SHORT COMMUNICATION

Relationships Among Raffinose Plasmids Determined by the Immunochemical Cross-reaction of their α -Galactosidases

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Plasmid-encoded α -galactosidase served as a marker enzyme for the recognition and comparison of raffinose (Raf) plasmids present in strains of *Escherichia coli*. Immunochemical relationships were established among Raf plasmids of 39 independent isolates from man and domestic animals (from three continents) by using antiserum against α -galactosidase. Immunodiffusion revealed three serological subclasses of α -galactosidase, which are correlated with the biological and geographical origin of the host strains. It is concluded that the *raf* determinants of all Raf plasmids tested have evolved from a common ancestor.

INTRODUCTION

Plasmids coding for utilization of raffinose (Raf) have been found in enterobacteria isolated from man (Magalhães & Vêras, 1977; Buissière *et al.*, 1977) and domestic animals (Smith & Parsell, 1975) under enteropathogenic conditions. Thus, porcine neonatal diarrhoea may be caused by strains of *Escherichia coli* which produce enterotoxin, encoded by Ent plasmids, and the surface antigen K88, which facilitates colonization in the anterior small intestine (Smith & Linggood, 1971; Jones & Rutter, 1972; So *et al.*, 1974; Sack, 1975; Smith, 1977). Genetic determinants of the K88 antigen have been found on plasmids together with *raf* genes (Smith & Parsell, 1975; Shipley *et al.*, 1978; Mooi *et al.*, 1979), thus explaining the Raf⁺ character of their hosts. A number of Raf plasmids bear determinants for hydrogen sulphide production (Hys) instead of K88 (Ørskov & Ørskov, 1974; Burkardt *et al.*, 1978).

Raf plasmids enable strains of *E. coli* to utilize the trisaccharide raffinose [*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranosyl β -D-fructofuranoside] as sole carbon source by the action of three inducible plasmid-coded functions, namely, a transport system (Raf permease), an α -galactosidase and an invertase. Their structural genes form an operon negatively controlled by a repressor gene (Schmitt *et al.*, 1977). In contrast to the chromosomal α -galactosidase (Mel α -gal) specified by the *mel* system of *E. coli* (Schmitt, 1968), Raf α -galactosidase (Raf α -gal) can be assayed in toluene-treated cells (Schmid & Schmitt, 1976). This test is useful for defining strains which harbour a Raf plasmid and for distinguishing them from Raf⁺ strains which do not (Cornelis *et al.*, 1978). Moreover, this marker enzyme is a valuable tool for immunochemical comparisons. In the present study, antiserum produced against purified Raf α -gal of *E. coli* DS25-91(pRSD2-1) (Schmid & Schmitt, 1976; Burkardt *et al.*, 1978) served to establish relationships among 39 Raf plasmids from various sources and to classify them according to their serotypes. These results are considered with respect to the possible phylogenetic relationship of the *raf* determinants.

METHODS

All *E. coli* Raf⁺ strains tested are listed in Table 1 together with their relevant markers and sources. In all cases where the Raf⁺ character was associated with toluene-resistant Raf α -gal, its determinant was localized on a plasmid as shown by conjugational transfer (Burkardt *et al.*, 1978) or by transformation (Shipley *et al.* 1978).

Tests of α -galactosidases were conducted either with the original *E. coli* strains or with K12 derivatives obtained after plasmid transfer (Table 1). The Raf⁺ character was determined on MacConkey agar base (Difco) containing 1% (w/v) raffinose (Merck). α -Galactosidase activity was assayed after 16 h induction of cells with 0.2% (w/v) raffinose or 0.2% (w/v) melibiose using the chromogenic substrate *p*-nitrophenyl- α -D-galactoside with and without toluene preincubation (Schmid & Schmitt, 1976). Hydrogen sulphide production was tested by stabbing single colonies into Kligler Iron Agar (Merck) and incubating overnight at 37 °C. Under these conditions Hys⁺ strains form black zones of ferrous sulphide. K88 agglutination tests were performed (Jones & Rutter, 1972) with K88 antiserum kindly provided by Dr J. van Embden.

