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MALDI TOF MASS SPECTROMETRY CAN BE USED TO IDENTIFY CHANGES IN THE PROTEIN COMPOSITION OF RUMEN BACTERIA: THE RESPONSE OF THE NOVEL RUMINAL BACTERIUM LPLR3-7 WHEN GROWN IN THE PRESENCE OF DIAMINOPROPIONIC ACID

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SUMMARY

The hypothesis that matrix assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry can be used to identify changes in the protein composition of rumen bacteria was tested. We used MALDI TOF mass spectrometry to identify the proteins responsible for the ability to degrade diaminopropionic acid (DAPA), a non-protein amino acid found in the forage legumes *Lathyrus* spp. and *Acacia angustissima*, by a novel ruminal bacterial isolate, LPLR3-7. LPLR3-7 was grown on 3 different growth media containing either nutrient supplements (nutrient) or 10mM DAPA (DAPA) or both (nutrient/DAPA). A MALDI TOF mass spectrometer was used to determine the protein composition of whole cells, of cell-free extracts and of cell debris, from cells grown on each of the different treatment media. LPLR3-7 altered its protein composition when grown in the presence of DAPA. A peak cluster at 9700 Da in the cell free extract showed a direct relation to the presence and absence of DAPA. MALDI TOF mass spectrometry was successfully used to examine protein composition in ruminal bacteria. The process allowed identification of changes in protein composition in this ruminal bacterium in response to a toxin. However, the profiles produced for entire cell protein composition by the MALDITOF technique are complex and difficult to interpret and compare between samples.

Keywords: ruminal bacteria, MALDI TOF, diaminopropionic acid, *Acacia angustissima*

INTRODUCTION

Several non-protein amino acids including 2,3-diaminopropionic acid (DAPA); 4-N-oxalyl-2,4-diaminobutyric acid; diaminobutyric acid; 3-N-oxalyl-2,3-diaminopropionic acid; and 2-amino-6N-oxalylureidopropionic; and acetylated forms of some of these amino acids have been detected in the fodder legumes *Lathyrus* spp. and *Acacia angustissima* (Evans *et a*l. 1993; Rasmussen *et al*. 1992). These amino acids are potential toxins but toxicity will depend upon their concentration in the plant (Johnston, 1971). LPLR3-7 is a novel rumen bacterium that has shown the ability to degrade and utilise diaminopropionic acid (DAPA) as a carbon source (C. McSweeney, 2001, pers comm).

By analysing the proteins produced by LPLR3-7, it may be possible to identify proteins that are involved in degrading and utilising DAPA. A change in protein composition may be found in the cytoplasm of the cell, or among proteins attached to inner surfaces of the cellular membranes. Alternatively, a change in protein composition may be detectable on the outer surface of the cell, indicating that novel or altered proteins are secreted to the cell exterior or to the outer surface of the cell membranes. To determine the cellular location of a protein of interest, bacterial cells can be analysed as whole cells or as sub-cellular fractions of lysed cells. To detect a protein of interest, the protein composition of bacterial cells can be determined by using two dimensional gel electrophoresis. However, it may be possible to determine protein composition more quickly and accurately using the Matrix Assisted Laser Desorption Ionisation Time Of Flight Mass Spectrometer (MALDI TOF MS; Cain *et al*., 1994). The MALDI TOF MS has not been used previously to study rumen bacteria but it has been used for some time to examine the protein composition of microorganisms that are important in medicine (Leenders *et al*. 1999; Lynn *et al*. 1999; Gustafsson *et al*. 2001).

An investigation into the effect of DAPA on protein composition of LPLR3-7 was undertaken in this project. It was hypothesized that the MALDI TOF MS can be used to identify changes in the protein composition of rumen bacteria and provide an indication of the proteins responsible for LPLR3-7's ability to degrade DAPA.

MATERIALS AND METHODS

Experimental design

Isolate LPLR3-7 was grown anaerobically in three different treatment media. The treatment media contained the same basal medium and either a nutrient mix, a nutrient mix plus DAPA, or DAPA as the sole carbon source for growth. MALDI TOF mass spectrometry was used to determine the protein composition in different cellular locations in LPLR3-7 by examining whole cells, cell-free extracts and the cellular debris.

Bacterial strain and culture methods

The bacterial isolate, LPLR3-7 was isolated from the rumen digesta of sheep adapted to the presence of *A. angustissima* in their feed. The basal rumen fluid medium for culture and experimentation was prepared under anaerobic conditions using the methods and recipes from Hungate (1966) and Lowe *et al* (1985). DAPA was obtained from Sigma (Australia). LPLR3-7 cells were grown to late exponential phase in three treatment media; basal medium with added nutrients (nutrient), basal medium with added nutrients plus 10 mM DAPA (nutrient/DAPA) and basal medium with 10 mM DAPA (DAPA). The nutrient mix added contained 2 g/L of cellobiose, 2 g/L glucose, 1 g/L of trypticase and 1 g/L of yeast extract.

