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Detection of small genotypic changes in *Escherichia coli* by pyrolysis mass spectrometry

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1. SUMMARY

Pyrolysis mass spectrometry (Py-MS) has been used to discriminate between four very closely related strains of *Escherichia coli*; a parent strain UB5021 and three derivatives each containing one of the antibiotic resistance plasmids, pBR322, pACYC184 or R388.

2. INTRODUCTION

The ability to use Py-MS to discriminate between closely related bacteria is already established. For example, the distinction between *Bacillus subtilis* and *Bacillus amyloliquefaciens* has been difficult to make using conventional methods but can be achieved using Py-MS [1]. This technique was also used to divide 25 strains of *B. cereus* into two groups; one of 12 strains with no history of involvement in food poisoning; the other of 13 strains isolated in connection with emetic-type food poisoning outbreaks [2].

In this paper the ability of Py-MS to distinguish between four *Escherichia coli* strains which differ only by the presence or absence of a single antibiotic resistance plasmid is reported. Such a high degree of discriminatory ability may be of use, for example, in monitoring release of engineered microorganisms, spread of plasmids in populations and loss of these elements from pure cultures in fermenters.

3. MATERIALS AND METHODS

3.1. Strains

The parent strain *E. coli* UB5201 was transformed with paCYC184, pBR322 and R388 by the method of Maniatis et al. [3]. The characteristics of these and the other strains used are shown in Table 1. All were maintained on Dorset egg slopes at room temperature.

3.2. Growth media

Strains were grown for 16 h at 37°C on a minimal salts supplemented medium (MSSM) containing (g/l): K_2HPO_4, 7.0; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.0; NaCitrate, 0.5; $MgSO_4$, 0.25; Casamino acids (Difco), 5.0; DL-Typtophan (BDH Chemicals Ltd.), 0.02; Glucose, 4; Agar

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Table 1

Strains and plasmids used

Strain/plasmid	Characteristics	Ref.
Escherichia coli		
UB5201	F^- *pro met recA56 gyrA*	[4]
UB5860	UB5201 (pBR322)	
UB6330	UB5201 (R388)	
UB6394	UB5201(pACYC184)	
HB101		
045 : K^-		
Other Gram-negative bacteria		
NCTC 10374	*Proteus mirabilis*	
NCIB 8704	*Pseudomonas aeruginosa*	
Plasmids		
pACYC184	Cm^r Tet^r	[5]
pBR322	Amp^r Tet^r *Inc* ColE *rop*$^+$	[6]
R388	Tp^r Su^r Tra^+ IncW	[7]

(Oxoid), 16.5. Strains containing an antibiotic resistance plasmid were grown as follows: UB5860 on MSSM + ampicillin (50 μg/ml), MSSM + tetracycline (25 μg/ml); UB6330 on MSSM + trimethroprim (10 μg/ml); UB6394 on MSSM + chloramphenicol (25 μg/ml), MSSM + tetracycline (25 μg/ml).

In the experiment using strains other than *E. coli*, all strains were grown on nutrient agar (Lab M Broth plus 2% Gibco Agar).

3.3. Sample preparation

Clean Ni-Fe foils (Horizon Instruments, Heathfield, Sussex) were inserted, with clean forceps, into a clean pyrolysis tube (Horizon Instruments) so that 6 mm was protruding from the mouth. Bacterial growth was picked up, avoiding the plate surface, using a disposable plastic loop and smeared on 5 mm of the protruding foil to give an uniform surface coating. The foil was then pushed into the tube using a clean stainless steel depth gauge to lie 10mm from the mouth. Vitron O-ring collars (Horizon Instruments) were placed on the tubes.

Each of the cultures was grown in duplicate and two samples were prepared from each plate, giving four replicates for each culture.

3.4. Pyrolysis mass spectrometry

The samples were analysed using a Horizon Instruments PYMS-200X pyrolysis mass spectrometer. Curie point pyrolysis was at 530 °C for 4 s in a vacuum. The expansion chamber, valve and collimating tube interfacing the pyrolysis tube with the mass spectrometer were heated at 150 °C. Fragments produced were ionized by collision with a cross beam of low energy (30 eV) electrons, and the ions were separated in a quadrupole mass spectrometer, which scanned the pyrolysate 160 times at 0.35-s intervals from initiation of pyrolysis. Integrated ion counts at unit mass intervals from 51 to 200 were recorded on floppy disk, together with pyrolysis sequence number and total ion count for each specimen [8] to produce a spectrum [1].

3.5. Mathematical analysis

Raw data were transferred to an IBM 3090 mainframe computer, running programs written in the GENSTAT statistical language. The methods have been described previously [9,10]. The first stage is data normalization to remove the effect of sample size difference, followed by principal component analysis [11] to reduce data for canonical variates analysis (CVA) [12], a form of discriminant analysis [13]. This analysis produces a generalized distance matrix which can be transformed to a percentage similarity matrix, using the Gower similarity coefficient S_G [14], to make these data amenable to average linkage cluster analysis and to allow the production of a dendrogram or a minimum spanning tree.

4. RESULTS AND DISCUSSION

The first analysis performed was on the four strains grown on MSSM, the resulting dendrogram is shown in Fig. 1a. It can be seen that the parent strain, *E. coli* UB5201, can be differentiated from derivatives carrying different plasmids. The second analysis was on the four strains grown separately on MSSM alone or with one of the four antibiotics (Fig. 1b). It can be seen that UB5860 and UB6330 are more similar to one another than to UB6394. This is also evident in Fig. 1a. UB6394, however, is now more similiar to UB5201.

