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Characterisation of intact microorganisms using electrospray ionisation mass spectrometry

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Abstract

We report the first application of electrospray ionisation mass spectrometry (ESI-MS) for the reproducible characterisation of strains of *intact* Gram-negative and Gram-positive bacteria. Electrospray ionisation was performed in both the positive and negative ion modes and the spectra obtained from *Escherichia coli* and *Bacillus cereus* were very information rich. Several of the observed negative mass ion fragments from *E. coli* could be assigned to specific fragmentation from bacterial phospholipids. Cluster analyses of these spectra showed that ESI-MS could be used to discriminate between these microorganisms to below species level. Therefore we conclude that ESI-MS constitutes a powerful approach to the characterisation and speciation of intact microorganisms.

Keywords: Electrospray ionisation; Mass spectrometry; Whole-organism fingerprinting

1. Introduction

In view of the increasing prevalence of microbial infections there is a continuing need for the rapid characterisation and speciation of bacterial cells. The ideal method would work on intact bacteria, and would be rapid, reproducible and reagentless. With recent developments in analytical instrumentation, these requirements are being fulfilled by physico-chemical spectroscopic methods, often referred to as 'whole-organism fingerprinting' [1].

There has been an explosion of interest in the use of soft ionisation methods such as matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI) for the analysis of biomacromolecules, as well as of small molecules, and such mass spectrometric (MS) methods are now important tools in proteomics and functional genomics [2]. MS is particularly well suited to the analysis of these complex biological systems because it has the ability to resolve single components based on their mass-to-charge (m/z) values allowing structural information to be obtained. Whilst pyrolysis MS has been shown to be useful for whole-microorganism fingerprinting [1,3,4], it does have the disadvantages that the highest m/z value reproducibly attainable is very small (only m/z 200) and the in vacuo thermal degradation step means that essentially all information on the structure or identity of the molecules producing the pyrolysate is lost. Recently, it has been demonstrated that characteristic profiles of in-

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Table 1
Strains studied

Species	Strain number	Source	Identifier on plots
Bacillus cereus	DSM31[a]	received as B0002 [25]	B1
Bacillus cereus	NRS721	received as B0550 [25]	B2
Bacillus cereus	V26	received as B0702 [25]	B3
Escherichia coli	HB101	[26]	E1
Escherichia coli	O45:K$^-$	[26]	E2
Escherichia coli	UB5201	[26]	E3

[a]Type strain.

tact microorganisms using MALDI-MS can be obtained [5,6], and although this technology is at the stage where it has been shown to work, MALDI-MS is not as yet entirely reproducible and robust [7].

More recently electrospray ionisation mass spectrometry ESI-MS has also been shown to be a valuable tool for the reproducible analysis of complex biological samples, including intact viruses [8], but to date the introduction of bacterial samples has been via specific cell lysates [9] and usually presented to the ESI-MS after liquid chromatographic (LC) separation [10]. In the present study we investigated the ability of electrospray ionisation in this context, and show for the first time that it may be used successfully to provide information-rich mass spectra from *intact* microbial cells.

2. Materials and methods

2.1. Organisms and cultivation

Details and origins of the six organisms analysed are given in Table 1. To ensure axenic cultures the bacteria were cultivated three times aerobically on LabM Malthus blood agar base (37 mg ml^{-1}) for 16 h at 37°C. To avoid the formation of any adducts from buffers, salts or detergents, with the ions created during the electrospray process, the biomass was carefully collected using sterile plastic loops and suspended in 1 ml aliquots of sterile MilliQ water; these harvesting and washing steps were done in triplicate, and the final biomass was adjusted to 250 ng ml^{-1} (dry weight; ca. 2.5×10^5 cells ml^{-1}).

