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The use of pyrolysis–mass spectrometry to detect the fimbrial adhesive antigen F41 from *Escherichia coli* HB101 (pSLM204)

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ABSTRACT

Escherichia coli HB101 is a plasmidless, non-fimbriated bacterium. The plasmid pSLM204, which encodes for the production of the adhesive F41 antigen found in enterotoxigenic *E. coli*, was sub-cloned into *E. coli* HB101. It is possible to remove the F41 antigen from *E. coli* HB101(pSLM204) by physical shearing and to monitor its removal by electron microscopy and mannose-resistant haemagglutination with guinea pig erythrocytes. Pyrolysis–mass spectrometry can differentiate between *E. coli* HB101, HB101(pSLM204) and the latter strain after the removal of F41 fimbriae. This study shows that the difference observed is due largely to F41 antigens on the surface of strain HB101(pSLM204).

Antigen; *E. coli* HB101; enterotoxin; fimbriae; pyrolysis.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) achieve their pathogenicity by the adherence of the pathogen to the small intestine of the host, where enterotoxins cause the secretion of fluid and electrolytes. Adherence is mediated by antigenic proteins which are fimbrial in nature and referred to as adhesins [1]. The F41 adhesin is found on ETEC of calves, lambs and piglets; very little is known of its specificity for mammalian receptors, apart from it being a mannose-resistance adhesin in haemagglutination. F41 has been purified and characterized by De Graaf and Roorda [2]; it is flexible, has a diameter of 3.2 nm, is comprised of protein sub-units of molecular weight 29500 and has a p*I* of 4.6. The F41 antigen is chromosomally

encoded and has been sub-cloned from a cosmid library to produce a 24 kb plasmid, pSLM204, which expresses the F41 antigen [3].

Pyrolysis–mass spectrometry (Py–MS) has been used to discriminate between closely related bacteria. For example, the distinction between *Bacillus subtilis* and *Bacillus amyloliquefaciens* has been difficult to make using conventional methods but can be achieved by Py–MS [4]. This technique has also been used to divide four genotypically related strains of *E. coli* K-12; a parent strain, UB5201, and three derivatives each containing one of the antibiotic resistance plasmids, pBR322, pACYC184 or R388 [5]. Other workers have demonstrated the ability of analytical pyrolysis to differentiate bacterial cell wall and polysaccharide antigens of streptococci [6,7].

In this study we investigate the ability of Py–MS to distinguish between a parent strain of *E. coli*, HB101, which lacks fimbriae, HB101 transformed with pSLM204, and HB101(pSLM204) after physical removal of the fimbriae.

MATERIALS AND METHODS

Bacterial strains

A parent strain of *Escherichia coli* K-12 HB101, which lacks F41 or K88 antigen on its surface, was used in this study. HB101 was transformed with pSLM204 and pBR322 by the method of Maniatis et al. [8]. The characteristics of these strains and others used in this study are shown in Table 1. All strains were maintained on Dorset egg slopes at room temperature.

TABLE 1

Strains and plasmids used

Strain/plasmid	Characteristics	Reference
Escherichia coli K-12		
HB101	F^- *hsdS20* ($r_B^- m_B^-$) *recA12 ara-14 proA2 lacYI galK2 rspl20* (Sm^r) *xyl-5 mtl-1 supE44* $lambda^-$	[8]
$045:K^-$	protrophic wild-type strain	[9]
UB5201	F^- *pro met recA56 gyrA*	[10]
Other Gram negative bacteria		
NCTC 10374	*Proteus mirabilis*	
NCIB 8704	*Pseudomonas aeruginosa*	
Plasmids		
pSLM204	$F41^+Amp^r$	[3]
pBR322	Amp^r Tet^r *IncColE rop*$^+$	[11]

Growth media

For the haemagglutination and Py–MS of HB101 and HB101(pSLM204), strains were grown for 16 h at 37 °C in an orbital shaker, shaking at 180 rev min^{-1}, in 10 ml nutrient broth (LabM). In addition, HB101 transformed with pSLM204 was grown on nutrient broth + ampicillin (50 μg/ml).

In the experiment using strains for Py–MS other than HB101 or HB101(pSLM204), all strains were grown on nutrient agar (LabM broth + 2% Gibco Agar).

