocal selections; the non-polar coinheritance frequencies will depend on both the entry of the markers into the recipient, and on the recombination of the markers into the recipient chromosome.

THE ORDER OF MARKERS ON THE CHROMOSOME OF Ecc AS CALCULATED FROM COINHERITANCE FREQUENCIES

The order of the markers on the Ecc chromosome was determined using coinheritance frequencies from KF1017 donors. Although thymine prototrophic chromosomal transconjugants which were additionally nalidixic acid or streptomycin resistant were also isolated, only chromosomal transconjugants which had been selected for thymine

Figure 12.The % coinheritance-frequencies (and 80% confidence limits) between markers on the Ecc SCRI193 chromosome mobilised from KF1017 donors.

Figure 12(a). Markers coinherited with $HisD1^+$.

Figure 12(b). $HisD1^+$ coinherited from other markers.

Figure 12(c). $Str\text{-}1^S$ coinherited from other markers.

Figure 12(a).

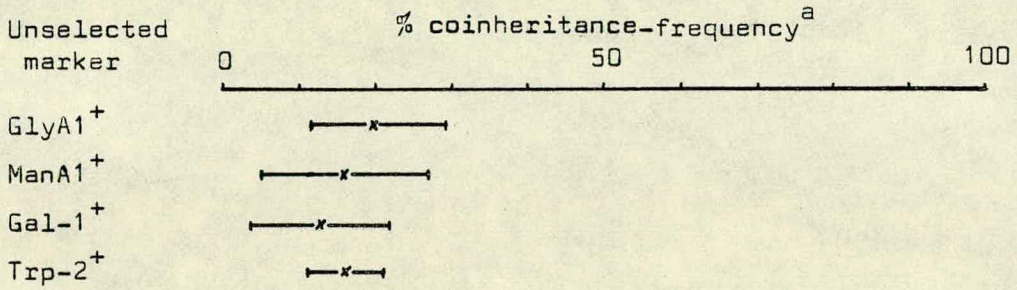


Figure 12(b).

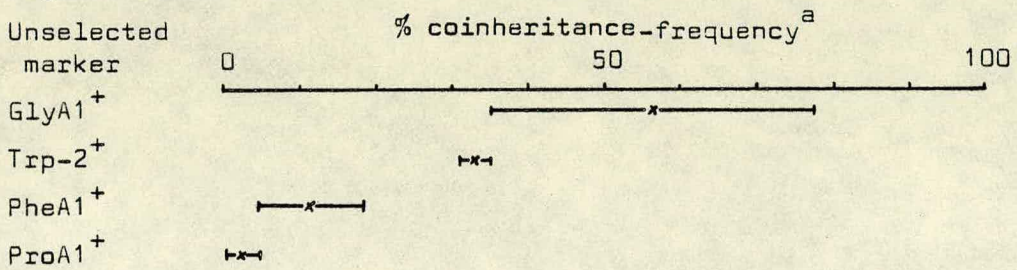
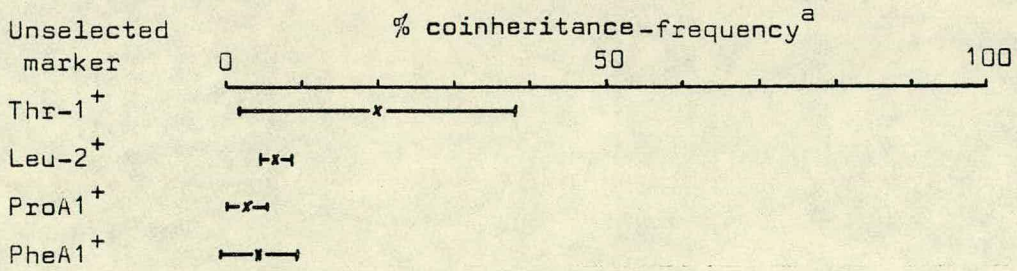


Figure 12(c).



a: % coinheritance-frequencies and 80% confidence limits were calculated from the results of two to four experiments; see Table 15 for further % coinheritance-frequencies.

Table 15. % coinheritance frequencies between Ecc SCRI193 chromosomal markers mobilised from KF1017 donors to multiply auxotrophic recipients.

Table 15(a). coinheritance of markers into KF1060.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1060: hisD1 leu-2 nal-1 str-1 thr-1

Counter-selection	Selected marker	n[a]	T[b]	% coinheritance of unselected markers[c]						
				His^+	$Leu\text{-}2^+$	$Nal\text{-}1^s$	$Rif\text{-}1^R$	$Str\text{-}1^s$	$Thr\text{-}1^+$	
Thy	His^+	3	333	-	<0.3	1.0[d]	<0.3	<0.3	<0.3	
	$Leu\text{-}2^+$	3	265	0.5	-	0.9[d]	0.5	6.8	23	
							* —— *			0.5[e]
								* — *		4.4
				* ——		—— *	—— *			0.2
	$Thr\text{-}1^+$	3	282	0.4	27	2.7[d]	2.9	20	-	
				* ——		——	——	—— *		0.4
					* ——	——	——	—— *		8.4
					* ——	—— *				2.4
					* — *					1.8
						* ——	——	—— *		0.9
						* — *				1.8
							* —	—— *		2.1
				* ——	* ——	——	——	—— *		0.2
					* ——	——	—— *	— *		2.1
					* ——	* — *				1.8
					* ——	* —	* —	—— *		0.9

Table 15 cont.

Table 15(b). Coinheritance of markers into KF1061.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1061: hisD1 nal-1 pheA1 ser-1 str-1

Counter-selection	Selected marker	n[a]	T[b]	% coinheritance of unselected markers[c]					
				$His^{+}D1$	$PheA1^{+}$	$Rif\text{-}1^{R}$	$Ser\text{-}1^{+}$	$Str\text{-}1^{s}$	
Thy	$HisD1^{+}$	2	251	-	<0.4	<0.4	<0.4	<0.4	
	$PheA1^{+}$	2	202	11	-	0.8	0.8	4.1	
									1.6[e]
									0.4
									0.8
									0.8
	$Ser\text{-}1^{+}$	2	205	<0.5	<0.5	<0.5	-	<0.5	

Table 15(c). Coinheritance of markers into KF1068.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1068: gal-1 hisD1 nal-1 proA1 str-1 trp-2

Counter-selection	Selected marker	n[a]	T[b]	% coinheritance of unselected markers[c]						
				$Gal\text{-}1^{+}$	$His^{+}D1$	$ProA1^{+}$	$Rif\text{-}1^{R}$	$Str\text{-}1^{s}$	$Trp\text{-}2^{+}$	
Thy	$HisD1^{+}$	2	263	14	-	<0.4	<0.4	<0.4	12	
										11[e]
	$ProA1^{+}$[f]	2	78	2.1	4.4	-	<1.3	2.1	2.1	
										2.1
	$Trp\text{-}2^{+}$	2	111	64	34	1.5	<0.9	<0.9	-	
										34
										1.5

Table 15 cont.

Table 15(d). Coinheritance of markers into KF1069.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1069: crp-1 hisD1 nal-1 proA1 str-1 trp-2

Counter-selection	Selected marker	n[a]	T[b]	% coinheritance of unselected markers[c]					
				Crp-1$^+$	HisD1$^+$	ProA1$^+$	Rif-1^R	Str-1^S	Trp-2$^+$
Thy	Crp-1$^+$	1	3	-	(<33)	(<33)	(<33)	(<33)	(<33)
	HisD1$^+$	2	220	<0.4	-	<0.4	<0.4	<0.4	21
	ProA1^{+f}	2	138	2.9	0.7	-	1.5	2.9[e]	2.9
									1.5[e]
	Trp-2$^+$	2	205	<0.5	32	<0.5	<0.5	<0.5	-

Table 15(e). Summary of coinheritance of markers into KF1068 and KF1069.

Counter-selection	Selected marker	% coinheritance of unselected markers[c]						
		Crp-1$^+$	Gal-1$^+$	HisD1$^+$	ProA1$^+$	Rif-1^R	Str-1^S	Trp-2$^+$
Thy	HisD1$^+$	(<33)	14	-	<0.2	<0.2	<0.2	16[e]
								11[e]
	ProA1$^+$	2.9	2.1	2.2	-	0.7	2.5	2.9
								1.5
								2.1
	Trp-2$^+$	(<33)	64	33[g]	0.7	<0.3	<0.3	-
								34
								0.7

Table 15(f). Coinheritance of markers into KF1064.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1064: aroB1 glyA1 hisD1 nal-1 str-1

Counter-selection	Selected marker	n[a]	T[b]	%coinheritance of unselected markers[c]				
				AroB1$^+$	GlyA1$^+$	HisD1$^+$	Rif-1^R	Str-1^S
Thy	HisD1$^+$	1	85	<1.2	22	-	<1.2	<1.2

Table 15 cont.

