SHORT COMMUNICATIONS

Characterization of Genes Specifying Ampicillin Resistance in Bacterial Isolates Using a Single-strand Specific Nuclease for Analysis of Plasmid DNA–DNA Duplexes

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(Received 26 May 1978; revised 1 August 1978)

INTRODUCTION

The analysis of molecular relationships among R factors and other plasmids of bacteria as well as the detection and characterization of extrachromosomal genes conferring resistance to antibiotics is possible by DNA–DNA hybridization. The amount of homologous polynucleotide sequences of plasmids can be assayed using the single-strand specific S1 nuclease of *Aspergillus oryzae* (Crosa *et al.*, 1973; Barth & Grinter, 1975). The endonuclease S1 method was used in this study to characterize genes specifying ampicillin resistance in bacteria isolated from hospital patients. The small plasmid RSF1030 which contains the TEM β -lactamase gene (Heffron *et al.*, 1975) served as a molecular probe.

METHODS

Bacterial strains and plasmids. The bacteria investigated were isolated from patients in the Department of Medicine at Göttingen University between January and July 1977 (strains 1 to 7 and 11 to 13 in Table 1). The *Haemophilus influenzae* strains KRE5367, FR16017, RSF0885 and Ve445 have been described by Laufs *et al.* (1979). The plasmids RSF1030, R16 and R455 (Heffron *et al.*, 1975), pMB8 and pMB8::TnA (Heffron *et al.*, 1977a) and RSF1010 and RSF1010::TnA (Heffron *et al.*, 1977b) were obtained from Dr Heffron.

Characterization of the plasmids in the clinical isolates. Media, assay of β -lactamase activity, preparation of unlabelled and labelled plasmid DNA, extraction of unlabelled whole-cell DNA, conjugation, transformation, agarose gel electrophoresis of plasmid DNA and determination of DNA contour length have been described by Laufs & Kaulfers (1977).

DNA–DNA duplex studies. The degree of polynucleotide sequence homology between plasmids was determined by the method of Crosa *et al.* (1973) using the single-strand specific S1 nuclease of *Aspergillus oryzae*. Purified [^3H]thymidine-labelled plasmid DNA was sheared (Branson sonicator model 140D for 4 min at 4 °C, output setting 3) to a molecular weight of about 250 000 and denatured by heating at 100 °C for 10 min. Most labelled plasmid preparations had a specific activity of approximately 0.5×10^6 to 1.5×10^6 c.p.m. μg^{-1} . Sheared and denatured unlabelled whole-cell DNA prepared from the clinical isolates was used as the source of unlabelled plasmid DNA in the duplex studies. The amount of plasmid DNA in such unlabelled unfractionated host materials was not known precisely but was estimated by Guerry & Falkow (1971) to be at least 2 to 4% of the total DNA in all cases. Before hybridization, labelled and unlabelled DNA were exhaustively dialysed against 0.42 M-NaCl. Approximately 0.001 to 0.01 μg labelled plasmid DNA (about 4000 c.p.m.) was mixed in a glass vial with 150 μg unlabelled whole-cell DNA in a total volume of 1.5 ml 0.21 M-NaCl. Reassociation was carried out at 70 °C for long enough to achieve essentially complete reassociation in the homologous reaction. The reassociation time necessary is dependent on the molecular weight of the plasmid DNA (Britten & Kohne, 1966) and was determined by calculating $c_0 t_{\frac{1}{2}}$ (Guerry & Falkow, 1971) for the DNA according to the empirical relationship (Falkow *et al.*, 1974): molecular weight, $M = c_0 t_{\frac{1}{2}} \times 377 \times 10^6$, where c_0 is the initial concentration of single-stranded DNA and $t_{\frac{1}{2}}$ is the half-time of the reaction. For the homologous reaction with the labelled probes, each mixture was incubated at least

