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Restriction enzyme fingerprinting of enterobacterial plasmids: a simple strategy with wide application

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SUMMARY

Restriction enzyme fingerprints were generated from purified plasmid DNA from 324 clinical isolates that belonged to 7 enterobacterial genera and 88 single plasmids in *Escherichia coli* K12 according to the following strategy.

Purified plasmid DNA was digested with *Pst*I. The number of fragments detected in a 0·8 agarose gel was used to determine which 2 of 6 restriction enzymes including *Pst*I was most likely to provide a fingerprint comprising sufficient fragments to ensure specificity but sufficiently few to allow easy visual assessment and minimize coincidental matching. When *Pst*I produced > 20 fragments, *Eco*RI and *Hind*III were used; when *Pst*I generated < 6 fragments *Bsp*1286 and *Ava*II were used and *Sma*I was employed when between 6 and 20 fragments were obtained from *Pst*I digests. Using a minimum of 12 fragments from a combination of 2 enzymes as the criterion for characterizing a strain/plasmid, satisfactory 2-enzyme fingerprints were obtained from 87% of the strains and plasmids studied using *Pst*I and no more than two additional enzymes per strain. Of the remaining 54 strains, 51 harboured only small plasmids (< 10 kb) and 3 produced satisfactory fingerprints when digested with a fourth enzyme.

INTRODUCTION

Thompson, Hughes & Broda (1974) recognized the value of restriction endonuclease fragmentation patterns and used *Eco*RI and *Ava*I to distinguish between the closely related plasmids R1, R100–1 and R6. More recently, this approach has been widely used to study the epidemiology of both plasmids and their host bacteria (Beul *et al.* 1985; Lyon *et al.* 1984; O'Brien *et al.* 1982; Platt, Chesham & Kristinsson, 1986; Rubens *et al.* 1981). The information provided by the application of these techniques is particularly useful for the study of genera for which no established typing scheme is available (Hawkey, Bennett & Hawkey, 1984) and the investigation of cryptic plasmids (Callihan, Young & Clark, 1983). Furthermore, the fingerprints may contain fragments characteristic of individual phenotypic traits specified by the plasmid (Platt, 1983).

However, plasmids differ considerably in the number of restriction sites they possess for a given enzyme, and different workers have employed a wide variety

of restriction enzymes to generate fingerprints from the plasmids harboured by diverse bacterial genera – for example O'Brien *et al.* (1982) used *Eco*RI in a study of salmonellae, Lyon *et al.* (1984), *Eco*RI, *Hpa*II and *Hind*III to investigate staphylococci, and Hawkey, Bennett & Hawkey (1984) digested *Providencia stuartii* plasmids with *Eco*R1 and *Hin*CII. Thus, it is not possible at present to compare plasmids from different genera or different laboratories without employing a wide range of enzymes, and the purification of large quantities of DNA is required from each strain.

Ideally any plasmid should be cleaved by two restriction enzymes (Richmond & Petrocheilou, 1978), each of which generates a sufficient number of fragments to ensure specificity but not so many that coincidental matching occurs. Furthermore, to be applicable to widespread use in epidemiology a minimum number of restriction enzymes should cleave a maximum number of plasmids to yield an optimum number of fragments.

Here we describe a simple sequential strategy which enables the construction of fingerprints from diverse enterobacterial genera both rapidly and economically.

MATERIALS AND METHODS

Organisms

Clinical isolates known to harbour plasmids were drawn from the departmental collection to represent diverse enterobacterial genera. Individual R-plasmids as transconjugants in *Escherichia coli* K12 were also studied, and these included representatives of various incompatibility groups. The original sources of both clinical isolates and plasmids are shown in Table 1.