Table 15(g). Coinheritance of markers into KF1072.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1072: aroB1 glyA1 hisD1 manA1 nal-1 str-1

Counter-selection	Selected marker	n[a]	T[b]	% coinheritance of unselected markers[c]						
				AroB1$^+$	GlyA1$^+$	HisD1$^+$	ManA1$^+$	Rif-1^R	Str-1^S	
Thy	GlyA1$^+$	1	22	(<4.5)	-	(36)	(27)[e]	(<4.5)	(<4.5)	(14)[e]
	HisD1$^+$	1	38	<2.6	10	-	8	<2.6	<2.6	8
	ManA1$^+$	1	15	(<6.7)	(47)	(47)	-	(<6.7)	(<6.7)	(27)

Table 15(h). Coinheritance of markers into KF1089.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1089: aroB1 glyA1 hisD1 manA1 nal-1 nal-2 str-1

Counter-selection	Selected marker	n[a]	T[b]	% Coinheritance of unselected markers[c]								
				AroB1$^+$	GlyA1$^+$	HisD1$^+$	ManA1$^+$	Nal-1^S Nal-2^S	Nal-2^S	Rif-1^R	Str-1^S	
Thy	GlyA1$^+$	2	125	<0.8	-	67	50	<0.8	50	<0.8	<0.8	38[e]
						*	*		*			35
						*	*					26
							*		*			19
						*	*		*			
	HisD1$^+$	2	174	<0.6	24	-	20	<0.6	45	<0.6	<0.6	15
					*		*		*			18
					*		*					12
							*		*			11
					*		*		*			
	ManA1$^+$	1	3	(0)	(100)	(100)	-	(0)	(33)	(0)	(0)	(100)
					*	*						(33)
					*	*			*			

Table 15 cont.

Table 15(i). Coinheritance of markers into KF1019.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1019: his-2 nal-3 str-1 Pec$^-$

Counter-selection	Selected marker	n[a]	T[b]	% Coinheritance of unselected markers[c]	
				Nal-3^s	Str-1^s
Thy	His-2$^+$	1	158	18	<0.6

Table 15(j). Summary of coinheritance of markers into KF1064,KF1072 and KF1089.

Counter-selection	Selected marker	% coinheritance of unselected markers[c]								
		AroB1$^+$	GlyA1$^+$	HisD1$^+$	ManA1$^+$	Nal-1^s	Nal-2^s	Rif-1^R	Str-1^s	
Thy	GlyA1$^+$	<0.7	-	57	43		50[e]	<0.7	<0.7	38[e]
				*			*			27
				*	*					26
					*		*			17
				*	*		*			
	HisD1$^+$	<0.3	20	-	16		45	<0.3	<0.3	15
			*				*			14
			*		*					12
					*		*			11
			*		*		*			

a: Number of experiments. b: Number of transconjugants tested. c: Frequencies in parenthesis were calculated from less than 30 transconjugants. d: From a single experiment, testing 100 transconjugants. e: The % coinheritance frequencies of the indicated (*) unselected markers from the selected marker. f: % coinheritance frequencies were corrected for Pro$^+$ revertants. g: The apparent discrepancy between these two % coinheritance frequencies is due to the amalgamation of the results: the Trp-2$^+$ HisD1$^+$ frequency being obtained from both Tables 15(c) and (d), and the Trp-2$^+$HisD1$^+$ Gal-1$^+$ being obtained only from Table 15(c).

prototrophy alone were used in the analysis below (Tables 15, 16). The other two classes of transconjugants gave similar frequencies of coinheritance of unselected markers (Appendix IV).

Crosses were first scored for pairs of markers which showed a large difference in the coinheritance frequencies from reciprocal selections. Such markers will have been mobilised by the polar mechanism; the unselected marker with the greater frequency of coinheritance being the first marker of the pair to be mobilised. The remaining markers in that cross were then located relative to these markers by three-point cross analysis. When there was no clear polarity of coinheritance (<2-fold) between pairs of markers, indicating that they had been mobilised by the non-polar mechanism, ordering was by three-point cross analysis. Comparisons of coinheritance frequencies of <2% have been avoided. Although the 80% confidence limits of the coinheritance frequencies shown in Figure 12 are in some cases quite large, the orders of markers obtained from individual experiments were always identical.

The results from crosses between KF1017 and KF1060 are given in Table 15(a). As can be seen, there is no polarity in the coinheritance of leu-2 and thr-1. Analysis of three-point crosses of these two markers with nal-1, rif-1 and str-1 gives the orders leu-2 - thr-1 - str-1, thr-1 - str-1 - rif-1 and thr-1 - str-1 - nal-1 which together give leu-2 - thr-1 - str-1 - nal-1/ rif-1. With the available data it is not possible to order nal-1 and rif-1 with respect to each other. There is probably a polarity of coinheritance between hisD1 and the leu-2 - thr-1 region.

The results of crosses between KF1017 and KF1061 are given in Table 15(b). The strong polarity of coinheritance between hisD1 and pheA1 implies that pheA1 is transferred late with respect to hisD1. Str-1 is probably located on the other side of pheA1 to hisD1 as the coinheritance of str-1 from pheA1 is less than that from pheA1 to hisD1 and close linkage of hisD1 and str-1 was not observed. This gives the order ori - hisD1 - str-1. The location of ser-1 is not clear as it does not show strong linkage to any of the markers used in this cross, though some linkage to pheA1 was detected (which was less than the linkage from pheA1 to str-1); ser-1 is, however, clearly not located in the ori - hisD1 - pheA1 - str-1 region of the chromosome.

The results from crosses between KF1017 and KF1068 are shown in Table 15(c). Strong polarity of coinheritance is shown by hisD1 and proA1 and by hisD1 and trp-2, giving the order ori - hisD1 - trp-2 - proA1. The very strong linkage between gal-1 and trp-2 (62%) implies that gal-1 is located close to trp-2; three-point cross analysis gives the order ori - hisD1 - gal-1 - trp-2 - proA1. As str-1 is linked only to proA1 its location must be ori - hisD1 - gal-1 - trp-2 - proA1 - str-1.

The results from crosses between KF1017 and KF1069 are shown in Table 15(d). The same gene order is obtained for the markers common to KF1068 and KF1069, that is ori - hisD1 - trp-2 - proA1 - str-1. The coinheritance of crp-1 in proA1 chromosomal transconjugants, some of which also coinherited rif-1 and str-1, gives ori - hisD1 - trp-2 - proA1 - crp-1 /str-1. The results of Tables 15(c,d) are summarised together in Table 15(e).

The coinheritance frequencies from crosses between KF1017 and KF1064, KF1072 and KF1089 recipients (Tables 15(f,g,h)) are summarised together in Table 15(j). The analysis of crosses between KF1017 and KF1060, discussed above, showed that nal-1 was linked to str-1 but not to hisD1; however as shown in Table 15(i), his-2 and nal-3 in KF1019 are linked, but are not linked to str-1. This raised the possibility that there were two nal loci, one linked to str-1 and the other to hisD1. A second mutation (nal-2) in the nal-1 KF1072, which conferred increased resistance to nalidixic acid, was shown (Table 15(h)) to segregate independently of nal-1 and str-1, but did show linkage to hisD1. It is probable therefore that nal-2 is a mutation at or near the nal-3 locus.

The polarity of coinheritance of glyA1 and hisD1 gives ori - hisD1 - glyA1. Analysis of three-point crosses extends this to ori - hisD1 - glyA1 - manA1. Nal-2 can be located between ori and hisD1, by analysis of three-point crosses between hisD1, glyA1, manA1 and nal-2. This gives the order nal-2,3 - his-1,2 - glyA1 - manA1; no linkage was detected between any of these markers and aroB1, str-1 or rif-1.

