Citrobacter freundii Mutants Deficient in Host Specificity Functions and Their Recipient Ability for Foreign Deoxyribonucleic Acid

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SUMMARY

The isolation and properties of mutants of Citrobacter freundii deficient in host specificity functions is described. Two mutant types have been isolated, one deficient in restriction (hsr) and another one deficient in both restriction and modification (hsr). These non-restricting mutants are good recipients for plasmid DNA in conjugation experiments with Escherichia coli donor strains. Transfer of chromosomal markers was observed in crosses between E. coli Hfr strains and non-restricting mutants of C. freundii, and in crosses between Salmonella typhimurium Hfr or S. abony Hfr strains and non-restricting C. freundii recipients. Recombinants from these crosses are unstable, partially diploid strains.

INTRODUCTION

One of the barriers that can operate in gene transfer between different species is host-specific restriction. Restriction results in a breakdown of invading foreign donor DNA in the recipient cell and contributes to the considerable reduction in the number of recombinants observed in crosses between bacteria of different strains and species (Arber, 1965; Arber & Linn, 1969). Host specificity has been studied in strains of Escherichia coli (reviewed by Arber & Linn, 1969; Glover, 1970; Hubacek & Glover, 1970), Salmonella typhimurium (Colson & Colson, 1967; Okada, Watanabe & Miyake, 1968; Colson, Colson & van Pel, 1970), Pseudomonas aeruginosa (Rolfe & Holloway, 1969) and Shigella flexneri (Falkow & Formal, 1969; Kętyi & Ørskov, 1970). In this paper the isolation and properties of mutants of Citrobacter freundii deficient in host specificity functions is described as part of a study on the taxonomic relationships between Citrobacter and the related genera Escherichia and Salmonella.

METHODS

Strains. The strains used are listed in Table 1. All Citrobacter freundii strains were preserved on slant agar containing (per l. distilled water): triple sugar iron (Difco), 65 g.; Casamino acids (Difco), 10 g.; and Bacto agar (Difco), 8 g. Storage on slants of nutrient agar (Oxoid) leads to a loss of viability in several strains. Citrobacter freundii strains were isolated from effluent by plating dilutions of sewage on plates containing 6.5 % (w/v) triple
sulphide in stab cultures on TSI slant agar (Davis sugar iron agar (TSI) (Difco). Although most of the C. freundii strains produced hydrogen sulphide in stab cultures on TSI slant agar, (Davis & Ewing, 1966), they failed to produce black colonies on TSIagar plates.

However, when the inoculated TSI agar plates were overlaid with 5 ml. melted soft agar, containing 0.8% (w/v) Ionagar no. 2 (Oxoid), C. freundii colonies blackened lightly after 18 h. at 37°. Black-grey colonies were isolated and their biochemical properties determined.

Table 1. Strains used

The nomenclature employed here conforms to the recommendations of Demerec, Adelberg, Clark & Hartman (1966). For host specificity functions the symbols proposed by Arber & Linn (1969) were used.