Protein estimation and sample preparation

The protein content of LPLR3-7 cultures from each growth condition was estimated using a BCA Protein estimation kit (Pierce, Rockford U.S.A) according to the manufacturer's guidelines for the test tube protocol. Cells from the three growth conditions were washed, resuspended in 2 ml sterile water and then split into 1ml samples to be analysed for their protein composition as whole cells, cell-free extract (sonicated & centrifuged) and cell debris (membranes and cell wall sediment from sonicated samples). Cell-free extract and cell debris samples were prepared by sonication of a 1ml sample from each growth condition using a Branson B-12 sonicator in a cycle of 15 sec sonication with a 15 sec rest, repeated four times. The samples were then centrifuged at 5000 x g for 5 minutes. The supernatant was removed as cell-free extract and the pellet of cell debris was resuspended in 1 ml sterile water.

MALDI TOF MS

The Voyager MALDI TOF MS (PerSeptive Biosystems, Framingham, MA) was used in linear positive ion mode with a nitrogen laser at a wavelength of 337 nm. Protein standards for MALDI TOF MS were: bovine serum albumin, chicken ovalbumin, lysozyme and aprotinin, (Sigma, Australia), and were prepared at a concentration of 50 pM. Sinapinic acid (3-5 dimethoxy-4 hydroxycinnamic acid; Sigma, Australia) was the matrix used. LPLR3-7 samples were prepared by the sandwich method (Galvani *et al*, 2001). The amount of sample used for MS analysis from each bacterial growth condition was adjusted to standardise the total protein content, according to the results of protein estimations. For each sample, including the standards, 14 spectra were accumulated from 7 replicates. Spectra were acquired in two ranges: 4000 – 20 000 Da and 10 000 - 80 000 Da. The baseline of each spectrum was corrected and the background noise removed using the Data Explorer program in the MALDI TOF MS software (PerSeptive Biosystems, Framingham, MA).

RESULTS

MALDI TOF MS and protein composition of LPLR3-7

The MALDI TOF MS produced spectra that indicated a change in protein composition of LPLR3-7 when it was grown in the presence of DAPA. Protein spectra varied between cell fraction and between the different growth media. However, there were strong similarities in the whole cells and cell-free extract from the nutrient and nutrient/DAPA media. There was also a strong similarity between the cell debris spectra for nutrient/DAPA and DAPA media. Some protein peaks were present in all samples from all growth media. For example, peaks at 5500 Da and at 7200 Da were present in all samples (Figure 1). In cell-free extracts, the samples from cells grown in DAPA medium showed a protein peak at 67 000 Da that was absent in other samples (data not shown). In addition, there was a cluster of peaks around 9700 Da that was particularly responsive to the presence or absence of DAPA. The peak at 9700 Da in particular showed increased relative concentration in nutrient/DAPA medium, rising markedly above the surrounding peaks. This peak was the only peak from a small cluster between 6000 – 10 000 Da that remained obvious in the cell-free extract from cells grown in DAPA. In general, the samples from the DAPA medium showed a reduced number of protein peaks in the

spectrum, compared with those from cultures where the nutrient mix was included. The differences observed were more obvious in the 4000 to 20 000 Da molecular mass range than in the 10 000 to 80 000 Da protein range. Figure 1 is an example of the output from the MALDI TOF MS, showing protein spectra for cell-free extract fractions of LPLR3-7 grown on the three experimental media.