for an equivalent of $10\ c_0\ t_{\frac{1}{2}}$. Renaturation was stopped by placing the vial in an ice bath. Samples (0.3 ml) of the reassociation mixture were removed into four tubes containing (in a total volume of 1 ml): 0.1 mM- ZnSO_4 , 0.15 M-NaCl, 30 mM-sodium acetate buffer pH 4.5 and 20 μg sheared denatured calf thymus DNA. S1 endonuclease [Miles Laboratories; 2500 U in 5 μl buffer containing 25% (v/v) glycerol, 0.05 M-NaCl, 0.01 M- KH_2PO_4 , pH 7.0] was added to two of the four tubes, and the mixtures were incubated at 50 °C for 20 min. The reaction was terminated in an ice bath by adding 1 drop of calf thymus DNA (2 mg ml⁻¹) and 0.25 ml ice-cold 5% (w/v) trichloroacetic acid (TCA). The precipitate was collected on membrane filters, and the radioactivity was determined in a liquid scintillation spectrometer. In the homologous reaction the TCA-insoluble material remaining after incubation with the S1 endonuclease represented 65 to 75% of the TCA-insoluble material in the nuclease-free controls. In each experiment, *Escherichia coli* strain 1485-1 F⁻ whole-cell DNA was included as a chromosomal control and showed 6 to 9% duplex formation. The homologous reaction corrected by subtraction of the chromosomal control was taken as 100%. The normalized values (recorded as percentage homology) were obtained by subtracting the chromosomal control (6 to 9%) and dividing by the homologous reaction.

RESULTS

Demonstration of plasmid-specified ampicillin resistance of the clinical isolates

All ten ampicillin-resistant bacterial isolates (isolates 1 to 10, Table 1) were resistant to 1000 μg ampicillin ml⁻¹. This suggested that the resistance was due to a β -lactamase. The relative rates of hydrolysis of various β -lactam antibiotics by extracts of the sonically disrupted bacteria were therefore measured and compared with those of the six ampicillin-sensitive control strains. The β -lactamase produced by the ten ampicillin-resistant bacterial isolates showed a high activity against ampicillin and penicillin G and low activity against oxacillin (data not shown). This substrate profile is characteristic for the TEM type β -lactamase (Heffron *et al.*, 1975). The extracts of the controls (isolates 11 to 16, Table 1) had no hydrolytic activity against any of the β -lactam antibiotics tested.

The ten ampicillin-resistant clinical isolates were examined together with the sensitive control strains for the presence of extrachromosomal DNA by agarose gel electrophoresis. The cleared lysates of all these strains showed one or more plasmid DNA bands in the gel. The molecular weights of the plasmids were determined according to their migration in the gel using standard plasmid DNAs as internal markers (Laufs & Kaulfers, 1977; Table 1). Plasmid DNA was isolated from the strains by direct visualization of the covalently closed circular DNA band in ethidium bromide-CsCl gradients under long-wavelength ultraviolet light. The size of the open circular DNAs found by electron microscopic analysis of the plasmid DNA corresponded well with those determined in the gel (Table 1).

The ampicillin resistance of the clinical isolates which carried plasmids with a molecular weight greater than 20×10^6 could be transferred to *E. coli* 1485 Nal^r by conjugation, and the recipients were shown to have acquired plasmids with molecular weights corresponding to those of the plasmids in the donors (Table 1). Together with the genes specifying ampicillin resistance other resistance markers were also transferred by conjugation. The transfer of the R factors was not reduced by the addition of DNAase I.

Preparations of purified covalently closed circular plasmid DNA from nine of the ten ampicillin-resistant clinical isolates were used for transformation. Exposure of the sensitive *E. coli* strain C600 to purified plasmid DNA resulted in all cases in the appearance of ampicillin-resistant clones (Table 1). The transformation frequency obtained using 5 μg DNA ranged between 10^1 and 10^3 ampicillin-resistant transformants per 10^8 bacteria.