In summary, the above linkage groups are : hisD1 - leu-2 - thr-1 - str-1 - nal-1/rif-1, ori - hisD1 - pheA1 - str-1, ori - hisD1 - gal-1 - trp-2 - proA1 - str-1, ori - hisD1 - trp-2 - proA1 - crp-1/rif-1/str-1, and ori - nal-2,3 - his-1,2 - glyA1 - manA1. To combine these groups together the coinheritance frequencies of markers from hisD1, and to hisD1 and to str-1 were compared (Figure 12). The frequencies of coinheritance of markers to hisD1 (Figure 12(a)) overlap and so cannot be used for ordering markers. The coinheritance

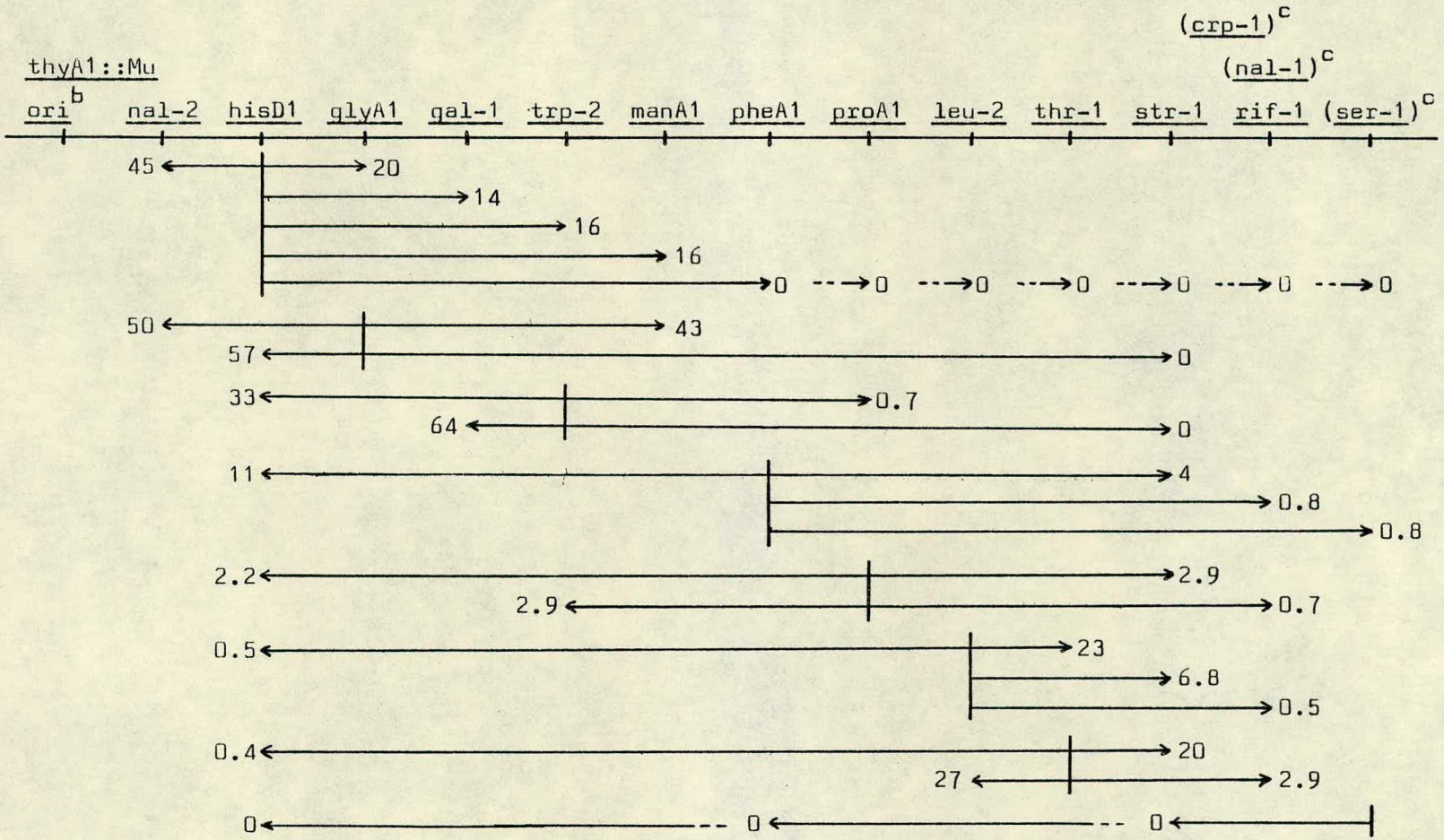


Figure 13. Summary of % coinheritance frequencies of markers mobilised from KF1017 donors[a].

a: Taken from Tables 15(a,b,e,j). b: Origin of polar chromosomal transfer. c: Markers in parenthesis are approximately located, relative to adjacent markers. d: % coinheritance frequencies are shown for pairs of markers; the selected marker indicated by a vertical line and the unselected marker indicated by an arrow-head.

frequencies of hisD1 from other markers (Figure 12(b)) gives the order hisD1 - glyA1 - trp-2 - pheA1 - proA1. From the coinheritance of str-1 from other markers (Figure 12(c)) the order proA1 - leu-2 - str-1 is obtained. Figure 13 combines all of these results as a single linkage map. It should be noted that manA1 cannot be mapped precisely by the above methods (but see Section 6)d)iv)).

ANALYSIS OF THE FREQUENCIES OF INHERITANCE OF MARKERS

The frequencies of inheritance of the markers from KF1017 donors (Table 16,a-i) are summarised together in Figure 14. The linkage map can be subdivided into three sections on the basis of the inheritance frequencies.

PheA1, proA1, leu-2 and thr-1 were all inherited at similar frequencies (c.10^{-5} transconjugants/initial donor) suggesting that they were all mobilised by the non-polar mechanism.

The frequencies of inheritance of hisD1, glyA1, gal-1, trp-2 and manA1. were in a gradient from a maximum of c.10^{-4} transconjugants/initial donor down to a minimum of c.10^{-5} transconjugants/initial donor, suggesting that they were mobilised by the polar mechanism. Two of the markers in this section gal-1 and manA1, were inherited at less than the expected frequency; indeed the inheritance of gal-1 could only be detected indirectly by its coinheritance from other markers. This reduced frequency of inheritance of gal-1 and manA1 must be because their direct selection is completely or partially lethal; in E.coli gal and manA are involved

Table 16. Mobilisation frequencies of Ecc SCRI193 chromosomal markers from KF1017 donors to multiply auxotrophic recipients.

Table 16(a). Chromosomal mobilisation from KF1017 to KF1060.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1060: hisD1 leu-2 nal-1 str-1 thr-1

Counter-selection	Frequency of marker inheritance[a]					
	HisD1$^+$		Leu-2$^+$		Thr-1$^+$	
	f[b]	n[c]	f	n	f	n
Thy	1200	3	74	3	94	3

Table 16(b). Chromosomal mobilisation from KF1017 to KF1061.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1061: hisD1 nal-1 pheA1 ser-1 str-1

Counter-selection	Frequency of marker inheritance[a]					
	HisD1$^+$		PheA1$^+$		Ser-1$^+$	
	f[b]	n[c]	f	n	f	n
Thy	1100	2	120	2	73	2

Table 16(c). Chromosomal mobilisation from KF1017 to KF1068.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1068: gal-1 hisD1 nal-1 proA1 str-1 trp-2

Counter-selection	Frequency of marker inheritance[a]							
	Gal-1$^+$		HisD1$^+$		ProA1$^+$		Trp-2$^+$	
	f[b]	n[c]	f	n	f	n	f	n
Thy	<0.7	2	780	2	82[d]	2	210	2

Table 16 cont.

Table 16(d). Chromosomal mobilisation from KF1017 to KF1069.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1069: crp-1 hisD1 nal-1 proA1 str-1 trp-2

Counter-selection	Frequency of marker inheritance[a]							
	Crp-1$^+$		HisD1$^+$		ProA1$^+$		Trp-2$^+$	
	f[b]	n[c]	f	n	f	n	f	n
Thy	2.1	2 860	2	100[d]	2	210	2	

Table 16(e). Summary of chromosomal mobilisation from KF1017 to KF1068 and KF1069.

Counter-selection	Frequency of marker inheritance[a]									
	Crp-1$^+$		Gal-1$^+$		HisD1$^+$		ProA1$^+$		Trp-2$^+$	
	f[b]	n[c]	f	n	f	n	f	n	f	n
Thy	2.1	2	<0.7	2	780	4	93[d]	4	210	4

Table 16(f). Chromosomal mobilisation from KF1017 to KF1072.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1072: aroB1 glyA1 hisD1 manA1 nal-1 str-1

Counter-selection	Frequency of marker inheritance[a]							
	AroB1$^+$		GlyA1$^+$		HisD1$^+$		ManA1$^+$	
	f[b]	n[c]	f	n	f	n	f	n
Thy	1.4	1 590	1	860	1	46	1	

Table 16 cont.

Table 16(g). Chromosomal mobilisation from KF1017 to KF1089.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1089: aroB1 glyA1 hisD1 manA1 nal-1 nal-2 str-1

Counter-selection	Frequency of marker inheritance[a]							
	AroB1$^+$		GlyA1$^+$		HisD1$^+$		ManA1$^+$	
	f[b]	n[c]	f	n	f	n	f	n
Thy	<2.3	1	155	2	275	2	2.1	1

Table 16(h). Chromosomal mobilisation from KF1017 to KF1019.