Immunodiffusion tests (Ouchterlony, 1967) with Raf α -gal antiserum were performed with crude extracts of sonicated cells previously induced with 0.2% (w/v) melibiose for at least 16 h to ensure high concentrations of α -galactosidase. Melibiose is an inducer of both plasmid-encoded and chromosomal α -galactosidases. Plates were prepared with 1.5% (w/v) Noble Agar (Difco) in 50 mM-sodium phosphate, pH 7.2. Antiserum (10 μ l) and cell extracts (10 μ l extracted from approx. 5×10^8 cells) were placed in equidistant wells in the agar (see Fig. 1) and allowed to diffuse for 16 h at 37 °C. Photographs of the precipitation lines were taken under dark-field illumination and used for documentation after contrast inversion. Antiserum (anti-A1*) was prepared against purified Raf α -gal from the Raf-constitutive strain DS25-91 (Schmid & Schmitt, 1976; Burkardt *et al.*, 1978). Rabbits were given subcutaneous injections each containing 0.5 mg enzyme protein dissolved in 0.5 ml saline and emulsified with 0.5 ml of complete Freund's adjuvant (Behringwerke, Marburg, F.R.G.). A booster injection containing the same amount of enzyme dissolved in saline and 0.5 ml incomplete Freund's adjuvant was given after 5 weeks. One week later blood was withdrawn from the ear vein of each rabbit. The blood samples were kept at 4 °C for 24 h, centrifuged twice at 3000 g for 10 min, and the supernatant serum was stored at -20 °C.

RESULTS AND DISCUSSION

A total of 39 strains of *E. coli* harbouring Raf plasmids from independent isolates were recognized by the detection of α -galactosidase in toluene-treated cells preinduced with melibiose (Table 1, column 3). Anti-A1* antiserum reacted with extracts of all 39 strains, thus indicating the close relationships of all α -galactosidases borne by Raf plasmids and suggesting their common origin. The α -galactosidases of *E. coli* K12 (M1900; Fig. 1), *E. coli* B/r, *E. coli* C and *Klebsiella pneumoniae* (data not shown) gave no reaction.

The immunodiffusion experiments indicated common antigenic determinants of the enzymes by fusing precipitation lines, but also revealed minor differences in antigenicity by spur formation (Ouchterlony, 1967). The 39 Raf α -gals were thus grouped into three classes (A1, A2 and A3) of increasing immunochemical divergence. Representatives of the three serotypes are shown in Fig. 1. The reference enzyme (DS25-91 = A1*) and an extract of D1148 (A1) show fusing precipitation lines indicating their immunochemical identity, whereas spurs are formed at the intersecting lines of A1/A2 (D1073), A2/A3 (F10) and A1*/A3. The position of these spurs indicates that the number of common antigenic determinants decreases from A1 to A3. Of the 39 strains, 31 produced A1-, 3 produced A2- and 5 produced A3-Raf α -gal (Table 1). Whereas serotype A1 appears to be widely distributed, A2 is restricted to samples from Sweden and A3 to samples from England and the U.S.A. (the latter isolated from man). Moreover, all strains producing A1-enzymes were linked either to K88 or to H₂S production, but never to both. A2-enzymes were linked to H₂S production, whereas A3-enzymes showed neither of these connections. These systematic linkages suggest that the three serotypes diverged from a common ancestral *raf* determinant after recombining with certain 'host-specific' traits, which led to biological isolation. The Raf⁺ phenotypes of *E. coli* F54, F65, F118, I3086, E_H12 and E 10400 were not mediated by Raf plasmids, since neither toluene-resistant Raf α -gal nor material reacting with the

Table 1. *Properties of Raf⁺ strains of E. coli and immunochemical comparison of their α -galactosidases*