Relationship between RSF1030 and the plasmids of the ampicillin-resistant clinical isolates

Plasmid RSF1030 (Heffron *et al.*, 1975) was used as a molecular probe for the detection of genes determining ampicillin resistance in the isolates. The [³H]thymidine-labelled RSF1030 plasmid DNA was hybridized for 2 h with unlabelled whole-cell DNA from each

Table 1. *Characterization of the clinical isolates*

Strain/plasmid	Resistance pattern*	Plasmid DNAs in agarose gel ($10^{-6} \times$ mol. wt)†	Transfer of Ap resistance by:		Relative DNA sequence homology with RSF1030 DNA (%)‡
			Conjugation	Transformation	
1. <i>Escherichia coli</i> 00118B	Ap, K, Tc, Cb	62	+	+	10
2. <i>E. coli</i> 00317B	Ap, Cm, K, Tc, Cb	66, 27, 16, 7.3, 5.7	+	+	33
3. <i>E. coli</i> 14798	Ap, Tc, Cb, SxT	61, 28, 9.6, 5.4	+	+	41
4. <i>E. coli</i> Th007/3	Ap, Cf, Cb	40, 9.5, 5, 3.3, 1.6 (39, 9.6, 5.1, 2.5, 1.2)	+	+	32
5. <i>E. coli</i> Ec0018C	Ap, Gm, Tc, Cb	60	+	ND	24
6. <i>Enterobacter cloacae</i> 0029B	Ap, Cm, K, Tc, Cb, SxT	65, 33 (35)	+	+	42
7. <i>Serratia marcescens</i> 17148	Ap, Cf, Cm, K, Cb	45	ND	+	35
8. <i>Haemophilus influenzae</i> KRE5367	Ap	31 (30)	+	+	56
9. <i>H. influenzae</i> RSF0885	Ap	4	—	+	21
10. <i>H. influenzae</i> Ve445	Ap	4.6 (4.4)	—	+	24
11. <i>E. coli</i> 4737	—	66	ND	ND	0
12. <i>E. coli</i> 5288	—	47, 4.6, 2.2	ND	ND	4
13. <i>E. coli</i> 1675A	—	43, 19.3	ND	ND	6
14. <i>H. influenzae</i> FR16017	Tc	34 (33)	+	+	3
15. RSF1010	Su, Sm	5.5	—	+	6
16. pMB8	—	1.8	ND	ND	5
17. RSF1010::TnA	Ap	8.7 (8.3)	—	+	51
18. pMB8::TnA	Ap	4.2	—	+	49
19. R16	Ap, Sm, Tc, Su	ND	ND	ND	2
20. R455	Ap, Sm, Tc, Cm, Su	ND	ND	ND	3

ND, Not done.

* Ap, Ampicillin; Cb, carbenicillin; Cf, cephalosporin; Cm, chloramphenicol; Gm, gentamicin; K, kanamycin; Su, sulphonamide; Sm, streptomycin; SxT, sulphamethoxazol trimethoprim; Tc, tetracycline. The ampicillin resistance of strains 1 to 10, 17 and 18 was shown to be due to the production of a TEM type β -lactamase, whereas strains 19 and 20 produced O type β -lactamase.

† Molecular weights determined by electron microscopy are indicated in parentheses.

‡ The degree of DNA-DNA duplex formation was assayed by the S1 endonuclease method as described in Methods. Each value is the average of two to four assays.

of the ten ampicillin-resistant isolates, two resistant strains (17 and 18, Table 1) known to contain the whole ampicillin resistance transposon as well as with the six ampicillin-sensitive controls and two strains (19 and 20, Table 1) known to produce an O type β -lactamase. The DNA of the ampicillin-resistant strains showed 21 to 56% base sequence homology with RSF1030 DNA with the exception of isolate 1, which showed only 10% homology. DNA from the sensitive control strains and from the O type β -lactamase-producing strain did not hybridize significantly with the ^3H -labelled RSF1030 DNA (Table 1). The reciprocal hybridizations between unlabelled whole-cell DNA from the strain carrying RSF1030 and [^3H]thymidine-labelled plasmid DNA of the clinical isolates were examined in some cases and the derived percentage homologies were in close agreement.