Vortexing procedure

Cultures from broth were placed on ice for 5 min to stop growth. Cells were centrifuged at 10000 g for 2 min and then resuspended in 750 μl cold phosphate-buffered saline (PBS). The HB101(pSLM204) strain was then vortexed for either 0, 10, 20 or 30 min using a Whirlimixer. The samples were then centrifuged at 10000 g for 2 min. The supernatant and pellet were retained. The pellet was washed twice in PBS, to extract any unattached antigen, and centrifuged at 10000 g for 2 min, the final volume of PBS added was 200 μl. The supernatant was centrifuged at 10000 g for 30 min to pellet any F41 antigen which may have been removed by physical shearing.

Mannose resistance haemagglutination (MRHA)

The method is essentially that described by Old et al. [12]. A bacterial suspension with an optical density at 675 nm of 2.0 was serially diluted in microtitre plates, using PBS containing 1% mannose, to give final volumes of 50 μl; 50 μl of 1% guinea pig erythrocytes was then added to the diluted suspensions. The plates were incubated at 4 °C for 2 h then haemagglutination assessed by eye.

Electron microscopy

A drop of bacterial or fimbrial sample was placed on a carbon-coated formvar grid and stained with ammonium molybdate (0.5% (w/v)). The micrographs were taken with a Phillips 201 transmission electron microscope operating at 80 kV.

Sample preparation

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

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 of the tube. Samples (5 μl) prepared from the broth, by the same method as used in the vortexing procedure, were pipetted on the protruding foil. When plate cultures were used, bacterial growth was picked up, avoiding the growth medium surface, using a disposable plastic loop and smeared on 5 mm of the foil to give a uniform surface coating. The sample was then vacuum desiccated. The foil was then pushed 10 mm into the tube using a clean stainless steel depth gauge, to locate the sample tube for pyrolysis. Viton O-rings (Horizon Instruments) were placed on the tubes.

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 0.2 s intervals from initiation of pyrolysis. Integrated ion counts at unit mass intervals from 51–200 were recorded on a floppy disk, together with pyrolysis sequence number and total ion count for each specimen [13] to produce a spectrum (Fig. 1).

Mathematical analysis

Raw data were processed on an OPUS PCV computer, running programs written in the GENSTAT statistical language. The methods have been

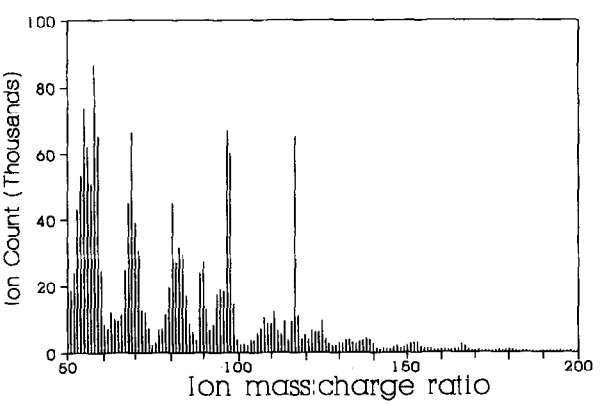

Fig. 1. A typical pyrolysis–mass spectrum of *E. coli* HB101 grown on nutrient agar. Quantitative ion counts for masses 51–200 (horizontal axis) are represented as vertical bars; the intensity at each mass may be read on the vertical axis.

TABLE 2

Mannose-resistant haemagglutination of guinea pig erythrocytes by F41 antigens

Strain	Vortex time (min)	Haemagglutination *
HB101 **	0	0
HB101 (pSLM204) **	0	256
HB101(pSLM204) ***	0	256
HB101(pSLM204) ***	10	128
HB101(pSLM204) ***	20	32
HB101(pSLM204) ***	30	32

* Reciprocal value of the highest dilution that showed a positive haemagglutination reaction.
** Grown in nutrient broth.
*** Grown in nutrient broth + 50 μg/ml ampicillin.

described previously [14,15]. The first stage is data normalization to remove the effect of sample size difference, followed by principal component analysis [16] to reduce data for canonical variates analysis (CVA) [17], a form of discriminant analysis [18]. In plots of CVA, circles are drawn which represent the 95% tolerance region constructed around each sample mean by the chi-squared distribution on two degrees of freedom [19]. This analysis produces a generalized distance matrix which can be transformed to a percentage similarity matrix, using the Gower similarity coefficient S_G [20], to make these data amenable to average linkage cluster analysis and to allow the production of a dendrogram or a minimum spanning tree.