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1019: his-2 nal-3 str-1 Pec$^-$

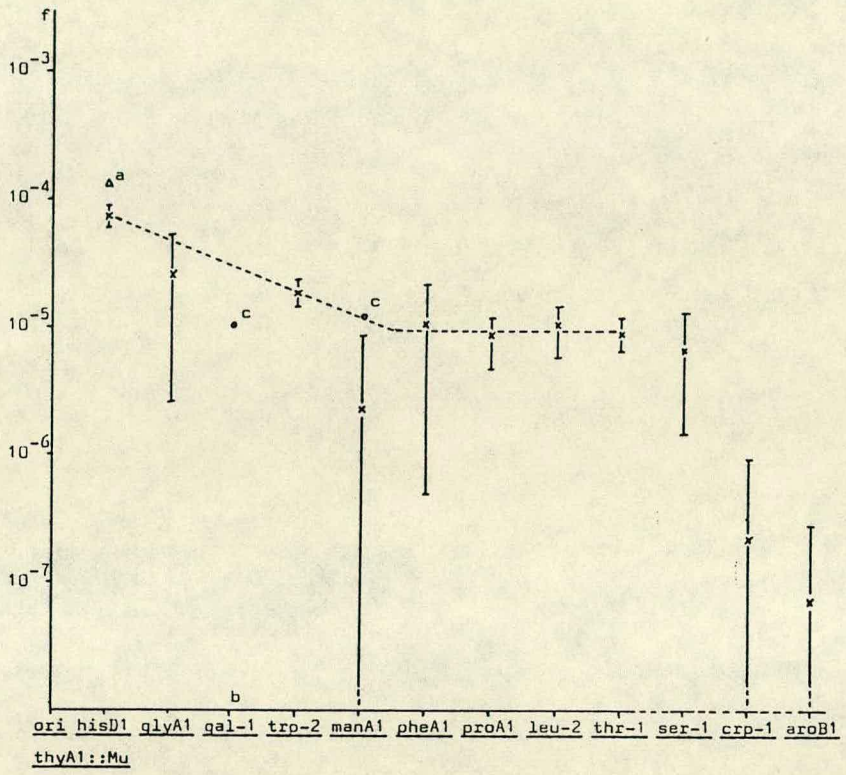
Counter-selection	Frequency of marker inheritance[a]	
	His-2$^+$	
	f[b]	n[c]
Thy	1500	1

Table 16(i). Summary of chromosomal mobilisation from KF1017 to KF1072 and KF1089.

Counter-selection	Frequency of marker inheritance[a]							
	AroB1$^+$		GlyA1$^+$		HisD1$^+$		ManA1$^+$	
	f[b]	n[c]	f	n	f	n	f	n
Thy	0.7	2	300	3	470	3	24	2

a: Membrane mating at 30^{o}C for 18h. b: Frequency of transfer per initial donor x10^7. c: Number of experiments. d: Frequency corrected for spontaneous Pro$^+$ revertants.

Figure 14. Frequency of inheritance of chromosomal markers from KF1017 donors.



f: Frequency of mobilisation of chromosomal markers per initial donor in 18h membrane matings at 30°C.

The order of the chromosomal markers between hisD1 and thr-1 is that calculated from the **coin**heritance frequencies (Figure 13); ser-1, crp-1 and aroB1 are ordered on the basis of their mobilisation frequencies. Frequencies are calculated from 2 - 4 experiments (hisD1, 12experiments). Error bars are the 80% confidence limits.

a: Frequency of mobilisation of His-2$^+$. b: The mobilisation of Gal-1$^+$ was not observed. c: The frequency of mobilisation of Gal-1$^+$ and ManA1$^+$ calculated from the product of the coinheritance frequency of the marker from HisD1$^+$ and the frequency of mobilisation of HisD1$^+$.

not only in carbohydrate catabolism but also in the synthesis of cell wall components. As growth on the mating membrane is slow (Appendix I) the transconjugants, while being genetically non-mutant, may well still be phenotypically mutant, and thus would be unable to grow on the selection medium. An estimate of the expected frequency of inheritance of gal-1 can be calculated from the product of the frequency of inheritance of hisD1 and the coinheritance frequency of gal-1 from hisD1; that is, 1.2×10^{-5} transconjugants /donor (i.e. $8.7 \times 10^{-5} \times 0.14$). This frequency will be an underestimate as some of the gal-1 transconjugants would not have inherited hisD1. Similarly the frequency of inheritance of manA1 can be calculated as 1.4×10^{-5} transconjugants /donor (that is $8.7 \times 10^{-5} \times 0.16$). These two estimated frequencies are very close to the frequencies which would have been predicted from the inheritance frequencies of the flanking markers (Figure 14).

Ser-1, crp-1 and aroB1 were all mobilised but, as noted above, had very low linkages with other markers (<1%). Ser-1 was inherited at a frequency typical of the non-polar mechanism. Crp-1 and aroB1 were inherited at frequencies below that expected even for the non-polar mechanism of transfer; this is probably a result of the linkage of these markers to thyA1::Mu which will reduce their apparent frequency of inheritance through the counter-selection against thymine requiring transconjugants. It is unlikely that there was a loss of recombinants through a delay in the expression of the non-mutant phenotype in these recombinants, as was the case for gal-1 and manA1, as crp-1 and aroB1 were not coinherited at high frequencies from other markers as was the case for gal-1 and manA1. Additionally aroB1 was mobilised efficiently

by F'Lac^{+}Tc (Section 5)d)).

6)d)iv) QUANTITATIVE ANALYSIS OF CHROMOSOMAL MOBILISATION FROM KF1092 DONORS INTO KF1068 RECIPIENTS

To order the markers in the KF1068 and KF1072 strains with respect to each other, thyAl::Mu and then pKF1 was crossed into KF1072 from KF1017, giving the donor strain KF1092 which will have the same chromosome mobilising characteristics as KF1017. Unfortunately plasmid and chromosome mobilisation from KF1092 was poor and many matings with this donor into different recipients failed to yield chromosomal recombinants.

The results of a successful cross between KF1092 and KF1068 are given in Table 17(a,b). The frequency of transfer of the plasmid, at 6.3×10^{-3}, was some ten-fold lower than was generally obtained from KF1017 donors. Trp-2 was inherited from KF1092 at a slightly greater frequency than it was from KF1017 donors. The coinheritance of markers from trp-2 (Table 17(b)) indicates greatest linkage between trp-2 and gal-1, and rather less linkage between trp-2 and both glyAl and manAl, implying that manAl and glyAl do not map between trp-2 and glyAl. Three-point cross analysis places glyAl on the other side of gal-1 to trp-2. Analysis of three-point crosses with manAl, and the lack of linkage between glyAl and manAl, suggests that manAl is located on the other side of trp-2 to gal-1; but with the few numbers of transconjugants available, the positioning of manAl must be treated with caution. The order glyAl - gal-1 - trp-2 - manAl obtained is in agreement with the orders calculated using KF1017 donors above.

Table 17. Chromosomal mobilisation in Ecc SCRI193 from KF1092 to KF1068.

Strains: KF1092: aroB1 glyA1 hisD1 manA1 nal-1 str-1 thyA1::Mu/ R68::Mu

KF1068: gal-1 hisD1 nal-1 proA1 str-1 trp-1

Table 17(a). Mobilisation frequencies of chromosomal markers.

Counter-selection	Frequency of plasmid inheritance[b]	Frequency of chromosomal marker inheritance[a,b]	
		Gal-1$^+$	Trp-2$^+$
Thy	6.3×10^{-3} [c]	$<8.0 \times 10^{-8}$	2.7×10^{-5}

Table 17(b). Cotransfer of markers into KF1068.

Counter-selection	Selected marker	T[d]	% coinheritance of unselected markers[a]						
			AroB1$^+$	Gal-1$^+$	GlyA1$^+$	ManA1$^+$	ProA1$^+$	Trp-2$^+$	
Thy	Trp-2$^+$	106	0	12	3.8	3.8 [e]	0	-	2.8[e]
									3.8

a: From a single mating (membrane at 30°C for 18h). b: Frequency of transfer per initial donor. c: Donors also counter-selected with Nal Str. d: Number of transconjugants tested. e: The % coinheritance frequencies of the indicated (*) unselected markers from the selected marker.