<table>
<thead>
<tr>
<th>Collection no.</th>
<th>Biotype</th>
<th>Properties</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>G 41</td>
<td>Escherichia coli row</td>
<td>Indicaor for colicin production</td>
<td>P. Fredericq</td>
</tr>
<tr>
<td>G 290</td>
<td>K 12</td>
<td>Hfr KL 96 thi</td>
<td>B. Low via P. G. De Haan</td>
</tr>
<tr>
<td>G 300</td>
<td>K 12</td>
<td>cys B trp C his str (F trp cys B colB colV)</td>
<td>P. Fredericq, strain 5 TD 4</td>
</tr>
<tr>
<td>G 302</td>
<td>Salmonella abony</td>
<td>Hfr H2. met aro str</td>
<td>H. Mäkelä, sw 1403</td>
</tr>
<tr>
<td>G 307</td>
<td>Salmonella typhimurium</td>
<td>Hfr Bz. pro 26</td>
<td>K. Sanderson</td>
</tr>
<tr>
<td>G 309</td>
<td>LT 2</td>
<td>Hfr SA 535-2 ser A 13</td>
<td></td>
</tr>
<tr>
<td>G 71</td>
<td>Wild-type</td>
<td></td>
<td>Effluent</td>
</tr>
<tr>
<td>G 310</td>
<td>nal-1</td>
<td></td>
<td>G 71</td>
</tr>
<tr>
<td>G 311</td>
<td>arg-1 nal-1</td>
<td></td>
<td>G 310</td>
</tr>
<tr>
<td>G 315</td>
<td>arg-1 trp-1 nal-1</td>
<td></td>
<td>G 311</td>
</tr>
<tr>
<td>G 316</td>
<td>arg-1 trp-1 nal-1 str-1</td>
<td></td>
<td>G 315</td>
</tr>
<tr>
<td>G 326</td>
<td>arg-1 trp-1 nal-1 hss (F trp cys B colB colV)</td>
<td>From mating G 300 x G 315</td>
<td></td>
</tr>
<tr>
<td>G 327</td>
<td>Citrobacter freundii</td>
<td>arg-1 trp-1 nal-1 (F trp cys B colB colV)</td>
<td></td>
</tr>
<tr>
<td>G 328</td>
<td>arg-1 trp-1 nal-1 hsr (F trp cys B colB colV)</td>
<td>Cured from G 326</td>
<td></td>
</tr>
<tr>
<td>G 329</td>
<td></td>
<td></td>
<td>Cured from G 327</td>
</tr>
<tr>
<td>G 330</td>
<td>arg-1 trp-1 nal-1</td>
<td></td>
<td>Cured from G 328</td>
</tr>
<tr>
<td>G 331</td>
<td>arg-1 trp-1 nal-1 hsr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G 335</td>
<td>arg-1 trp-1 trp-1 nal-1 str-3 hss</td>
<td></td>
<td>G 332</td>
</tr>
<tr>
<td>G 337</td>
<td>arg-1 trp-1 his-1 nal-1 str-3 hss</td>
<td></td>
<td>G 332</td>
</tr>
<tr>
<td>G 342</td>
<td>arg-1 trp-1 nal-1 str-5 hss</td>
<td></td>
<td>G 329</td>
</tr>
</tbody>
</table>

Citrobacter freundii strains have to meet the properties described by Davis & Ewing (1966). Strain G 311 did not grow on minimal medium supplemented with ornithine or citrulline from which we infer that the arg-1 locus is argE or argF (Gorini, Gunderson & Burger, 1961; Maas, Maas, Wiame & Glansdorff, 1964; Vogel, Bacon & Baich, 1963).

Media. The following media were used. Nutrient broth: Lab Lemco broth (Oxoid), 8.0 g.; NaCl, 5.0 g.; distilled water, 1 l. Nutrient agar: blood agar base no. 2 (Oxoid, 4% (w/v). Soft nutrient agar: brain heart infusion (Oxoid), 37.0 g.; Ionagar no. 2 (Oxoid), 8.0 g.; distilled water, 1 l. A synthetic minimal medium for Citrobacter freundii consisted of the minimal medium described by Hadjipetrou, Gerrits, Teulings & Stouthamer (1964) supplemented (per ml. medium) with methionine, 20-0 μg.; lysine, 20-0 μg.; glutamic acid, 200-0 μg. and thiamine, 3.0 μg.

Curing experiments. Treatment with acridine orange at pH 7-6, by the method of Hirota (1960) and Stouthamer, de Haan & Bulten (1963), was used for curing.
Host specificity mutants of Citrobacter

Isolation of mutants. Mutants were isolated after treatment with N-methyl-N'-nitroso-N-nitroguanidine (Koch–Light Laboratories Ltd, Colnbrook, Buckinghamshire) by the method of Adelberg, Mandel & Chen (1965).

Conjugation experiments. Conjugation was carried out by the methods of Matney & Achenbach (1962) and Ozeki, Stocker & Smith (1962) unless otherwise stated. When transfer of F trp cys colB colV factor was studied selection was made for tryptophan independence and for resistance to streptomycin (200 μg./ml.) or naladixic acid (150 μg./ml.) (Winthrop, Haarlem, The Netherlands). For transfer of chromosomal markers selection was made for resistance to streptomycin (200 μg./ml.) or naladixic acid (150 μg./ml.). Recombination frequencies are expressed as recombinants/donor/ml. mixture.