2.2. Electrospray ionisation mass spectrometry

ESI-MS was performed using a Micromass Ltd. (Floats Road, Wythenshawe, Manchester, UK) LCT mass spectrometer. Spectra were collected in both the positive (ES$^+$) and negative (ES$^-$) ion modes. For ES$^+$ the bacteria were suspended in 50% acetonitrile/water (1% v/v formic acid), and for ES$^-$ the samples were analysed in 50% isopropanol/water. The acetonitrile and isopropanol were used to decrease the surface tension of the carrying solvent and thus aid electrospray. For ES$^+$ formic acid was used to enhance the sensitivity by aiding protonation of basic molecules, and although not employed ammonium hydroxide is often used to aid deprotonation in ES$^-$. These samples were then loaded into a 100 μl volume Hamilton gastight 7000 series syringe and introduced to the LCT mass spectrometer using a Harvard Apparatus Pump 11 operating at a flow rate of 5 μl min^{-1}.

To optimise the positive and negative ESI spectra the sample cone voltage set was varied from 30 to 150 V in 10 V steps; the optimum cone voltage was 70 V for ES$^+$ and 90 V for ES$^-$; whilst the capillary voltage set was 3000 V for ES$^+$ and 2500 V for ES$^-$. For both source polarities the extraction cone voltage was 10 V, the source and desolvation temperatures were both 80°C, and the desolvation and nebuliser gas flow rates were 650 l h^{-1} and 90 l h^{-1} respectively.

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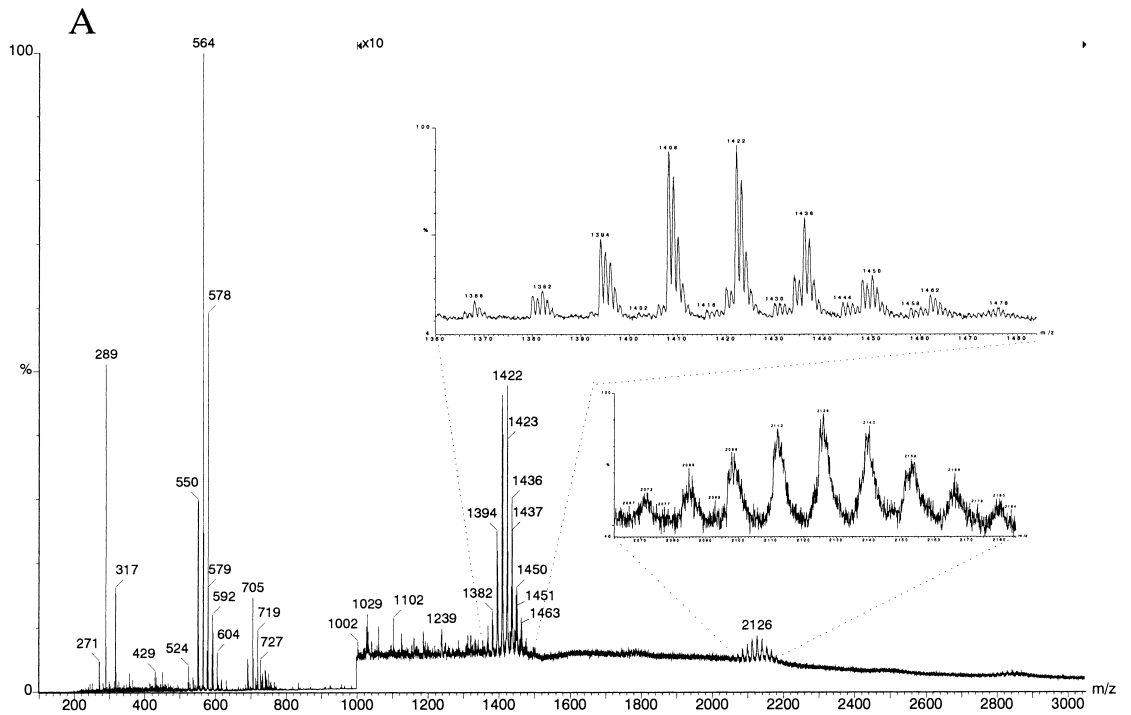
Fig. 1. Positive ionisation mass spectra of *E. coli* HB101 (A) and *B. cereus* DSM31 (B). Spectra are normalised to % base peak. Total ions counts 2.7×10^7 (A) and 3.5×10^7 (B). Note that the scale is multiplied $\times10$ over m/z = 1000 (A) and $\times6$ over m/z = 780 (B).