6)e)CONCLUSIONS

The use of plasmid::Mu cointegrate vectors to mobilise the chromosome of *Ecc* has allowed the construction of a linkage map of 15 ordered markers and two approximately mapped markers (Figure 15), but which cannot be circularised from the available data. This map was constructed using donors from which there were two modes of chromosomal transfer. One of these, a non-polar mechanism, required only the presence of an RP4 or R68 plasmid which carried a Mu prophage. The other, a polar mechanism, also required such a cointegrate plasmid in addition to a chromosomal Mu prophage. These two mechanisms were both acting simultaneously in the KF1017 donors to mobilise the chromosome into the multiply auxotrophic recipients. The contribution of each mechanism to the transfer of a marker depended on the location of the marker on the chromosome: markers close to the origin, *ori* (*thyA1::Mu*), for example *hisD1*, were mobilised more frequently than markers which were located further away from the origin, for example *leu-2*, *thr-1* and *str-1*.

The mapping of *Ecc* SCRI193 with plasmid::Mu cointegrates would have been facilitated had this strain been more amenable to mutagenesis with Mu: either by being sensitive to infection by Mu or by being more readily susceptible to mutagenesis by zygotic or temperature induction of plasmid::Mu cointegrates. This would have allowed the study of chromosomal transfer from more than one origin.

The role of the plasmid itself in these cointegrate vectors could not be readily studied as all of the cointegrates available in *Ecc* SCRI193

were of the IncP group and these proved to have some unusual characteristics in this strain (in general the transconjugants were resistant to the donor-specific phage PRR1),in addition to being poorly transferable.

Time did not permit the construction of a linkage map using donors without a chromosomal Mu prophage, however this would clearly be of use in clarifying the mechanism by which the chromosome of Ecc was mobilised in such cases. The mechanism of the non-polar chromosomal mobilisation, which occurred at a higher frequency in Ecc than would be the case in E.coli, is probably the result of the spontaneous transposition of the plasmidic Mu, and its associated R68 vector, into random sites on the host chromosome.

The ampicillin resistant derivative of Mu, MupApl (Leach and Symonds,1979), cointegrated with an IncF plasmid may be of use in the isolation of chromosomal Mu insertions; as selection for ampicillin resistant transconjugants which were also resistant to IncF donor-specific phage would circumvent the necessity of isolating Mu induced chromosomal mutations as such. Mini-Mu (Faelen et al.,1978), a Mu phage with a large internal deletion, which is temperature inducible for transposition but which is not zygotically induced on transfer into Mu free recipients, is available on the IncF plasmid F'Lac$^+$ProA$^+$ (Toussaint et al.,1981) and the IncP plasmid RP4 (Van Gijsegem and Toussaint,1982). These cointegrate plasmids mobilise the chromosome randomly, following induction of the donor, into Mu free recipients. The use of this latter system would avoid the isolation of chromosomal Mu insertions at all, and may help in more fully

understanding the non-polar mode of chromosomal transfer observed here.

SECTION 7

A LINKAGE MAP OF THE Ecc CHROMOSOME AND ITS RELATIONSHIP TO THE LINKAGE MAPS OF OTHER Enterobacteriaceae SPECIES

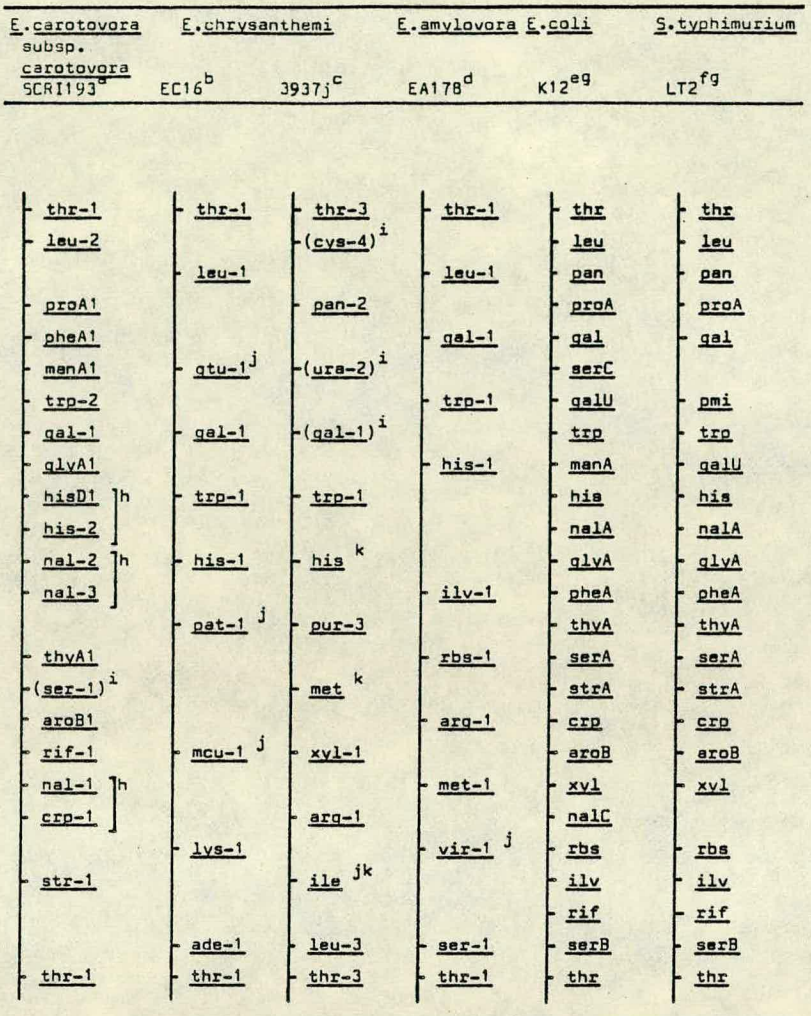
7)a) LINKAGE MAP OF THE Ecc CHROMOSOME

From the chromosomal linkage maps of Ecc SCRI193 constructed by conjugation with the plasmids F'Lac^{+}Tc (Figure 11) and R68::Mu (Figure 15) a single linkage map can be constructed (Figure 16). This circular map consists of seventeen ordered mutations and one approximately located mutation. Most of the mutants used have been characterised biochemically and physiologically such that they can be compared with analogous mutant genes in other species.

a: Origin of polar chromosomal mobilisation. b: Crp-1 is linked to str-1 and rif-1. c: Nal-1 is closely linked to rif-1. d: AroB1 shows no linkage to the markers tested. e: Ser-1 is not strongly linked to other markers but does show weak linkage to hisD1 and pheA1.

Figure 15. Chromosomal linkage map of Ecc SCRI193: mobilisation from KF1017 and KF1092 donors.

Figure 16. Chromosomal linkage maps of Erwinia species, E.coli and S.typhimurium.



a: This work b: Chatterjee and Starr (1977). c: Kotoujansky et al.(1892). d:Pugashetti et al.(1978). e: Bachman and Low (1980). f: Sanderson and Hatrman (1978). g: Only those genes which have been mapped in an Erwinia species, and which do not have different loci dispersed over the chromosomes of either E.coli or S.typhimurium are shown. h: Mutations not ordered with respect to each other. i: Mutations in parenthesis are approximately mapped with respect to adjacent mutations. j: D-galacturonate utilisation (gtu), isoleucine requirement (ilvA=ile), multiple carbohydrate utilisation - Crp^- phenotype (mcu), polygalacturonic acid trans-eliminase (pat), pmi = manA of E.coli, plant virulence (vir). k: Several mutations of this phenotype have been mapped to this location.

7)b) A COMPARISON OF THE LINKAGE MAPS OF Ecc, Erwinia SPECIES, AND Enterobacteriaceae SPECIES

To facilitate comparison, all of the linkage maps have been drawn linearly from the thr locus and in the same orientation (Figure 16).

A comparison of the linkage map of Ecc with those of strains of the closely related E.chrysanthemi, determined by Chatterjee and Starr (1977) and Kotoujansky et al. (1982) (Figure 16), between which there are differences of gene order, shows that there are both conserved and dispersed gene sequences between these two species. The linkage map of E.amylovora (Pugashetti et al., 1978) also shows similarities to the Ecc linkage map. Unfortunately detailed comparisons between these different species is not possible because there are insufficient common markers, partly as a result of the poor characterisation of the mutants used in the other mapping studies.

