Identification of Diaminopimelic Acid in the Legionnaires Disease Bacterium

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Diaminopimelic acid was found to be a component of the cell wall of the Legionnaires disease bacterium, thus providing additional evidence that the organism is a bacterium. The presence of this amino acid was determined by gas-liquid chromatography and confirmed by gas chromatography-mass spectrometry.

There has been considerable interest in the characterization and classification of the Legionnaires disease bacterium (LDB) Legionella pneumophila (1) since it was isolated in 1976 (8) as the causative agent in outbreaks of upper respiratory disease. Although some of the difficulties in culturing and identifying LDB have been overcome (2, 4, 13), there is much interest in determining the chemical composition of the bacterium, since this information is useful for taxonomic and identification purposes. Determination of the cellular fatty acid composition of LDB by Moss et al. (11) showed that its profile was different from other gram-negative bacteria and could be used to confirm the identity of isolates of this organism.

At the International Symposium on Legionnaires’ Disease held in Atlanta, Ga., on 13 to 15 November 1978, Finnerty et al. (5) reported additional studies of the cellular lipids of LDB. Progress in the determination of amino acids and sugars present in the peptidoglycan of the Philadelphia 1 strain of LDB was reported by Keel et al. (7). These authors reported the presence of alanine-glutamic acid and muramic acid-glucosamine in molar ratios of 2:1 and 1:1, respectively. They also reported high concentrations of lysine, but no diaminopimelic acid (DAP) was detected. DAP is of interest because it is a unique amino acid of the bacterial cell wall and is largely restricted to certain bacteria and blue-green algae (cyanobacteria) (14, 17). It can also serve as an intermediate in the synthesis of lysine, and both DAP and lysine are known to coexist in the cell walls of several gram-negative bacteria (17).

In this report, the gas-liquid chromatographic determination of DAP in seven strains of LDB and confirmation of this amino acid by gas chromatography-mass spectrometry are described. DAP and lysine were determined, and the ratio of these two amino acids was calculated to show the change in molar distribution between the whole cell and cell wall fraction.

MATERIALS AND METHODS

The strains of LDB examined were Pontiac 1, Knoxville 1, Philadelphia 2, 3, and 4, Flint 1, and Bellingham 1. The cultures were obtained from the stock culture collection of the Analytical Bacteriology Branch at the Center for Disease Control. They were maintained on charcoal-yeast extract (4) agar slants and transferred at biweekly intervals. Growth from a 48-h charcoal-yeast extract slant was removed from the agar with a sterile cotton swab and used to inoculate the surface of three charcoal-yeast extract plates. The plates were incubated in a candle extinction jar at 35°C for 48 h. The cells were removed from the agar surface with sterile distilled water, centrifuged, washed one time, and frozen at −20°C until processed.

Samples of fresh or frozen whole cells were hydrolyzed with 6 N HCl in a nitrogen atmosphere at 105°C for 16 h. The hydrolysate was extracted with an equal volume of diethyl ether-hexane (1:1, vol/vol), filtered, and evaporated under nitrogen in a steam jacket to remove excess HCl. The residue containing the amino acids was washed twice with distilled water to further reduce the HCl concentration. The amino acids were converted to the N-heptafluorobutyryl-n-propyl ester derivatives by a procedure similar to that described previously (9, 10). In the present study, however, the propylation step was repeated a second time.

Cell wall fractions were isolated by a method which is essentially that described by Schleifer and Kandler (15), except we used a 16-h period instead of a 2-h period of trypsinization (12). Samples of fresh or frozen cells were suspended in 2 ml of 10% trichloroacetic acid and heated at 100°C for 20 min. The samples were centrifuged and washed twice with distilled water. The residue was mixed with 0.5 ml of trypsin (0.2 mg/ml) in a 0.1 M phosphate buffer, pH 7.9, and incubated at 37°C on a rotary shaker for 16 h. The solution was checked for clarity and centrifuged at 27,000 × g for 30 min; the sedimented cell wall fractions were washed twice with distilled water. The cell
wall residue was hydrolyzed, processed, and derivatized in the same manner as the whole cells.

The N-heptafluorobutyryl-n-propyl ester derivatives were analyzed on a model 990 gas chromatograph (The Perkin-Elmer Corp., Norwalk, Conn.) equipped with a flame ionization detector and a recorder with a disk integrator. The injection port temperature was 235°C, and the detector temperature was 280°C. Samples were analyzed on a glass column (3.7 m by 4-mm inside diameter) packed with 3% OV-101 on 100/120-mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pa.). The temperature used for the separation was 130°C for 6 min, followed by a temperature increase of 6°C/min to 270°C; the nitrogen carrier gas flow was 45 ml/min. The amino acids in the samples were identified by comparing retention times with those of highly purified amino acid standards (Beckman Instruments, Inc., Fullerton, Calif., or Sigma Chemical Co., St. Louis, Mo.). Identities were confirmed by analysis on a 15% Dextral gas-liquid chromatographic column and also by gas chromatography-mass spectrometry. The relative concentrations of each amino acid to the total amino acids were calculated by using response factors and peak areas or peak heights. The concentrations on a weight basis were determined by adding known amounts of 2,4-diaminobutyric acid as an internal standard to the samples.