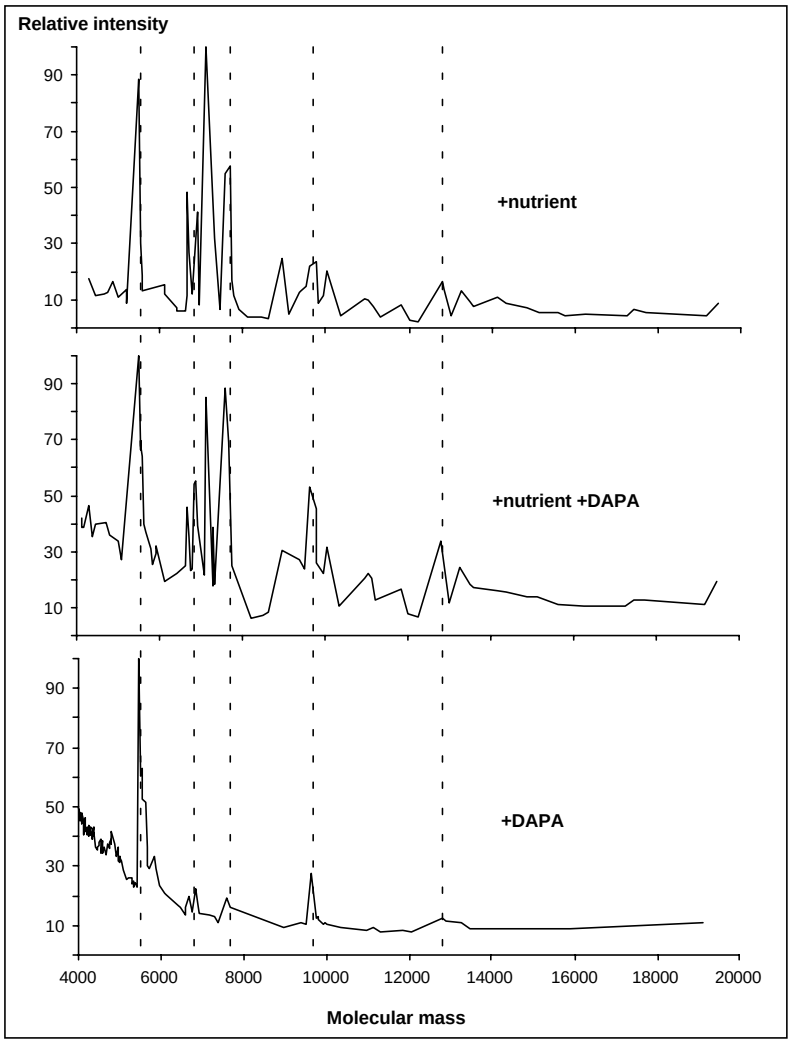

Figure 1. Protein spectra from cell-free extract of LPLR3-7 grown in nutrient, nutrient/DAPA and DAPA media, in the 4000 Da to 20 000 Da range. The solid and dotted lines indicate matching peaks between samples. The solid line indicates a peak at 9700 Da, which showed increased relative concentration in nutrient/DAPA medium compared to the surrounding peaks.

Protein estimation

Protein concentration varied according to the growth media. Samples taken from bacteria grown in DAPA medium had the lowest protein concentrations. The estimates of protein content were used to ensure samples of approximately equal protein content were used for analysis from each growth condition.

DISCUSSION

The MALDI TOF MS identified changes in protein composition in the novel ruminal bacterium LPLR3-7. By using the MALDI TOF MS it was possible to show that the protein composition of LPLR3-7 cells varied when the cells were grown in the presence and absence of DAPA. This supports the hypothesis that protein composition of the LPLR3-7 cells would change when DAPA was present in the medium. Complete reliance on DAPA as a nutrient source (basal medium + DAPA) appeared to reduce the intensity of many proteins in the LPLR3-7 cells and the overall total protein content of the culture. Thus changes in protein composition of LPLR3-7 grown in the DAPA medium were most probably a result of the combination of nutritional deficiency in the medium as well as the effect of DAPA.

The spectra obtained from the MALDI TOF MS indicated that the presence of 10 mM DAPA in the growth media induced a change in protein composition. However, it is possible that the exact same number of cells were not present in all of the samples. Therefore, the variations in protein intensity between samples need to be compared with caution. However, within samples the protein intensity relative to others can be compared. The intensity of the protein peak at 9700 Da (Figure 1), compared to the two peaks directly adjacent to it, was altered greatly (approximately doubled in height) when DAPA was included in the medium. It is notable that this was the only peak of the cluster that remained present when cellular proteins were depleted by reliance upon DAPA as an organic carbon source. Therefore, this protein peak may have an important role in the response of LPLR3-7 to DAPA.

The MALDI TOF MS can be used as a fast and simple method of obtaining a general protein fingerprint of bacteria. The limited knowledge of protein content in isolate LPLR3-7 makes it difficult to interpret the spectra, particularly so because of the reduced accuracy of protein peaks over large size ranges. This suggests that future analyses of LPLR3-7 should assess a number of narrower protein ranges. With optimization, this technique could provide a rapid way of identifying changes in protein composition and could help to focus more complex studies that use two dimensional gel electrophoretic analyses of proteins.

An exciting result of this study was the successful application of proteomics in the assessment of a bacterial response to a non-protein amino acid. The use of the MALDI TOF MS to determine differences in protein composition of LPLR3-7, in response to DAPA, introduced a novel technique for rapid identification of the responses of rumen bacteria to their surrounds.

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