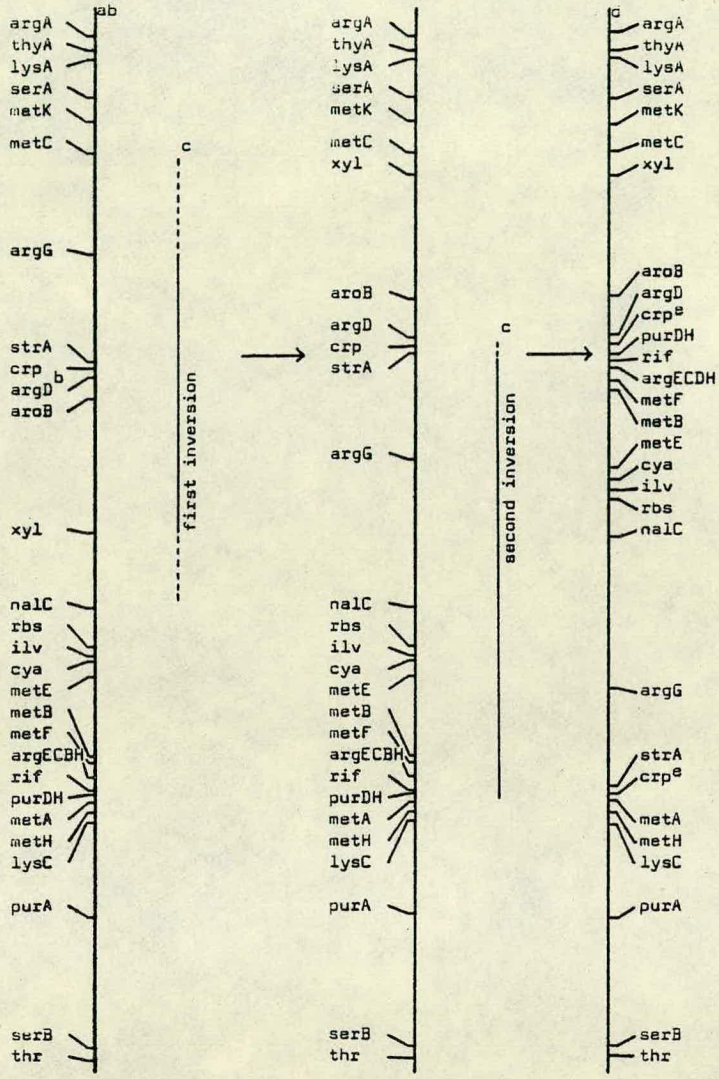
A detailed comparison of the Ecc linkage map with those of other species of the Enterobacteriaceae is similarly limited to those studies which utilised well-characterised mutants. Comparisons with the distantly related Proteus mirabilis (Coetzee, 1979), Proteus morganii (Beck et al., 1982) and Yersinia pseudotuberculosis (McMahon, 1973) are thus not possible, whilst comparison to the closely related Klebsiella pneumoniae and Serratia marcescens are similarly limited. Comparison with the extensively mapped E.coli K12 (Bachman and Low, 1980) and S.typhimurium LT2 (Sanderson and Hartman, 1978), which are closely related to each other but distantly related to Erwinia, with the members of this latter genus is possible.

The order manA1 - trp-2 - gal-1 found in Ecc is also found in S.typhimurium. Between E.coli K12 and S.typhimurium LT2 there is a 10min chromosomal inversion of this region which includes trp and galU. In E.coli, manA maps immediately to the right of this inversion, while in S.typhimurium, pmi (=manA) maps in the centre of the inversion. The galE,K,T operon of both species maps to the left of the inversion. If gal-1 is a mutation of galU (although it could be a mutation of galT - see Section 4)c)iii)) then the gene order of Ecc is closer to that of S.typhimurium than to that of E.coli.

The aroB1 - rif-1 - nal-1/crp-1 - str-1 sequence in Ecc differs from that in E.coli and S.typhimurium where the order, which is reversed with respect to the rest of the chromosome, is strA - crp - aroB - nalC - rif. Inversions of this region, relative to the chromosomes of E.coli and S.typhimurium, may also be present in one isolate of E.chrysanthemi (ile - arg, Kotoujansky et al., 1982) and in E.amylovora (ilv - rbs, Pugashetti et al., 1978) - see Figure 16. These apparent differences in the linkage maps of the Erwinia spp. compared to those of E.coli and S.typhimurium can be reconciled by inverting two overlapping regions of the chromosomes of the latter two species - Figure 17. It should be noted, however that had more of the mutants mapped by other workers in the Erwinia spp. been better characterised, then such a solution may no longer be tenable; alternative solutions which involve the translocation of several of the genes in this region would also give a possible solution, though this would require more mutational steps.

In Ecc, pheA1 mapped near to proA1, which is far from its location to

Figure 17. Chromosomal inversions and the linkage maps of E.coli and Erwinia species.



The above figure shows the extents of the two inversions of the E.coli K12 (or S.typhimurium LT2) chromosome in the region from 60 to 100 minutes which result in a rearranged linkage map compatible with the linkage maps of all the Erwinia species so far mapped (Figure 16). a: Linkage map of the E.coli K12 chromosome in the region from 60 to 100 minutes. b: The linkage map of the S.typhimurium LT2 chromosome is identical to that of E.coli K12 in this region other than an inversion of crp and argD. c: Extent of inversions (solid lines) with the end-points in the regions indicated (dotted lines). d: Rearranged chromosome which has the same gene order as all of the Erwinia species so far mapped. e: The location of crp is most probably adjacent to strA but could alternatively be adjacent to purDH.

the right of thyA in E.coli and S.typhimurium. In Ecc, glyA1 mapped to the left of his, and not as in E.coli and S.typhimurium to the right of his and the left of thyA. Riley and Anilionis (1978) noted that there were insertions or deletions or both in the pheA - glyA region of the chromosomes of E.coli and S.typhimurium, and as Ecc is unable to metabolise sorbitol (srl mapping between thyA and pheA in E.coli and S.typhimurium) it is possible that there are chromosomal rearrangements of this region in Ecc compared to E.coli and S.typhimurium.

As discussed in Section 5, the use of E.coli F-prime plasmids to mobilise the Ecc chromosome allows the determination of the orientation of homologous chromosomal regions between these two species. The F'Lac^{+}Tc plasmid (which codes for c.1min of the E.coli chromosome contiguous with lac) mobilised the Ecc chromosome in the same orientation as it did in E.coli (orientation of transfer in E.coli determined in this work).

The genus Erwinia is closely related to the tribe Klebsielleae which includes the genera Klebsiella and Serratia, and so it would be expected that the chromosomal maps of these species would show greater homology than they would to the more distantly related E.coli and S.typhimurium species. Unfortunately genetic maps of Klebsiella and Serratia strains are few in number, and do not cover all of the chromosome. Thus in Serratia marcescens only two pairs of genes have been mapped - and had the same linkages as in E.coli (Hedges, 1980). The Klebsiella pneumoniae chromosomal mapping study by Matsumoto and Tazaki (1970, 1971) did not employ donors and recipients of a single

isolate and, as few of their mutants were well characterised, interspecific comparison is difficult; these workers suggested that there was extensive homology between K.pneumoniae and both E.coli and S.typhimurium. Other workers have mapped four sections of the K.pneumoniae chromosome, by generalised transduction, which together account for some 10% of the genome. These linkage groups are: rbs - glnA - rha - metB - ppc - argH - ilvA, and tyrA - nadB - guaB (Streicher et al., 1975), and pyrF - trp - gdh (Bender et al., 1976), and gal - hut - bio - chlA (Goldberg and Magasanik, 1975). With the exceptions of the translocation of ilvA from its position in E.coli and S.typhimurium between rbs and glnA (this translocation is apparently of the same type as those selected artificially by Hill and Harnish (1982) in E.coli by recombination between the ribosomal RNA (rrn) genes), the location of gdh at a different site in E.coli, and E.coli being histidine non-utilising, these sequences are identical in all three species. Unfortunately the paucity of markers common to Ecc and K.pneumoniae prevents interspecific comparison, and mutants in other Erwinia spp. have not been sufficiently well characterised to allow comparisons here either. The inversions postulated above which reconcile the maps of Erwinia spp. with E.coli and S.typhimurium in the region strA - nalC - rif, are also consistent with the gene orders available in K.pneumoniae.

As noted above, differences have been observed between the E.coli, S.typhimurium group and both K.pneumoniae and Erwinia spp., with the apparently more extensive chromosomal rearrangements of the Erwinia spp. as compared to K.pneumoniae, suggesting that the Erwinia spp. have diverged more from E.coli and S.typhimurium than has

K.pneumoniae. Such a divergence between Erwinia spp. and K.pneumoniae may partly be a reflection of the different analytical methods used - transduction of small sections of the chromosome for K.pneumoniae, and conjugation of the whole chromosome for the Erwinia spp.. Thus where two species have diverged by inversions and translocations of large sections of the chromosome, the differences observed in the gene orders of the two species will appear more radical for maps constructed by conjugation (of the whole chromosome), than will be the case with maps constructed by transduction (of small chromosomal sections).

SECTION 8

GENERAL DISCUSSION

Having first studied several E.carotovora strains for functions of interest in the general sphere of genetics; plasmid complement, bacteriocin production, resistance to antibiotics; a more detailed study of genetic systems in E.carotovora was undertaken. The primary aim of this was to establish gene transfer systems in E.carotovora, and to initiate the construction of a genetic map of the chromosome.