The gas chromatography-mass spectrometry instrument used for confirming the identity of the amino acid derivatives was a model 21-491B (Du Pont Co., Wilmington, Del.) equipped for both electron impact ionization and chemical ionization. The reagent gas for chemical ionization was isobutane. The mass spectrometer was coupled to a Varian model 2700 gas-liquid chromatograph through an all-glass system with a jet separator. A glass column (2 m by 2-mm inside diameter) of 3% OV-101 on 100/120-mesh Gas-Chrom Q was used and programmed from 100°C to 275°C at 8°C/min for separation of amino acids for mass spectrometry.

RESULTS AND DISCUSSION

The amino acids detected in the whole cells were the same as those usually found in other bacteria; in addition, DAP was present. Tryptophan was not detected, since this amino acid is destroyed during acid hydrolysis. The whole cells and the cell wall fractions had the same qualitative amino acid composition, which helps to confirm earlier data that LDB is a gram-negative bacterium (8, 14, 16). When the quantitative amino acid results were compared, the most striking changes were observed in the content of DAP and lysine. Table 1 summarizes the amounts of these two amino acids expressed as the molar percentage of total amino acids and also as the molar ratio of DAP to lysine. In most instances, the values are the average results of several determinations.

The average molar percentage of DAP increased from 1.5 in the whole cells to 5.1 in the cell wall fractions, whereas the average molar percentage of lysine decreased from 7.0 in the whole cells to 3.3 in the cell wall fractions. The ratio of DAP to lysine in the whole cells was 0.2, and that in the cell wall fraction was 1.5, a sevenfold increase in molar ratio. This marked increase strongly indicates that DAP is an integral component of the cell wall.

The amounts of DAP and lysine in the whole cells of the Knoxville strain were also determined on a dry weight basis. The whole cells contained 0.23% (by weight; 0.012 μmol/mg) DAP and 2.9% (by weight; 0.20 μmol/mg) lysine. In addition, the isomers of DAP in acid hydrolysates of whole cells of the Knoxville strain of LDB were separated by one-dimensional paper chromatography, meso-DAP or DD-DAP or both were present; Ll-DAP was absent.

Several methods for isolating the cell wall fractions were evaluated, and trypsin digestion was used because of its simplicity, safety, and frequency of use in other studies (12, 14, 15). Park and Hancock (12) have reported that the cell wall mucopolypeptides recovered after trypsin digestion are contaminated with less than 5% of the cytoplasmic proteins. In addition, neither muramic acid nor DAP is released during trypsin digestion (14); however, the digestion conditions must be adjusted to the specific organism and are usually assessed on the basis of the disappearance of turbidity in the trypsinsized sample (12). In our study, the LDB suspension had minimum turbidity after approximately 16 h; at this time, the maximum molar ratio of DAP to lysine was reached.

The concentrations of DAP found in the cell wall fractions of LDB agree with the results reported by others for gram-negative bacteria. Salton (14) tabulated the amount of DAP present in the cell walls of Aerobacter cloaca as 0.20 μmol, that in Pseudomonas aeruginosa as 3.2 g/100 g, and that in Salmonella bethesda as

<table>
<thead>
<tr>
<th>Strains</th>
<th>Whole cells</th>
<th>Cell wall fractions</th>
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<tbody>
<tr>
<td></td>
<td>DAP</td>
<td>Ly-sine</td>
</tr>
<tr>
<td>Pontiac 1</td>
<td>2.4</td>
<td>7.1</td>
</tr>
<tr>
<td>Knoxvile 1</td>
<td>0.84</td>
<td>7.0</td>
</tr>
<tr>
<td>Philadelphia 2</td>
<td>1.5</td>
<td>7.5</td>
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<td>Bellingham 1</td>
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<td>6.7</td>
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<tr>
<td>Philadelphia 4</td>
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<td>—</td>
</tr>
<tr>
<td>Avg</td>
<td>1.5</td>
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* — Not determined.
2.1 g/100 g. These values correspond to 3.7, 5.6, and 4.7 molar percent, respectively. Much higher concentrations of DAP were found in the cell walls of gram-positive bacteria. The values reported by Salton (14) for Lactobacillus casei, by Kato et al. (6) for Corynebacterium diphteriae, and by Cummins et al. (3) for Mycobacterium lepraeum correspond to 19.4, 9.1, and 12.9 molar percent, respectively.

DAP and lysine were well separated from each other and from other amino acids by gas-liquid chromatography, and their identities were confirmed by gas chromatography-mass spectrometry in each of the seven strains. The mass spectra of a reference standard of DAP obtained by chemical and electron impact ionizations are shown in Fig. 1. In the chemical ionization mode, the major peak found at m/e 667 represented the protonated molecular ion (M + H\(^+\)) of the DAP derivative. The mass fragments of DAP that correspond to the primary peaks obtained by electron impact ionization are tabulated in Table 2. The presence of the other amino acids was also confirmed by mass spectrometry.

DAP was found in all seven strains of LDB, with significantly higher concentrations present in the cell wall fraction than in whole cells. This indicates that DAP is concentrated in the LDB cell wall as a structural component and thus provides additional evidence that the causative agent of Legionnaires disease is a gram-negative bacterium.

The gas-liquid chromatographic method described for amino acid analysis of whole cells and cellular components is relatively simple and clear-cut. After the techniques were optimized, reliable and reproducible results were obtained. The method should be useful for rapid analysis of cell wall amino acids in other microorganisms.

### ACKNOWLEDGMENTS

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### LITERATURE CITED