DNA preparation and digestion

DNA was extracted from 10 ml overnight cultures in Brain Heart Infusion Broth (Oxoid: CM225) as previously described (Platt, Chesham & Kristinsson, 1986) or from about 20 colonies harvested from nutrient agar (Oxoid: CM3) plates. Purified DNA was dissolved in 60 μl of TE buffer (Tris 10 mM, EDTA 1 mM; pH 8·0). *Eco*RI, *Hind*III, *Pst*I, *Sma*I and *Ava*II were obtained from Gibco-BRL (Paisley, Scotland) and *Bsp*1286 from New England Biolabs (CP Laboratories Ltd, Bishops Stortford, Herts, England). Digestion buffers were as recommended by the manufacturer; the reaction mixture comprised 15 μl DNA, 20 units restriction enzyme, 5 μl × 10 buffer and sterile distilled water to 50 μl. Incubation was at 37 °C for 4 h (except *Sma*I which was incubated at 30 °C). Electrophoresis was in 0·8 % agarose gels run at 18 mA overnight in tris-borate buffer containing 0·3 μg/ml ethidium bromide (Platt, Chesham & Kristinsson, 1986). Each gel was calibrated with a *Pst*I digest of bacteriophage lambda (λ) DNA and controlled with a lambda digest of the enzyme under study. The number of fragments seen in each track was recorded with a lower limit, normally defined by the 1093 bp *Pst*I fragment of λ. Throughout, the term 'enzyme combination' indicates individual enzymes used separately and not as 'double digests'.

Digestion strategy

Purified plasmid DNA was initially digested with *Pst*I. The choice of subsequent enzyme(s) was based on the number of fragments produced by *Pst*I such that:

*Pst*I: > 20 fragments: *Eco*RI and *Hind*III

*Pst*I: 6–20 fragments: *Sma*I

*Pst*I: < 6 fragments: *Bsp*1286 and *Ava*II

The initial choice of individual enzymes was based on their recognition of different DNA base sequences and the production of a wide range of fragments from standard DNA (λ, ϕx174, SV40). The selection of between 6 and 20 as the optimal number of fragments was essentially arbitrary. A minimum of 12 fragments with 2 enzymes was considered adequate to ensure specificity, and since the probability of coincidental matching of fragments increases with the number of fragments, 20 fragments was normally taken as the upper limit. However, when fragments were clearly detected between 805 and 1093 bp λ *Pst*I fragments these were included and the upper limit raised to 25 fragments.

RESULTS

The enzyme combinations used to fingerprint 324 clinical isolates that harboured plasmids and 88 single plasmid transconjugants are shown in Table 1. Eighty-seven per cent of strains/plasmids were satisfactorily fingerprinted using *Pst*I and no more than two further enzymes. Of the remainder, 12% produced too few (< 12) fragments to ensure specificity; none harboured a plasmid > 10 kb. One per cent (three clinical isolates) were satisfactorily fingerprinted with a fourth enzyme.

In general when *Pst*I generated few fragments, more were produced by *Bsp*1286 and *Ava*II and conversely when *Pst*I produced > 20 fragments *Eco*RI and *Hind*III gave rise to between 6 and 20. However, there were exceptions to this. One strain of *E. coli* that harboured two plasmids gave the following results: *Pst*I, 1 fragment; *Sma*I, 20 fragments; *Eco*RI, 17 fragments; *Hind*III, 1 fragment and *Bsp*1286, 34 fragments.

Of the 87% of strains/plasmids that produced satisfactory fingerprints with no more than three enzymes, there were notable differences in the distribution of strains of different genera among them. Thus, all of the plasmids from *Proteus* spp. produced few fragments after digestion with *Pst*I and required *Bsp*1286 and *Ava*II, whereas *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* sp. and salmonellas were mostly fingerprinted with *Pst*I/*Sma*I. However, *Salmonella typhimurium* differed from other salmonella serotypes in that a larger number (23% and 10% respectively) were fingerprinted using *Eco*RI/*Hind*III, whereas among *E. coli* this enzyme combination accounted for the largest group of strains.

In contrast to the above strains, which often harboured multiple plasmids, the 88 single plasmids as transconjugants were more evenly distributed among the three pairs of enzyme combinations. The three representatives of *Inc* P (RP4, R702, R751) were all fingerprinted with *Bsp*1286/*Ava*II; each fingerprint was different but a number of fragments were common to each plasmid.