The two small plasmids pMB8 (1.8 Mdal) and RSF1010 (5.5 Mdal) were also used as radioactive probes for base sequence homologies with the plasmid DNAs of the clinical isolates. In contrast to plasmid RSF1030, base sequences in clinical isolates related to those of the plasmids pMB8 and RSF1010 were quite frequent. The plasmids in three out of four enteric isolates examined with pMB8 showed 11, 17 and 62% base sequence homology, and DNA from only three out of ten enteric isolates examined did not hybridize significantly with RSF1010.

DISCUSSION

All ampicillin-resistant clinical isolates analysed in this study produced a TEM type β -lactamase. This finding is consistent with other reports that, regardless of the species of origin or the geographical source of the R factor, the TEM β -lactamase is the most common R factor β -lactamase (Hedges *et al.*, 1974). For nine of the ten isolates it is concluded that the structural gene for the demonstrated TEM β -lactamase is plasmid-borne since plasmid DNA extracted from them transformed a sensitive *E. coli* strain to ampicillin resistance.

To detect the TEM β -lactamase genes in the clinical isolates by DNA-DNA hybridization, the 5.5 Mdal plasmid RSF1030 was used as a molecular probe. We found that 150 μg unlabelled, sheared whole-cell DNA prepared from the clinical isolates which carried the TEM β -lactamase genes contained sufficient plasmid DNA to form DNA-DNA duplexes with approximately 0.001 μg ^3H -labelled RSF1030 DNA. The DNA-DNA duplex studies showed that nine of the ten ampicillin-resistant isolates displayed between 21 and 56% DNA sequence homology with RSF1030. Strains RSF0885 and Ve445, which are known to contain only 30 to 40% of the ampicillin resistance transposon (De Graaff *et al.*, 1976; Laufs *et al.*, 1979), showed 21 and 24% base homology, respectively, with ^3H -labelled RSF1030 DNA and strains KRE5367 (Kaulfers *et al.*, 1978) and RSF1010::TnA (Heffron *et al.*, 1977b), which carry the whole ampicillin resistance transposon, showed 56 and 51% base sequence homology, respectively, with ^3H -labelled RSF1030 DNA. These findings are in good agreement with the theoretical predictions considering the fact that 58% of the base sequences of the ^3H -labelled RSF1030 probe represent the ampicillin resistance transposon. The reason why one of the TEM β -lactamase-producing strains showed only 10% DNA sequence homology with RSF1030 is not known.

The whole-cell DNA of the ampicillin-sensitive control strains, which all contained plasmids, did not hybridize significantly with the RSF1030 probe; the average hybridization value was 3.6%. In addition, the two *E. coli* strains which were ampicillin-resistant because they carried plasmids mediating production of an O type β -lactamase also showed no base sequences in common with the RSF1030 probe. These findings confirm the observation of Heffron *et al.* (1975) that RSF1030 displays DNA sequence relationships only with those plasmids of other R factor incompatibility groups which carry genes determining a TEM β -lactamase. Although an absolute control to determine possible common sequences between RSF1030 and the plasmids in the ampicillin-resistant isolates other than those associated with the TEM sequence was not practical, we believe that the common sequences demonstrated in the S1 endonuclease assays are probably restricted to the TEM sequences.

Plasmid pMB8, a 1.8 Mdal ColE1 derivative, and the 5.5 Mdal plasmid RSF1010 are both available with the addition of the ampicillin resistance specifying transposon as pMB8::TnA and RSF1010::TnA and appeared therefore to be good candidates for molecular probes which could be used in parallel to exclude common base sequences not due to the TEM β -lactamase gene. However, high homology values between both pMB8 and RSF1010 and the plasmids in the clinical isolates were quite frequent. For this reason pMB8::TnA and RSF1010::TnA are not as suitable as RSF1030 as molecular probes for the detection of TEM β -lactamase genes in survey work with clinical isolates.

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, W. Germany. We would like to express our gratitude to J. H. Crosa, University of Washington, Seattle, U.S.A., for his most helpful advice for this study. The technical assistance of Mrs I. Richter was appreciated. The data reported here form part of the thesis by one of us (R.F.).

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