A
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x10
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289
550
317
579
592
705
719
604
727
271
429
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1102
1002
1239
1382
1394
1422
1423
1436
1437
1450
1451
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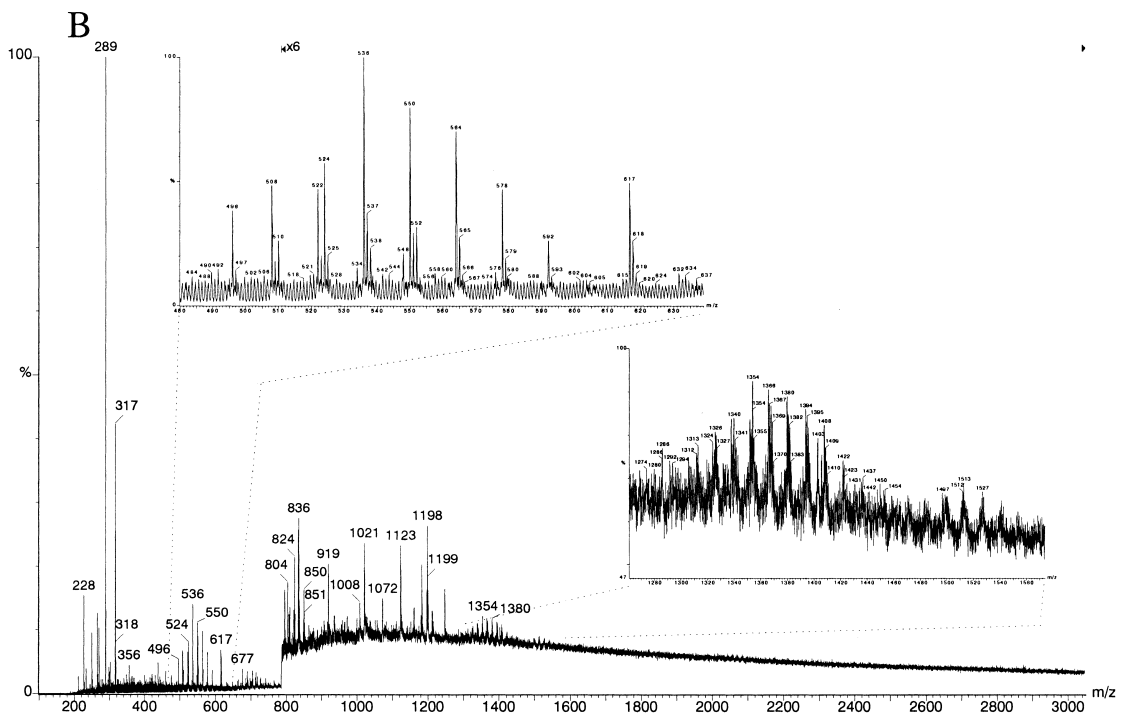
1800
2000
2200
2400
2600
2800
3000
m/z