Test for colicin production. Colicin production was tested by the method described by de Graaf, Tieze, Wendelaar Bonga & Stouthamer (1968).

RESULTS

Growth requirements of Citrobacter freundii

Most Citrobacter freundii strains did not grow on a synthetic medium with glucose as sole carbon source. After supplementation with methionine, lysine, glutamic acid and thiamine, 221 of 257 different strains of C. freundii grew within 48 h. There were some strains that needed fewer supplements. However, strain G 71 used here grew on a synthetic medium supplemented with methionine, glutamic acid and thiamine within 48 h.

Isolation of Citrobacter freundii strains containing F trp cys colB colV factors

Citrobacter freundii accepts F' factor F trp cys colB colV from Escherichia coli K 12 strain G 300 at a frequency of less than 3 × 10⁻⁸. In conjugation experiments with G 315, trp+ strains were isolated which were sensitive to male specific phages μ and MS2 and produced colicin, indicating the presence of the F trp cys colB colV factor. The F trp cys colB colV factor is stable in C. freundii strains, and spontaneous loss of tryptophan independence, colicin production and sensitivity to μ and MS2 was never observed. After acridine orange treatment of C. freundii F trp cys colB colV strains colonies were isolated which were insensitive to μ and MS2, tryptophan-requiring and did not produce colicin, indicating a loss of the F trp cys colB colV factor. This means that the F' factor is present as a plasmid and is not integrated into the chromosome (Hirota, 1960; Stouthamer et al. 1963).

Donor ability of Citrobacter freundii F trp cys colB colV strains

Citrobacter freundii F trp cys colB colV strains are able to transfer the F trp cys colB colV factor to strain G 316 (a streptomycin-resistant derivative of G 315) with a frequency varying from 10⁻⁸ to 10⁻⁸. These C. freundii F trp cys colB colV strains are further able to act as donors for chromosomal markers in homologous C. freundii matings (unpublished results). From these results we conclude that transfer frequencies in matings of C. freundii F trp cys colB colV × C. freundii F⁻ are considerably higher than in matings of Escherichia coli G 300 × G 315. This suggests that E. coli DNA encountered restriction in the C. freundii recipients.

Restriction and modification in Citrobacter freundii strains

Restriction and modification of foreign DNA can be measured by assaying the plating frequencies of some bacteriophages (Colson, Glover, Symonds & Stacey, 1965) or by assaying transfer frequencies in bacterial matings (Arber & Morse, 1965; de Haan, Stouthamer, Felix & Mol, 1963; Glover, Schell, Symonds & Stacey, 1963; Hoekstra, 1966).
To investigate the occurrence of restriction in *Escherichia coli F’ × Citrobacter freundii F trp cys colB colV* recombinants derived from the mating G 300 × G 315 were studied. For this purpose the F trp cys colB colV factor was first eliminated from the three *C. freundii* F trp cys coZB COW strains by treatment with acridine orange. Subsequently the cured derivatives were used as recipients in crosses with *E. coli F trp cys colB colV*. The transfer frequencies in these crosses are shown in Table 2. Two of them, G 329 and G 331, accepted F trp cys colB colV with a much higher frequency than wild-type *C. freundii*, from which we infer that they are non-restricting.

<table>
<thead>
<tr>
<th>Donor Escherichia coli G 300</th>
<th>Transfer frequencies*</th>
<th>Recipient Citrobacter freundii strain</th>
<th>Recipient Citrobacter freundii G 316 (restricting)</th>
<th>Host specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>G 329</td>
<td>1.8 × 10⁻⁴</td>
<td>G 326</td>
<td>1.5 × 10⁻⁵</td>
<td>R⁻ m⁻ hss</td>
</tr>
<tr>
<td>G 330</td>
<td>&lt;3 × 10⁻⁸</td>
<td>G 327</td>
<td>5.1 × 10⁻⁸</td>
<td>R⁺ m⁺ Wild-type</td>
</tr>
<tr>
<td>G 331</td>
<td>1.8 × 10⁻⁴</td>
<td>G 328</td>
<td>1.4 × 10⁻²</td>
<td>R⁺ m⁺ hsr</td>
</tr>
<tr>
<td>G 315</td>
<td>&lt;3 × 10⁻⁸</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Selection was made for resistance to streptomycin.
† Selection was made for resistance to naladixic acid.
‡ r = restriction. m = modification.
§ The nomenclature of Arber & Linn (1969) is used for genotypes of host specificity mutants.