RESULTS

Haemagglutination

The ability of *E. coli* HB101 and HB101(pSLM204) to haemagglutinate guinea pig erythrocytes by F41 antigens in the presence of 1% mannose was determined (Table 2). F41 antigen exhibited a strong mannose-resistant haemagglutination activity, vortexing HB101(pSLM204) for 10, 20 or 30 min showed that the level of haemagglutination decreased, implying that the number of F41 antigens had been reduced.

Electron microscopy

Negatively stained preparations of *E. coli* HB101(pSLM204) vortexed for 10, 20 or 30 min and HB101 grown in nutrient broth at 37 °C for 18 h were examined by electron microscopy. HB101 showed no F41 fimbriae on its surface. Unvortexed HB101(pSLM204) revealed F41 fimbriae on 30–40% of the bacteria (Fig. 2(a)). After *E. coli* HB101(pSLM204) had been vortexed

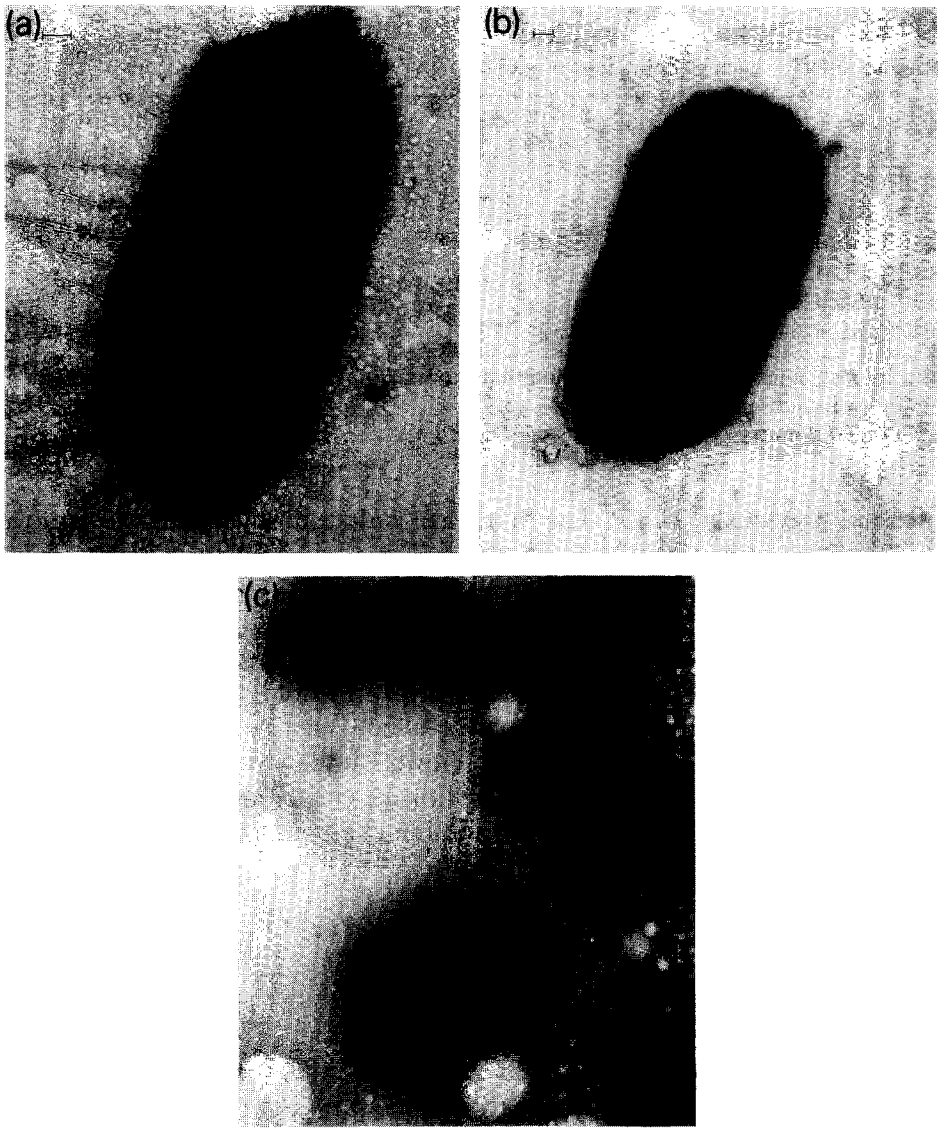

Fig. 2. (a) Transmission electron micrograph of *E. coli* HB101(pSLM204) showing F41 fimbriae (bar, 100 nm); (b) transmission electron micrograph of *E. coli* HB101(pSLM204) after vortexing for 20 min, showing absence of fimbriae (bar, 100 nm); (c) transmission electron micrograph of a crude preparation of F41 antigens showing their slight curvature (bar = 100 nm).

for 20 min or more, extensive searching of the samples in an electron microscope, led to the conclusion that less than 1% of bacteria possessed fimbriae. No stumps were visible (Fig. 2(b)). On examination using negative staining of the F41 fimbriae preparation (Fig. 2(c)), from 20 min vortexed cultures, slightly curved filamentous structures, approximately 3 nm in diameter, were observed.