B
100
%
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289
317
228
318
356
496
524
536
550
617
677
804
824
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850
851
919
1008
1021
1072
1123
1198
1199
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2000
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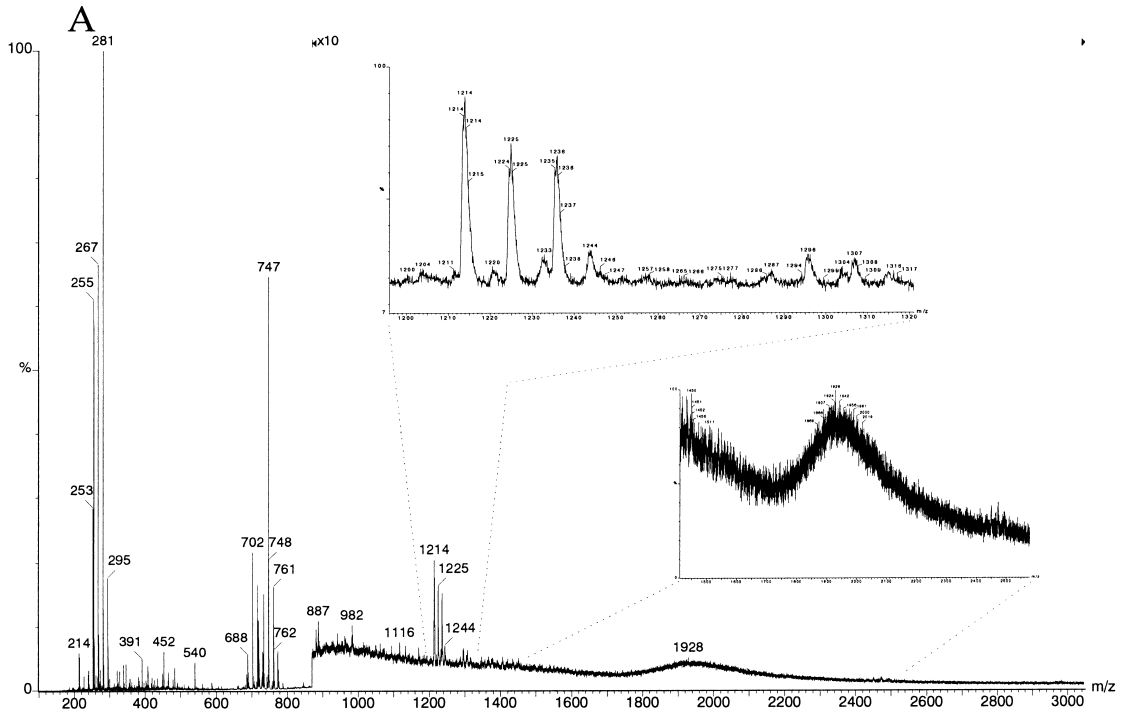
2800
3000
m/z


A
x10
281
267
255
253
747
702
748
761
295
214
391
452
540
688
762
887
982
1116
1214
1225
1244
1928
%
m/z