The similarity differences seen could be due to plasmid DNA. This seems unlikely as the amount

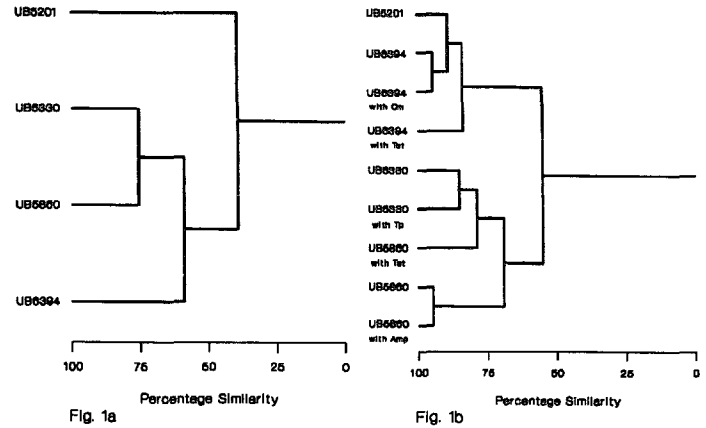

Fig. 1. Dendrogram produced by GENSTAT showing the similarity of the *Escherichia coli* strains grown on MSSM (a) and MSSM or MSSM containing antibiotics (b)

of DNA introduced is small compared to that already in the host cell. A more likely explanation is that the gene products of these plasmids caused the differences (Table 1). In all instances the products causing antibiotic resistance are constitutively produced [15,16]: ampicillin resistance is due to the production of β-lactamase [16], chloramphenicol resistance to chloramphenicol acetyltransferase [16] and trimethroprim resistance is achieved by the production of a greater amount of dihydrofolate reductase, which is the antibiotic target site [17]. Thus either an additional protein or additional amounts of existing protein is present in the bacteria into which plasmids have been introduced.

In Fig. 1b it can be seen that there is a greater difference in UB6330 cultures when grown in the presence of trimethroprim than in UB5860 or UB6394, when grown with ampicillin and chloramphenicol. This may possibly be due to more dihydrofolate reductase being produced than β-lactamase or chloroamphenicol acetyl transferase, but we have no evidence of this. Growth with tetracycline leads to a more marked effect on the spectra of UB5860 and UB6394 (Fig. 1b), than does growth with ampicillin or chloramphenicol. Tetracycline resistance is due to constitutive efflux protein located in the cytoplasmic membrane [16]. The action of this protein depends on metabolic energy and a possible explanation for the larger differences observed in the tetracycline resistant derivatives, when grown with tetracycline, is an increase in the quantity of the cellular components involved in energy generation.

The introduction of the antibiotic resistance plasmids into *E. coli* UB5201 does not, however, alter the position of the derivatives in CVA plots relative to either the other *E. coli* strains, HB101 and 045 : K^-, or two other Gram-negative bacteria (Fig. 2). On examination of just the data for *E. coli* strains, the UB5201 strains and its plasmid derivatives were all grouped together in the CVA plot, but were separated from the other *E. coli* strains (Fig. 3). Thus, the inclusion of plasmids into *E. coli* UB5201 does not modify its taxonomic position relative to other *E. coli* strains.

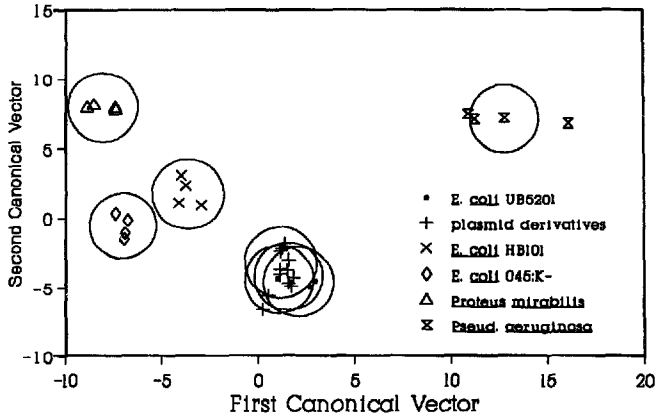

Fig. 2. Canonical variates plot produced by GENSTAT showing the similarity of the UB5201 *Escherichia coli* strains, with its derivatives, alongside *E. coli* HB101 and 045 : K^-, *Pseudomonas aeruginosa* NCIB 8704 and *Proteus mirabilis* grown on nutrient agar. The circles represent the 95% tolerance region constructed round each sample mean by the χ^2 distribution on two degrees of freedom [18].

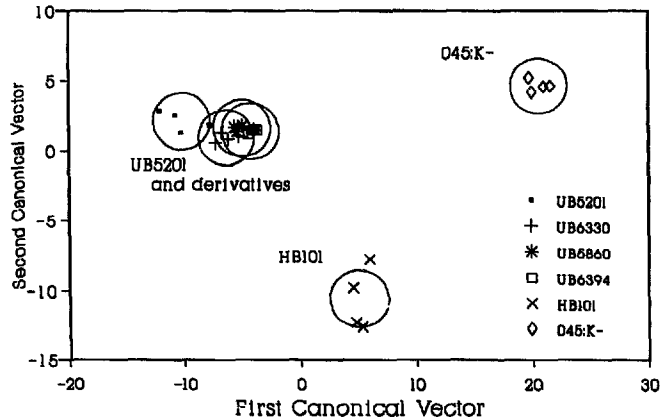

Fig. 3. Canonical variates plot produced by GENSTAT showing the similarity of the UB5201 *Escherichia coli* strains, with its derivatives, alongside *E. coli* HB101 and 045 : K^- grown on nutrient agar. The circles represent the 95% tolerance region constructed round each sample mean by the χ^2 distribution on two degrees of freedom [18].

In conclusion, this demonstration that the discriminatory ability of Py-MS is sufficient to detect very small genotypic changes in bacteria, is another example of the usefulness of this powerful technique.

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