To determine the modification properties of G 326, G 327 and G 328 transfer frequencies were compared in mating experiments with two recipients, G 316 (restricting) and G 342 (a streptomycin-resistant derivative of the non-restricting G 329) (Table 2). With G 327 and G 328 the transfer frequencies with G 316 and G 342 recipients were about the same, from which we infer that in these strains modification is present. However, with G 326 the frequency of transfer of F trp cys colB colV to G 342 was much higher than that to G 316. Thus in G 326 modification is absent. Two mutant types have therefore been isolated: G 326 non-restricting and non-modifying, with the presumptive genotype hss (Arber & Linn, 1969; Glover, 1970), and G 328 only deficient in restricting functions, with the presumptive genotype hsr (Arber & Linn, 1969; Glover, 1970). Strain G 327 accepted F trp cys colB colV with the same low frequency as wild-type *C. freundii*, which shows that F trp cys colB colV factor can enter restricting *C. freundii* strains. These restricting *C. freundii F trp cys colB colV* strains may have arisen by epidemic spread of the F trp cys colB colV factor from non-restricting *C. freundii F trp cys colB colV* strains of the presumptive genotype hsr, as is shown by the high transfer frequency of the mating G 328 × G 316.

**Citrobacter freundii strains as recipients for heterologous chromosomal markers**

In mating experiments with *Escherichia coli K 12, Salmonella typhimurium* and *S. abony Hfr* strains as donors and restricting *Citrobacter freundii* strains as recipients, recombinants are rarely formed. Experimental conditions which improve the efficiency of mating pair formation (Matney & Achenbach, 1962) or the acceptor ability for foreign DNA (Uetake, Toyama & Hagiwara, 1964; Eisenstark, 1965; Holloway, 1965; Schell & Glover, 1966) did not have any effect.

However, non-restricting mutants of *Citrobacter freundii* are better recipients for foreign DNA. In matings between *Escherichia coli G 290* (which transfers his as an early marker)
and \textit{C. freundii} G 337, \textit{his}^+ recombinants arose with a frequency varying from $10^{-2}$ to $10^{-5}$. These recombinants were unstable and segregate \textit{his}^- clones, indicating that they may be unstable partial diploids. Transfer of the \textit{trp} locus in these matings with \textit{E. coli} Hfr donors was never observed. In conjugation experiments with Salmonella Hfr donors and non-restricting \textit{C. freundii} strains, transfer of chromosomal markers was observed in some cases, e.g. in mating experiments between \textit{Salmonella typhimurium} G 307 and \textit{C. freundii} G 337 \textit{his}^+ recombinants arose with frequencies varying from $10^{-6}$ to $10^{-8}$. The same frequency was found for the \textit{tyr} marker in a mating \textit{S. typhimurium} G 309 $\times$ \textit{C. freundii} G 335. Transfer of longer chromosomal regions was also observed in these matings. In a mating \textit{S. typhimurium} G 309 $\times$ \textit{C. freundii} G 335 \textit{arg}^+, \textit{trp}^+, inositol-fermenting (\textit{inf}^+) recombinants were isolated which were unstable and segregated \textit{inf}^-. In a mating \textit{S. typhimurium} G 302 (which transfers the chromosome in a clockwise direction with \textit{his} as an early marker) $\times$ \textit{C. freundii} G 337 \textit{arg}^+ \textit{his}^+ \textit{trp}^+ \textit{inf}^+ recombinants were isolated which were unstable for the \textit{arg}, \textit{his} and \textit{trp} regions and segregated clones which lacked these three markers simultaneously. The \textit{arg}^- mutation in G 337 is either in the \textit{argE} or \textit{argF} locus (see Methods). From the transfer of the \textit{arg}^- marker in this mating it is evident that about half the Salmonella chromosome can be transferred (Sanderson, 1970).