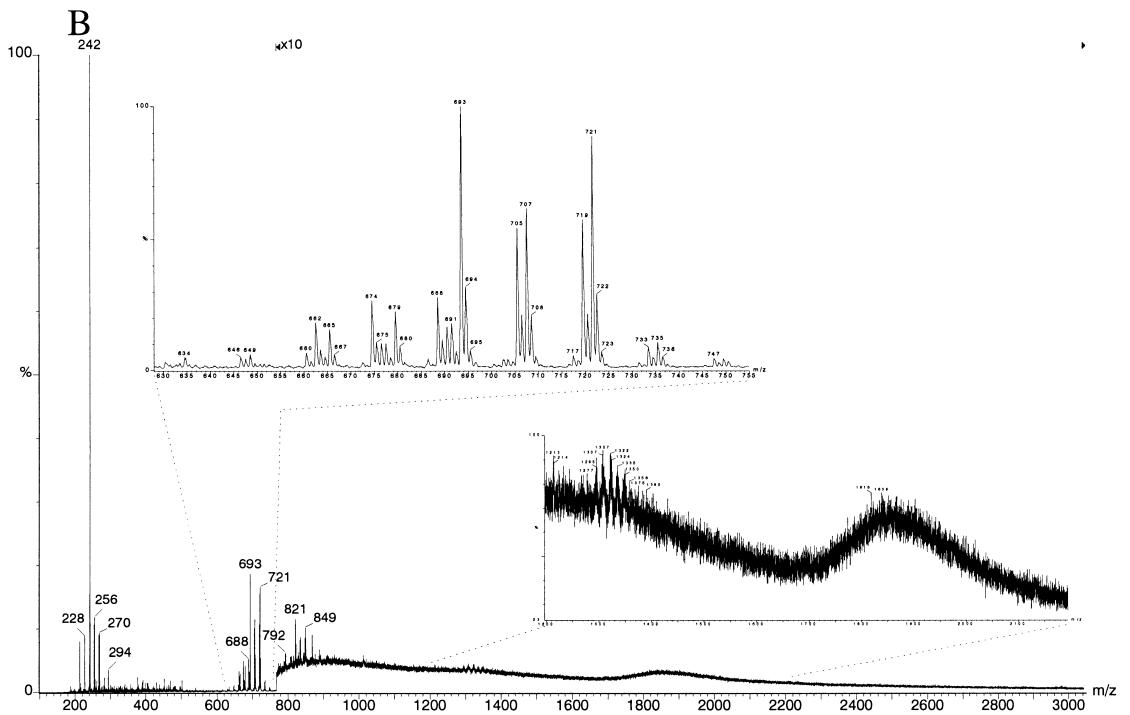

B
x10
242
256
228
270
294
693
721
688
792
821
849
%
m/z

Fig. 2. Negative ionisation mass spectra of *E. coli* HB101 (A) and *B. cereus* DSM31 (B). Spectra are normalised to % base peak. Total ions counts 1.7×10^7 (A) and 1.7×10^7 (B). Note that the scale is multiplied $\times10$ over m/z=870 (A) and $\times10$ over m/z = 770 (B). ←

To obtain high mass accuracy the LCT employs a reflectron time-of-flight (TOF) analyser [11], and in this study the mass range was set to 100–3050 m/z and spectra were collected every 1.5 s for 10 min; these were then summed. The instrument was calibrated such that nominal mass measurement was achieved over the entire mass range, and each spectrum consisted of 53 662 ion intensity measurements. Typical ES^+ and ES^- spectra normalised to percent base peak (highest) of *Escherichia coli* HB101 and *Bacillus cereus* DSM31 are shown in Figs. 1 and 2 respectively.

2.3. Cluster analyses

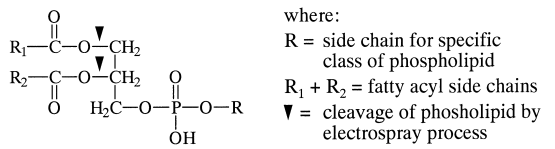
Data were exported from the MassLynx program version 3.2 (software provided by Micromass and running under Microsoft Windows NT on an IBM-compatible PC) and imported into Matlab version 5.0.0.4069 (The MathWorks, Inc., 24 Prime Par Way, Natick, MA, USA), which also runs under Microsoft Windows NT on an IBM-compatible PC. Before analyses the spectral data were normalised to percent total ion count.

The cluster analysis process is based on that developed by Gutteridge et al. [12] to analysis PyMS data, and has been adapted in-house for the analyses of high dimensional infrared [13] and Raman spectra [14]. Briefly, the initial stage involved the reduction of the dimensionality of the ESI-MS data by principal components analysis (PCA) [15]. PCA is a well known technique for reducing the dimensionality of multivariate data whilst preserving most of the variance, and PCA was performed according to the NIPALS algorithm [16]. Discriminant function analysis (DFA; also known as canonical variates analysis (CVA)) then discriminated between groups on the basis of the first five retained principal components, which accounted for 88.7% of the total explained variance, and the a priori knowledge of which spectra were replicates, and thus this process does not bias the analysis in any way [17]. Finally, the Euclidean distance between a priori group centres in DFA space was used to construct a similarity measure, with the Gower similarity coefficient S_G [18], and these distance measures were then processed by an agglomerative clustering algorithm to construct a dendrogram [17].