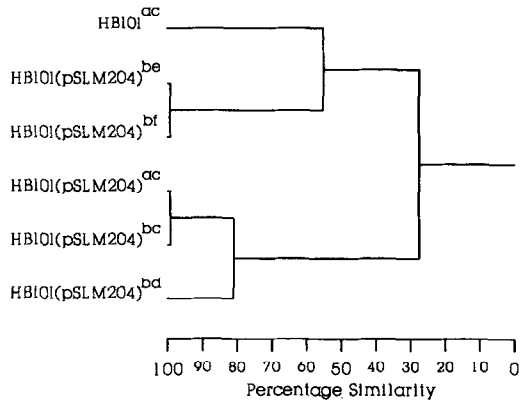

Fig. 3. Dendrogram produced by GENSTAT showing the similarity of *E. coli* HB101 and HB101(pSLM204) grown on nutrient broth (a) or nutrient broth with ampicillin (b), vortexed for either 0 (c), 10 (d), 20 (e) or 30 (f) min.

Pyrolysis mass spectrometry

The first analysis performed was on *E. coli* HB101 and HB101(pSLM204) grown on nutrient broth alone or with ampicillin (50 μg/ml), the HB101(pSLM204) strain was vortexed for 0, 10, 20 or 30 min, the resulting dendrogram is shown in Fig. 3. The second analysis was on *E. coli* HB101 and its plasmid carrying derivatives, HB101(pSLM204) and HB101(pBR322), and two other *E. coli* strains, 045 : K^- and UB5201; this is displayed as a

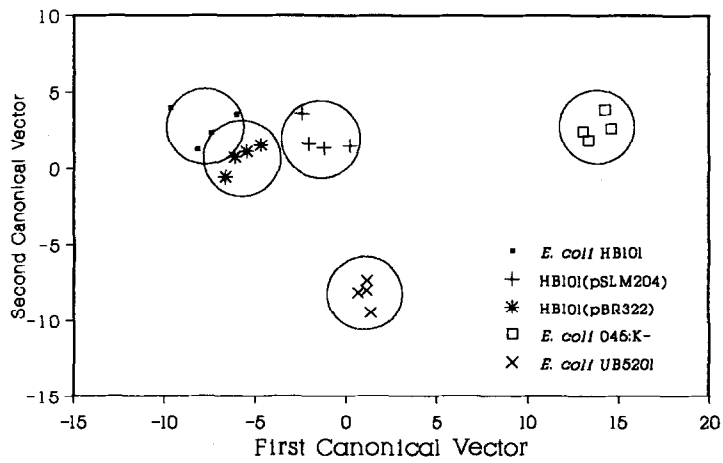

Fig. 4. Canonical variates plot produced by GENSTAT showing the similarity of the HB101 *E. coli* strain with its derivatives, alongside 045 : K^- and UB5201, grown on nutrient agar.

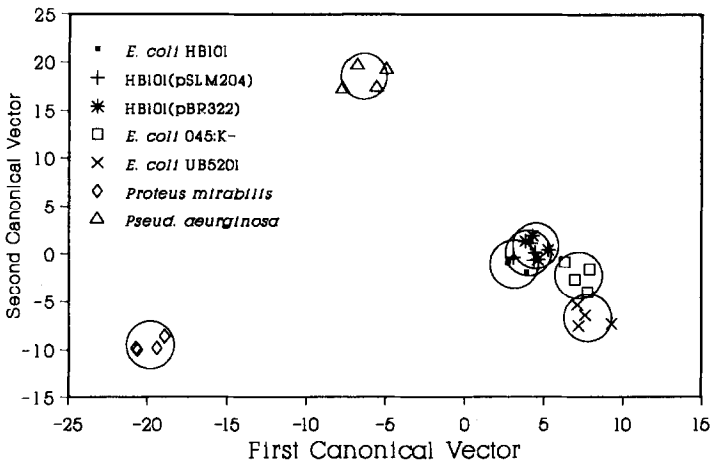

Fig. 5. Canonical variates plot produced by GENSTAT showing the similarity of the HB101 *E. coli* strain with its derivatives, alongside 045 : K^- and UB5201, *Pseudomonas aeruginosa* NCIB 8704 and *Proteus mirabilis* NCTC 10374 grown on nutrient agar.