Table 1. *Combinations of restriction enzymes that gave satisfactory and sub-optimal fingerprints of plasmids from 324 clinical isolates of enterobacteria and 88 plasmids in* E. coli *K12*

			Satisfactory fingerprints			Sub-optimal fingerprints		
Source/reference	Organism (number)		*Eco*RI *Hind*III	*Pst*I *Sma*1	*Bsp*1286 *Ava*II	A	B	C
	E. coli	(99)	44	38	12	0	2 (*Sma*I/*Eco*2)	3
Coliforms from patients with vascular disease after the exclusion of multiple strains from the same patient	*Klebsiella* sp. *Enterobacter* sp. *Citrobacter* sp.	(27)	3	14	4	0	0	6
	Proteus sp.	(20)	0	0	13	2 (*Pst*I/*Bsp*1286)	0	5
Strains obtained from SSRL* after exclusion of multiple isolates from epidemiological episodes	*S. typhimurium*	(96)	22	53	5	0	1 (*Sma*I/HindIII)	15
	Salmonella (other serotypes)	(70)	7	36	11	0	0	16
Platt & Sommerville (1981)	*Serratia* sp.	(13)	3	0	1	9 (*Pst*I/*Bsp*1286)	0	0
Trimethoprim-resistant transconjugants (Kraft, Platt & Timbury, 1983; 1984)	*E. coli* K12	(73)	18	28	21	0	0	6
Representatives of 13 incompatibility groups including 3 belonging to *Inc* P	*E. coli* K12	(15)	3	6	5	1 (*Pst*I/*Bsp*1286)	0	0

A, Atypical combination of enzymes (but not more than three) required to obtain a satisfactory fingerprint.
B, Satisfactory fingerprint obtained but a fourth enzyme required.
C, Fewer than six fragments produced by each of *Pst*I, *Bsp*1286 amd *Ava*II.
* SSRL, Scottish Salmonella Reference Laboratory.

DISCUSSION

The results presented suggest that the strategy described offers considerable flexibility, in that it can be applied not only to clinical isolates that harbour multiple plasmids, for epidemiological purposes, but also to single-plasmid transconjugants as an adjunct to the investigation of their molecular relatedness. Since the strategy successfully fingerprinted 88% of the organisms and plasmids studied (including those strains that required a fourth enzyme), one application is the construction of a fingerprint library for prospective epidemiological studies. Although this would necessitate rigid adherence to, and perhaps further definition of the strategy, in individual circumstances less stringent criteria may be employed. For example the six trimethoprim-resistance plasmids, which did not fulfil the criteria for establishing a 2-enzyme fingerprint, all specified the same resistance phenotype and each produced 2, 4, 2, and 3, fragments (all matching) in *Pst*I, *Bsp*1286, *Ava*II and *Hind*III digests respectively. Thus it is highly probable that these plasmids are identical, although the specificity of the fragment patterns is relatively poor. Furthermore, it could be argued that, in this particular situation, few fragments from a wider range of enzymes confers adequate specificity. However, the use of more than three enzymes is also contrary to the aim of the strategy if it is avoidable.

The scheme described here does not preclude the possibility that other combinations of enzymes will also yield suitable fingerprints. Among the salmonellas, 39 strains produced > 20 fragments with *Pst*I and were fingerprinted with *Eco*RI and *Hind*III. However, *Sma*I digestion also produced between 6 and 20 fragments in most cases and thus *Eco*RI/*Sma*I and *Hind*III/*Sma*I could be considered as alternative combinations. However, we consider that the routine use of additional enzyme combinations is likely to confuse both procedures and analysis and that secondary combinations should be reserved for situations which occur (albeit rarely) when bacterial strains or plasmids do not produce the requisite number of fragments with the primary enzyme combinations. This situation occurred with 2 *E. coli*, 2 *Proteus*, and 1 of the 96 strains of *S. typhimurium* (Table 1). With regard to those strains that harbour only small plasmids that were not amenable to cleavage with the enzymes used here, satisfactory fingerprints can often be obtained using *Hae*III (Platt, Chesham & Kristinsson, 1986; and unpublished).

Although in this report we have restricted this approach to fingerprinting enterobacterial plasmids, preliminary studies suggest that its application may extend to other genera such as *Haemophilus* and *Staphylococcus*.

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