3. Results and discussion

The initial stage of the study was to observe the effect of varying the cone voltage on the formation of molecular and fragment ions, and the cone voltage was therefore altered from 30 to 150 V in 10 V steps. At 30–50 V few ions were seen and by contrast, at voltages above 100 fragmentation of some higher mass ions to produce lower mass fragment ions was seen. The optimum cone voltage was 70 V for ES^+ and 90 V for ES^-, and this was determined by visual inspection as a balance between the maximum number of ions seen and the stability of fragmentation, that is to say small alterations ($\sim \pm 5$ V) in cone voltage had very little effect on the spectrum.

Figs. 1 and 2 show typical ESI-MS spectra of *E. coli* HB101 and *B. cereus* DSM31 collected in the positive and negative ion mode, respectively. Both the ES^+ and ES^- spectra are very information rich, and they contain several clusters of ions. Ions



Calculated masses for R_1 and R_2

Number of unsaturated bonds	Number of carbon atoms in fatty acyl chain							
	13	14	15	16	17	18	19	20
0	213	**227**	**241**	**255**	269	283	297	311
1	211	225	**239**	**253**	**267**	**281**	**295**	309
2	209	223	237	251	265	279	293	307

masses in **bold** are those observed in ES^- of *E. coli*

Fig. 3. The structure of bacterial phospholipids and calculations (based only on ^{12}C) of possible masses of the fatty acyl substituents R_1 and R_2 containing between 13 to 20 carbon atoms, which are either saturated (0), or contain one or two unsaturated C=C bonds.

in each cluster have a periodicity of 14 daltons and are likely to be formed from a homologous series of fatty acids, or fatty acid conjugates, produced by the breakup of the once intact bacteria as they enter the MS vacuum system (vide infra).

It can be seen in the expanded area between m/z 1380 and m/z 1485 of the ES^+ spectrum of *E. coli* HB101 (Fig. 1A, top insert), in addition to the 14 dalton periodicity between the highest peaks in the four most intense clusters (1394, 1408, 1422 and 1436), that as well as the overall peak shape being similar, more importantly the peaks within each of these clusters are separated by single dalton. This suggests that the majority of the ions that are being produced by the electrospray process are carrying a single positive charge. This was also observed in the ES^+ spectrum of *B. cereus* DSM31 (Fig. 1B, lower insert) in the expanded region from m/z 1270 to m/z 1570.

ESI-MS is a powerful method for the analysis of very large molecules and in particular this technique allows precise measurement of mass from protonated peptides and proteins carrying *multiple* charges [2,19]. It is noteworthy, that in the present study we did not seem to observe at the gross level any positively charged fragments with multiple charges as characteristic of proteins in the ES^+ spectra.

It can be seen that in the ES^- spectrum of *E. coli* (Fig. 2A) m/z 747 is one of the most abundant mass ions, and from studies on purified bacterial phospholipids [20] and on eukaryotic membranes [21] using LC-ESI-MS this can be assigned to the deprotonated molecular ion of phosphatidylglycerol. The same authors also found that m/z 255 and 281 were carboxylate anions from C16:0 and C18:1 fatty acyl chains created from the electrospray process on phospholipids. If one examines this cluster in more detail, fragments with masses of 227, 239, 241, 253, 267 and 295 are also observed, which from mass calculations presuming single charge (Fig. 3), may be assigned to the carboxylate anions from C14:0, C15:1, C15:0, C16:1, C17:1, and C19:1 fatty acyl side chains.