CVA plot in Fig. 4. The same set of *E. coli* strains was analysed with two other Gram negative bacteria (Fig. 5).

DISCUSSION

Mannose-resistant haemagglutination has been used in this study to follow the removal of F41 from *E. coli* HB101(pSLM204) by vortexing (Table 2). Strain HB101 shows no haemagglutination with guinea pig erythrocytes. However, when the plasmid pSLM204 is sub-cloned into HB101 haemagglutination was observed in cells grown in nutrient broth in the presence or absence of ampicillin (50 μg/ml). Haemagglutination decreased slightly if HB101(pSLM204) was vortexed for 10 min, but a greater reduction was seen when cells were vortexed for 20 min or longer, implying that a vortexing time of 20 min or above is enough to remove most F41 fimbriae from the surface of *E. coli* HB101(pSLM204). Shearing off of F41 antigens is evident from electron microscopy of the supernatant from a 20 min vortexed culture. The fimbriae are approximately 3 nm in width and slightly curved (Fig. 2(c)). This is in agreement with De Graaf and Roorda [2].

The dendrogram in Fig. 3 displays the similarity, as judged by pyrolysis–mass spectrometry, of untreated and vortexed cultures of *E. coli* HB101 and HB101(pSLM204). Vortexing for 10 min alters only slightly HB101(pSLM 204) grown on nutrient broth containing ampicillin and it still has a high level of similarity with HB101(pSLM204). After vortexing for 20 or 30 min the culture of HB101(pSLM204) is identified with *E. coli* HB101, the parent

strain, which lacks F41 fimbriae on its surface. Furthermore, the cultures vortexed for 20 and 30 min group together, showing that there is little or no more shearing off of F41 fimbriae after 20 min; this is also evident from haemagglutination studies (Table 2). *E. coli* HB101(pSLM204) grown in the presence or absence of ampicillin also cluster together; this implies that there has not been any significant alteration to the pyrolysis mass spectrum of HB101(pSLM204) when the organism is exposed to ampicillin. It might be thought surprising that an increase of the pSLM204 plasmid copy number or of the amount of β-lactamase, encoded by this plasmid and produced constitutively [21], did not cause detectable changes in the spectrum. Similar lack of spectral change was, however, noted in a study by Goodacre and Berkeley [5] with *E. coli* UB5201 using the drug-resistance plasmids pACYC184, pBR322 and R388.

Removal of F41 fimbriae from the surface of *E. coli* HB101(pSLM204) by vortexing alters the identification of the cell from HB101(pSLM204) to HB101. Thus the molecular basis for the original mass spectral differences between HB101 and HB101(pSLM204) seen in the CVA plot (Fig. 4) is due largely to F41 fimbriae, although products of other genes on the pSLM204 plasmid (Table 1) may also contribute a little to the changes observed.

The introduction of plasmids pSLM204 and pBR322 into *E. coli* HB101 does not alter the position in the CVA plot of the derivatives HB101(pSLM204) or HB101(pBR322), relative to other *E. coli* strains, UB5201 and $045:K^-$, or to two other Gram negative bacteria, *Proteus mirabilis* and *Pseudomonas aeruginosa* (Fig. 5). The CVA plot of just the *E. coli* strains (Fig. 4) shows that HB101 and its derivatives cluster together, but were grouped separately from the other *E. coli* strains. *E. coli* HB101(pSLM204) groups slightly apart from the HB101 and HB101(pBR322) cluster, as a result of the F41 fimbriae on its surface causing a greater change in the Py–MS spectrum than that produced by the introduction of pBR322 into *E. coli* HB101. Thus the inclusion of plasmids into HB101 does not modify its taxonomic position relative to other *E. coli* strains, but it is still possible to discriminate between the parent strain and its derivatives.

In conclusion, this study indicates that the discrimination by Py–MS between HB101 and HB101(pSLM204) is due largely to the existence of F41 fimbriae on the surface of the latter. Furthermore, it shows that Py–MS has the ability to discriminate between bacteria which differ genotypically only in a very small way.

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