**DISCUSSION**

Colson & Colson (1967) described \textit{fer} mutants of \textit{Salmonella typhimurium} which were non-restricting. Okada, Watanabe & Miyake (1968) described non-restricting mutants of \textit{S. typhimurium} with a high recipient ability for \textit{F'} factors, \textit{R} factors and for chromosomal markers of \textit{Escherichia coli} donor strains. The isolation of mutants of \textit{S. typhimurium} LT 2 with a high recipient ability for \textit{E. coli} K 12 genetic material was performed by selecting for \textit{R}^+ sexductants in matings between \textit{E. coli} K 12 \textit{R}^+ $\times$ \textit{S. typhimurium} \textit{R}^- and by eliminating the \textit{R} factor with acridine orange (Okada & Watanabe, 1968). For the isolation of mutants of \textit{Citrobacter freundii} defective in host specificity functions the same method was used. The non-restricting mutants of \textit{C. freundii} also had a better recipient ability for \textit{F'} factors in conjugation experiments with \textit{E. coli} donor strains. However, unlike the non-restricting mutants of \textit{S. typhimurium} stable integration of chromosomal markers from \textit{E. coli} Hfr strains has never been observed with the non-restricting \textit{C. freundii} mutants. Reduced integration or absence of integration of chromosomal markers in matings between heterologous strains has generally been ascribed to differences in nucleotide sequence between donor and acceptor chromosome (Baron, Gemski, Johnson & Wohhlieter, 1968; Verhoef, 1968; Falkow & Formal, 1969). The influence of such differences has been studied extensively by Verhoef (1968) in matings between \textit{E. coli} K 12 Hfr and \textit{E. coli} C or restrictionless strains of \textit{E. coli} B. It is known that there exist only minor differences between these strains with respect to the location of the genes on the chromosome map and, furthermore, the extent of duplex formation between single-stranded DNA of \textit{E. coli} B with that of \textit{E. coli} K 12 is 98\% (McCarthy & Bolton, 1963). In homologous crosses of \textit{E. coli} K 12 Hfr $\times$ \textit{E. coli} K 12 \textit{F}^- the incorporation frequency of a donor fragment is 0.5 (Jacob & Wollman, 1961; De Haan & Gross, 1962; Verhoef, 1968). However, in heterologous crosses between \textit{E. coli} K 12 Hfr and restrictionless strains of \textit{E. coli} B \textit{F}^- the incorporation frequency is only 0.019, whereas in \textit{E. coli} K 12 Hfr $\times$ \textit{E. coli} C \textit{F}^- a value of 0.11 is found (Verhoef, 1968).

In crosses between \textit{Escherichia coli} K 12 Hfr and hybrids of \textit{E. coli} K 12 and \textit{E. coli} C the incorporation frequency becomes higher when the amount of \textit{E. coli} K 12 genetic material in the hybrid is higher. Another possibility which explains the reduced integration of donor
chromosome segments in heterologous crosses is to postulate differences in the specificity of recombination enzymes (Baron et al. 1968). However, this possibility has been disputed by Verhoef (1968). Certain observations suggest that there exists some homology between the chromosomes of Citrobacter freundii and those of E. coli and Salmonella typhimurium. For instance, the order of genes in a part of the linkage map of C. freundii is very similar to that in E. coli and S. typhimurium (unpublished results). From the absence of stable integration of heterologous donor material in restrictionless C. freundii recipients we must conclude, however, that the divergence in nucleotide sequence between E. coli or S. typhimurium and C. freundii is so extensive that generally integration is impossible. Therefore recombinants in these heterologous crosses are all unstable partial diploids and resemble the E. coli–S. typhosa and E. coli–Proteus mirabilis hybrids which have been described by Baron et al. (1968) and Gemski, Wohlhieter & Baron (1967).

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REFERENCES


Host-specificity mutants of Citrobacter