Strain*	Relevant phenotypic markers†	Raf α -gal‡	Sero-type §	Origin	Reference/source
DS25-91 (pRSD2-1)	Raf Hys	+	A1	Domestic fowl (India)	Schmid & Schmitt (1976)
D1022	Raf Hys	+	A1		Ørskov & Ørskov (1973)
D1148	Raf Hys Scr	+	A1		
D1152	Raf Hys	+	A1		
D1073	Raf Hys	+	A2	Domestic fowl (Sweden)	Ørskov & Ørskov (1973)
D1121	Raf Hys	+	A2		
D1122	Raf Hys	+	A2		
P11	Raf K88	+	A1	Piglet (England)	H. W. Smith (Houghton, U.K.)
P15	Raf K88	+	A1		
P31	Raf K88	+	A1		
P107	Raf K88	+	A1		
P109	Raf K88	+	A1		
P155	Raf K88	+	A1		
P195	Raf K88	+	A1		
P233	Raf K88 Scr	+	A1		
P237	Raf K88	+	A1		
P309	Raf K88	+	A1		
P310	Raf K88	+	A1		
P323	Raf K88	+	A1		
P325	Raf K88	+	A1		
P328	Raf K88	+	A1		
P337	Raf K88	+	A1		
V15	Raf K88	+	A1		
V149	Raf K88	+	A1		
V200	Raf K88	+	A1		
V218	Raf K88	+	A1		
V220	Raf K88	+	A1		
V256	Raf K88	+	A1		
X92	Raf K88	+	A1		
X26	Raf —	+	A3	Domestic fowl (England)	H. W. Smith (Houghton, U.K.)
F10	Raf — Scr	+	A3		
F54	Raf — Col	—	0		
F65	Raf Hys Scr Col	—	0		
F118	Raf — Col	—	0		
F170	Raf —	+	A3		
F173	Raf —	+	A3		
SF711(139)	Raf K88	+	A1	Piglet (Canada)	Shipley <i>et al.</i> (1978)
SF711(152)	Raf K88	+	A1		
I3086	Raf — Scr Hly	—	0	Piglet (France)	Buissière <i>et al.</i> (1977)
I3131	Raf K88 Scr Hly	+	A1		
P _F 1164	Raf K88 Hly	+	A1		
P _F 1981	Raf K88 Hly	+	A1		
E _H 12	Raf — Ent	—	0	Man (U.S.A.)	J. G. Wells (Atlanta, U.S.A.)
E _H 14	Raf — Ent	+	A3		
E _H 10400	Raf — Ent	—	0	Man (Bangladesh)	A. Audremont (Tours, France)
M1900 (K12)	Raf ⁻	— (Mel α -gal ⁺)	0		Schmitt (1968)

* Strains with indices D, I, P_F and E_H are original isolates; all other strains are *E. coli* K12 derivatives obtained by conjugal transfer or transformation of plasmids from an independent isolate.

† Raf, Raffinose fermentation; Scr, sucrose fermentation; Hys, hydrogen sulphide production; Hly, haemolysin production; Col, colicin production; Ent, enterotoxin production; K88, antigen K88 production.

‡ Tested after toluene treatment (Schmid & Schmitt, 1976): +, present; —, absent.

§ Determined by immunodiffusion (Fig. 1). 0, No reaction.

Fig. 1. Agar plate double-diffusion of Raf α -gals from *E. coli* strains. Antiserum against pure Raf α -gal of DS25-91 (anti-A1*) was applied to the centre well. Crude extracts of strains were placed in the outer wells as indicated and incubated for 16 h at 37 °C. Reference strain: DS25-91 (A1*); test strains: M1900 (0), D1148 (A1), D1073 (A2) and F10 (A3).

antiserum was detectable in cell extracts (Table 1). As has been proposed by Cornelis *et al.* (1978), these strains may accumulate raffinose through lactose permease provided either by the chromosomal *lac* system or by a lactose plasmid. Enzymic breakdown of internal raffinose is accomplished by the chromosomal Mel α -gal and, in strains F65 and I3086, by invertase encoded by sucrose plasmids.

As has been recently shown in this laboratory (Mattes *et al.*, 1978), the *raf* and *hys* genes of pRSD2 are located on a 27 megadalton element flanked by direct repeats of IS1. Similar results were obtained for the *raf*/K88 markers of SF711 (S. Falkow, personal communication; R. Mattes, unpublished results) suggesting that the respective combination of markers has spread to various replicons by IS1-mediated translocation.

In summary, we have shown immunochemical relationships among all 39 Raf α -gals tested suggesting a common origin of the *raf* determinants encoded by Raf plasmids. The three serological subclasses of Raf α -gal are correlated with the presence or absence of Hys and K88. The *raf* regions of various plasmids are part of a transposable element flanked by direct repeats of IS1, thus explaining the widespread occurrence of closely related Raf α -gals.

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