In both the ES^- spectra (Fig. 2), and others collected, a broad baseline 'hump' (see inserts) can be seen between m/z 1700 and m/z 2200. It is likely that these are from nucleic acids present in the cells. No defined peak shape was observed because (1) there

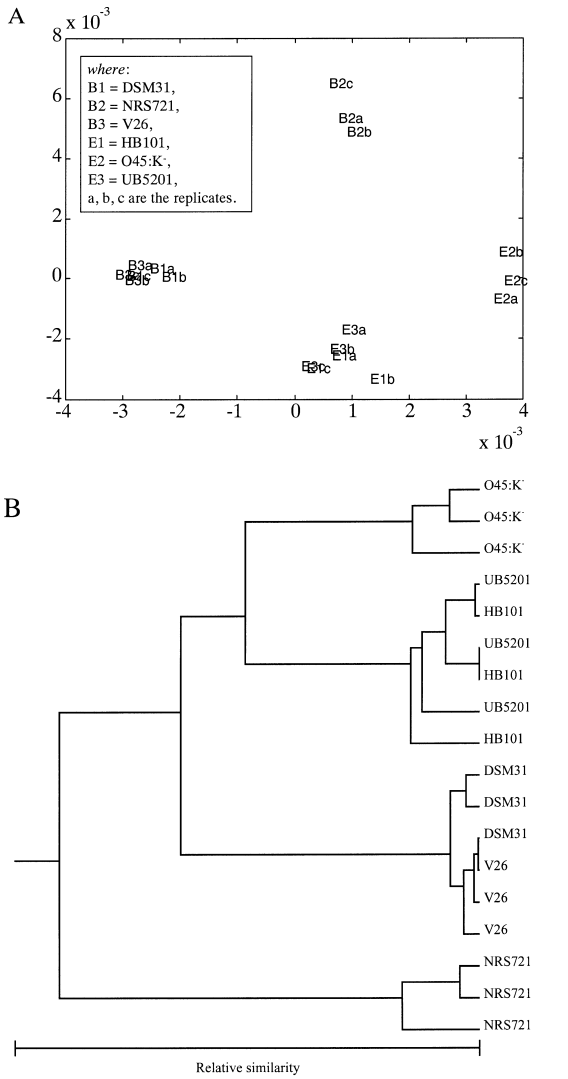


Fig. 4. Discriminant function analyses plot (A) and dendrogram (B) based on ESI-MS data collected in the positive ion mode showing the relationship between the three *B. cereus* and three *E. coli* strains.

are many 1000s of different RNA molecules (the complete genome sequence of *E. coli* K-12 revealed 4288 protein-coding genes [22]) which would result in spectral overlap and (2) nucleic acids have a high affinity to Na^+ and thus salt adducts, which are often difficult to resolve, would be formed during the electrospray process.

To assess both whether these spectra were reproducible and could be used to differentiate these bac-

teria, discriminant function and hierarchical cluster analyses were used to cluster ES^+ spectra and the resulting biplot and dendrogram are shown in Fig. 4. This figure demonstrates that ESI-MS can be used to differentiate between these Gram-negative and Gram-positive bacteria, and more importantly that discrimination of these microorganisms is easily possible to below species level. Moreover, this high level of differentiation is very similar to that observed in previous studies using pyrolysis MS to discriminate among strains of *E. coli* [23] and *B. cereus* [24].

In conclusion, rather than perform the tedious, labour intense sample preparation involved in the production of purified cell lysates, followed by their LC separation prior to MS analysis, the results we present provide very strong evidence that ESI-MS of intact bacteria is realistic. As expected the fragmentation varies with cone voltage, although voltages can be chosen which produce reproducible spectra. The spectra obtained from electrospray ionisation in both the positive and negative ion modes were very information rich, and several of the negative mass ion fragments could be assigned to specific molecular fragments from bacterial phospholipids. Cluster analyses of the ESI-MS spectra gave similar results compared with those groupings from a very different mass spectrometric whole-organism fingerprinting technique. We therefore conclude that ESI-MS presents itself as a powerful new approach for the characterisation of intact microorganisms.

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