Several wild strains of the two E.carotovora subspecies were tested for the presence of plasmids, for production of bacteriocins and for resistance to high levels of antibiotics. This survey showed that plasmids of diverse sizes were present in several strains of both Ecc and Eca but were by no means ubiquitous, within the limitations of experimental technique, in either subspecies. Bacteriocin production was only observed from some of the Ecc strains tested; none of the Eca strains tested produced bacteriocins detectable with the indicator strains used. This restriction of bacteriocin production to Ecc has been noted by Pérombelon and Kelman (1980). None of the plasmid-carrying strains tested, carried high-level resistance to any of the 14 antibiotics on which they were tested.

Ecc SCRI193 was used for most of the genetic work as it is a typical member of the subspecies, it is plasmid-free, and preliminary genetic work by M.C.M. Pérombelon had shown that IncFI and IncP plasmids could be conjugated into it. As no genetic work had been done in Eca the choice of strains was rather wider; two plasmid-free strains were

chosen, both of which had been originally isolated from potatoes, the usual plant host of the subspecies, namely SCRI8 and SCRI13.

Mutations were isolated in the E.carotovora strains which conferred antibiotic resistances, auxotrophy, or defects in carbohydrate metabolism. From similar work in E.coli and S.typhimurium, mutants were selected in E.carotovora which were easily classified biochemically. All of the mutants isolated in Ecc had biochemically analogous, if not indeed homologous, mutations in E.coli and S.typhimurium; the Eca mutants were not characterised in detail.

Methods of conjugational mobilisation were sought in E.carotovora, principally in Ecc, as these allow the mapping of widely-spaced chromosomal markers. Preliminary attempts to establish generalised transduction using phage P1 failed, not apparently because the phage was unable to infect suitably mutant cells, but because the phage was unable to maintain itself in a viable form (at least in the case of Ecc SCRI193). Further work on this instability, with cointegrates of the phage and a conjugative plasmid (Iida, 1980) or with recombinant Mu phages which have a P1 host range (Toussaint et al., 1978), would be of interest.

Two basic types of conjugational gene transfer suggested themselves for use in E.carotovora both of which relied on homology between a conjugative plasmid and the chromosome. Recombination between such homologous sequences results in chromosome mobilisation from a fixed origin, thus giving 'time of entry' mapping data on the location of chromosomal markers, in addition to cotransfer information, which is

all that plasmids such as R68.45 can provide. The two basic conjugational gene transfer systems used here yielded different information. By using F-prime plasmids from E.coli it was possible to crudely compare the Ecc chromosome with that sequence of the E.coli chromosome borne on the F-prime; information was thus gained about recombinational homology between chromosomal DNA sequences of the two species (from the frequency and gradient of chromosomal marker inheritance) about the orientation of the sequence on the chromosomes of the two species, and about the order of markers on the Ecc chromosome. Alternatively, by using transposable elements, either transposons or phage Mu, as regions of homology, it is possible to create origins of transfer where required.

Few F-prime plasmids were successfully conjugated into Ecc SCRI193 and it is suggested that as those which were transferred were stably inherited and expressed all of their markers in Ecc, the difficulty of transferring in other F-prime plasmids was a result of their restriction on transfer from E.coli into Ecc. This inability to transfer in many F-prime plasmids severely limited the genetic systems that could be employed in Ecc. Thus the F'Rep[Ts114]Lac$^+$::Tn10 plasmid which was so successfully used for the construction of Hfr's (Kotoujansky et al., 1982) and for transposon mutagenesis (A. Kotoujansky, pers. comm.) in E.chrysanthemi, and the Mu, Tn10 bearing F-prime pKF4, constructed here, could not be used. Plasmid F'Lac$^+$Tc, which was conjugated into Ecc enabled studies to be made of the efficiency of transfer of this F-prime under different mating conditions. Similarly, the transfer of F-prime plasmids into Eca was limited; evidence was obtained that F'Lac$^+$Tc was not maintained in Eca

SCRI13 and that the Tc determinant could transpose onto the Eca chromosome. From the frequency and gradient of chromosomal inheritance by plasmid F'Lac^{+}Tc in Ecc it was concluded that there was recombinational homology between the Ecc chromosome and the 40kb of E.coli chromosomal DNA carried on F'Lac^{+}Tc, and that this region was in the same orientation on the chromosomes of both species. Linkage information on Ecc chromosomal markers was also obtained.

Phage Mu was also used as a region of homology between a conjugative plasmid and the Ecc chromosome. As Ecc SCRI193 is resistant to infection by phage Mu, the phage was transferred into the strain with conjugative, cointegrate plasmids. The transfer of the IncFI cointegrate plasmid pKF4 into Eca SCRI193 was unsuccessful, while the IncP plasmid R68 (or RP4) carrying either Muc$^{+}\Delta$445-7 (a phage unable to invert its G-segment) or Mucts62 (a phage with a temperature sensitive repressor) were both readily conjugated into the strain. All attempts to isolate Mu insertions on the Ecc chromosome in this strain as had been done by Pérombelon and Boucher (1978) failed; however chromosomal mapping studies with the Ecc chromosomal Mu insertion of Pérombelon and Boucher (1978), mobilising from two different Ecc SCRI193 mutant strains, allowed the construction of a linkage map of 17 mapped mutations.

As the two linkage maps of Ecc SCRI193 constructed with plasmids F'Lac^{+}Tc and R68::Muc$^{+}\Delta$445-7 were in complete agreement with each other, it was possible to construct a single, circular linkage map of the strain (Figure 16). The homology and divergence of this map to the maps of other members of the Erwinia and the Enterobacteriaceae has

been discussed, however the following are of note. The Ecc gene order was found to be more similar to the S.typhimurium gene order than to the E.coli gene order, with respect to the large 10min S.typhimurium / E.coli inversion. Other inversion(s) were also observed in Ecc (relative to E.coli and S.typhimurium) in the rightmost third of the linkage map (which includes strA and rif), and it is observed that two overlapping inversions of the E.coli / S.typhimurium linkage maps, in this region, results in a gene order compatible with all of the Erwinia spp. linkage maps available. The locations of pheA and glyA in Ecc were different to those found in E.coli and S.typhimurium.

The present study should assist future genetic studies of E.carotovora in several ways. The chromosomal linkage map of Ecc, comprising 17 mapped mutations, will allow the accurate location of other mutations. In this regard however, it should be said that the methods of chromosome mobilisation used here were not very efficient and further improvements here would be of great use. Of the conjugative plasmids used in this study, F appeared to be the more suitable for chromosomal mobilisation. The difficulty of conjugating some F-primes into Ecc, however, proved to be a problem; this might be circumvented by the use of transposable elements rather than chromosomal DNA as regions of homology. The study of the behaviour of phage Mu transposition in Ecc suggests that the incidence of new insertional mutations, in this strain at least, is rare. The development of a transposon based, insertional mutagenesis system, may therefore prove less difficult. Such transposon mutagenesis would require a suitable vector - a possible approach might be to use a plasmid unstable in Ecc, either by

virtue of its incompatibility group or of a non-permissive mutation, or alternatively loss of the plasmid could be selected or enhanced by curing or by infecting with an appropriate male-specific phage. Such transposon-induced mutants would be ideal both for chromosomal mobilisation and the mapping of poorly selectable mutations, as for example mutations in pathogenesis. Such a transposon-based genetic analysis would be easily developed in Eca SCRI13 as IncFI plasmids (specifically F'Lac^{+}Tc) have been shown to be unstable in this strain. It may also be possible to isolate an Hfr of Eca SCRI13 by selecting for the integration of an IncFI plasmid into the chromosome, as has been done in E.chrysanthemi (Chatterjee and Starr, 1977) and in S.typhimurium (using R100; Chumley et al., 1979).

APPENDIX I

GROWTH RATES OF Ecc SCRI193 AND Eca SCRI13 STRAINS

The growth rates of Ecc SCRI193 and Eca SCRI13 strains were determined under various growth conditions.

In shaken liquid culture at 28°C Ecc SCRI193 had a generation time of 50min in LB and of 65min in glucose MM, while Eca SCRI13 had a generation time of 60min in LB and 70min in glucose MM -determined by viable count. The generation times as determined from the optical density (measured through a 1cm light path at a wavelength of 550nm using a Beckman DB Spectrophotometer) of the cultures were found to be slightly longer in all cases (5min longer for Ecc SCRI193 and 10min longer for Eca SCRI13). It was noted that the optical density of a log-phase, LB culture, of either strain, was related to the viable count as one optical density unit being equivalent to 1.7×10^8 bacteria /ml.

The generation times of several mutant Ecc SCRI193 strains were determined for growth on membranes incubated on LB agar at 30°C:

Strain	Number of Experiments	Mean generation time (hours)
KF1017	15	2.6
KF1033	3	1.4
KF1037	3	1.5
KF1060	4	3.8
KF1061	4	4.7
KF1068	2	4.6
KF1069	2	6.2
KF1072	1	4.9
KF1089	2	6.9

APPENDIX II

SPONTANEOUS MUTATIONS TO ANTIBIOTIC RESISTANCES IN E.carotovora STRAINS

The frequencies of spontaneous mutation to resistance to different antibiotics was determined for several strains. Log-phase cultures of the strains were tested for viable-count (on glucose MM) and for mutation frequency (on glucose MM plus antibiotic).

Strain	Frequency of spontaneous mutation to antibiotic resistance $\times 10^8$						
	AMP[a] 20	CML 20	KAN 20	KAN 40	NEO 20	KAN 20 NEO 20	TET 20
Ecc							
SCRI193	<2	<2	690(290[b])	26	240[b]	46[b]	<2
SCRI238	<2	<2	1300	13	-	-	<2
Eca							
SCRI8	<3	40[c]	-	-	-	-	<3
SCRI13	<3	<3	1500	25	-	-	<3
SCRI31	<10	<3	440	-	-	-	<3

a:Concentration in μg/ml. b: Tested on LB medium. c: The chloramphenicol resistant colonies required 6d incubation to form 2mm wide colonies on the initial selection medium; the chloramphenicol resistance was lost following further growth on non-selective medium.

APPENDIX III

PHAGE P1 IN E.carotovora STRAINS

The host-range of the generalised transducing phage P1 (review: Sternberg and Austin, 1981) has been extended from E.coli using phage which carry resistances to chloramphenicol (Rosner, 1972) or kanamycin (Goldberg et al., 1974) by selecting for (mutant) bacteria which have acquired the appropriate antibiotic resistance after phage infection.

Phage P1clr100 Km was found to be unsuitable for the isolation of P1 sensitive mutants in Eca strains SCRI13 and SCRI31 and in Ecc strains SCRI193 and SCRI238 as all of these strains showed a high frequency of spontaneous mutation to kanamycin resistance (10^{-5} - 10^{-7} kanamycin resistant mutants /cell; Appendix II). Bacteria treated with P1clr100 Km did not show an increased frequency of kanamycin resistance over and above the spontaneous mutation rate. Ecc SCRI193 strain KF1033 (which mutates spontaneously to chloramphenicol resistance at $<2\times10^{-8}$/cell (Appendix II)) was treated with P1clr100 Cm as described in Materials and Methods and chloramphenicol resistant colonies were recovered at 10^{-9}/cell. The ten resistant colonies isolated were stable for the antibiotic resistance and on further testing were all found to be unable to form plaques on the sensitive E.coli ED8874 although seven had become temperature-sensitive for growth and might therefore be expected to be lysogenic for the phage. Curing the temperature sensitivity from these seven isolates by culturing at 37°C was not observed ($<10^{-6}$cured cells /cell), nor was the lysis of heat shocked cells in liquid culture observed. The isolation of chloramphenicol resistant transductants which do not

apparently carry phage functions might be expected as this resistance marker is carried on the transposon Tn9 on this phage. The maintenance of apparently defective phage in Ecc, or possibly the repression of the phage lytic pathway, was also observed by Goldberg et al. (1974) who isolated P1c1r100 Km lysogens of the Ecc strain ATCC495 but could not obtain phage release from them.

APPENDIX IV

APPENDIX IV(a). % COINHERITANCE FREQUENCIES BETWEEN Ecc SCRI193 CHROMOSOMAL MARKERS MOBILISED FROM KF1017 DONORS TO MULTIPLY AUXOTROPHIC RECIPIENTS, AND COUNTER-SELECTED FOR THYMINE PROTOTROPHY AND EITHER STREPTOMYCIN OR NALIDIXIC ACID RESISTANCE

COINHERITANCE OF MARKERS INTO KF1060

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1060: hisD1 leu-2 nal-1 str-1 thr-1

Counter-selection	Selected marker	n[a]	T[b]	% coinheritance of unselected markers				
				Leu-2^{+}	Nal-1^{S}	Rif-1^{R}	Str-1^{S}	Thr-1^{+}
Thy Str	Leu-2^{+}	1	244	-	<0.4	<0.4	-	16
	Thr-1^{+}	1	273	14	<0.4	<0.4	-	-

COINHERITANCE OF MARKERS INTO KF1061

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1061: hisD1 nal-1 pheA1 ser-1 str-1

Counter-selection	Selected marker	n[a]	T[b]	% coinheritance of unselected markers[c]				
				HisD1^{+}	PheA1^{+}	Rif-1^{R}	Ser-1^{+}	Str-1^{S}
Thy Nal	HisD1^{+}	1	125	-	<0.8	<0.8	<0.8	<0.8
	PheA1^{+}	1	22	(<4.5)	(<4.5)	(<4.5)	(<4.5)	(<4.5)
	Ser-1^{+}	1	20	(<5.0)	(<5.0)	(<5.0)	-	(<5.0)

APPENDIX IV(a) cont.

COINHERITANCE OF MARKERS INTO KF1068

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1068: gal-1 hisD1 nal-1 proA1 str-1 trp-2

Counter-selection	Selected marker	n[a]	T[b]	% coinheritance of unselected markers[c]					
				Gal-1$^+$	HisD1$^+$	ProA1$^+$	Rif-1^R	Str-1^S	Trp-2$^+$
Thy Str	HisD1$^+$	1	149	14	-	<0.7	<0.7	-	14
				——————————————————————————					10[d]
	ProA1$^+$	1	119	<0.8	<0.8	-	<0.8	-	<0.8

COINHERITANCE OF MARKERS INTO KF1064

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1068: aroB1 glyA1 hisD1 nal-1 str-1

Counter-selection	Selected marker	n[a]	T[b]	% coinheritance of unselected markers[c]				
				AroB1$^+$	GlyA1$^+$	HisD1$^+$	Nal-1^S	Str-1^S
Thy Str	GlyA1$^+$	1	100	<1.0	-	52	<1.0	-
	HisD1$^+$	1	100	<1.0	37	-	<1.0	-

APPENDIX IV(a) cont.

COINHERITANCE OF MARKERS INTO KF1072

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1072: aroB1 glyA1 hisD1 manA1 nal-1 str-1

Counter-selection	Selected marker	n[a]	T[b]	% coinheritance of unselected markers[c]					
				AroB1$^+$	GlyA1$^+$	HisD1$^+$	ManA1$^+$	Rif-1^R	Str-1^S
Thy Nal	GlyA1$^+$	1	22	(<4.5)	-	(54)	(41)	(<4.5)	(<4.5)
						*———	———*		(32)[d]
	HisD1$^+$	1	43	<2.3	20	-	7	<2.3	<2.3
					*———————		———*		5

a: Number of experiments. b: Number of transconjugants tested. c: Frequencies in parenthesis were calculated from <30 transconjugants. d: The %-coinheritance frequencies of the indicated (*) unselected markers from the selected marker.

APPENDIX IV(b): INHERITANCE FREQUENCIES OF Ecc SCRI193 CHROMOSOMAL MARKERS FROM KF1017 DONORS IN MULTIPLY AUXOTROPHIC RECIPIENTS, AND COUNTER-SELECTED FOR THYMINE PROTOTROPHY AND EITHER STREPTOMYCIN OR NALIDIXIC ACID RESISTANCE.

CHROMOSOMAL MOBILISATION FROM KF1017 TO KF1060

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1060: hisD1 leu-2 nal-1 str-1 thr-1

Counter-selection	Frequency of marker inheritance[a]					
	$HisD1^+$		$Leu-2^+$		$Thr-1^+$	
	f[b]	n[c]	f	n	f	n
Thy Str	1000	1	220	2	320	2
Thy Nal	1000	2	380	2	140	2

CHROMOSOMAL MOBILISATION FROM KF1017 TO KF1061

Strains: KF1017: deo-1 rif-1 thyA1::Mu / R68::Mu

KF1061: hisD1 nal-1 pheA1 ser-1 str-1

Counter-selection	Frequency of marker inheritance[a]					
	$HisD1^+$		$PheA1^+$		$Ser-1^+$	
	f[b]	n[c]	f	n	f	n
Thy Nal	440	2	17	2	20	2

a: Membrane mating at $30^{\circ}C$ for 18h. b: Frequency of inheritance per initial donor $x10^7$. c: Number of experiments.

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