Modified Ammonia Electrode Method to Investigate D-Asparagine Breakdown by Campylobacter Strains

MOHAMED A. KARMALI,* MARGARET ROSCOE, AND PETER C. FLEMING

Department of Bacteriology and the Research Institute, The Hospital for Sick Children, Toronto, Ontario MSG 1X8, and the Department of Microbiology, University of Toronto, Toronto, Ontario, Canada

Received 1 July 1985/Accepted 11 December 1985

An ammonia electrode method has been developed for investigating the deamination of amino acids by bacteria. It consists of incubating a standard inoculum of organisms in an amino acid solution and then measuring the amount of ammonia evolved by the electrode. Two hundred and twelve Campylobacter strains (118 C. jejuni and 94 C. coli) were tested for their ability to break down D-asparagine by this method. Organism control (bacterial suspension in buffer alone) values ranged from 0.44 to 2.0 (mean 0.93 ± 0.24) ammonia concentration (AC) units (one AC unit is equal to 10⁻³ mol of ammonia per liter), whereas test values ranged from 0.60 to 46.0 units. Test ACs of <2 units (97 strains) were considered negative, whereas ACs of ≥10 (77 strains) were considered positive for D-asparaginase; 38 (18%) strains with ACs between 2 and 10 units were provisionally assigned an intermediate status. The amount of ammonia produced by strains with ACs of ≥10 increased greatly when the inoculum size was increased, whereas this was not a feature of strains with ACs of <2 units. The presence or absence of an inoculum effect was instrumental in classifying strains with intermediate ACs and allowed a breakpoint to be defined. When the ammonia electrode method was repeated, 97.6% of the 212 strains gave the same positive or negative reaction that they did on the first occasion. Thus the test was highly reproducible. Five strains (all porcine C. coli from Germany) were unclassifiable because they repeatedly gave either a weak-positive or negative reaction. Overall, 12.7% of C. jejuni strains and 86.2% of C. coli strains were positive for D-asparaginase. The ammonia electrode method was found to be simple and reliable for separating strains on the basis of D-asparaginase activity.

The number of proposed taxons within the genus Campylobacter has increased dramatically (2, 5, 8–10, 12, 13) ever since the first comprehensive taxonomic study on this subject was published in 1973 (14). A major problem in classifying and separating them has been the lack of a sufficient number of reliable phenotypic markers; this is because campylobacters do not utilize carbohydrates and are inert in most conventional biochemical tests (2,12). Campylobacters use amino acids as major nutritional substrates (11). We have argued that heterogeneity among species and strains might be reflected in their ability to break down different amino acids (3), but suitable methods for investigating this in a systematic manner are not available. To overcome this, we have developed a method with an ammonia electrode (AE) that allows strains to be tested for their ability to deaminate a wide range of amino acids and amides. A preliminary method (3) provided clear cut evidence of strain discrimination with D-asparagine as a substrate, but had a number of limitations including the lack of a properly defined breakpoint; this made it difficult to classify several strains which gave intermediate values. In this report, a modified AE method is described in which the earlier developmental problems have been overcome.

MATERIALS AND METHODS

Bacterial strains. A total of 212 strains from our collection, comprising 118 Campylobacter jejuni and 94 C. coli, were studied. All of the C. jejuni strains were clinical isolates from patients with diarrhea who came to our hospital. The C. coli strains comprised 19 clinical isolates from cases in our hospital; 69 isolates (15 human, 14 animal, and 40 of unknown source) from C. Adenyi Jones, R. M. Bannatyne, J. P. Butzler, J. M. S. Dixon, T. Itoh, R. Kaplan, T. Kosunen, W. D. Lears, N. Leuchtefeld, J. F. Prescott, H. Richardson, M. B. Skirrow, V. Stich Gröth, M. Tischler, S. Toma, R. Vanhoof, and J. L. Whitby; and 6 reference strains (7077, 7078, 7079, 7080, 7081, and 715) from the Collection Institut Pasteur. The strains were characterized according to previously reported criteria (2). C. jejuni was differentiated from C. coli by the rapid hippurate hydrolysis method of Hwang and Ederer (1) according to the guidelines suggested by Lior (4). Strains MK87 (D-asparaginase-positive C. coli), and MK463 (D-asparaginase-negative C. jejuni) were selected as controls and run with each new batch of strains tested.

AE method. D-Asparagine was made up as a 0.001 M solution in sodium phosphate buffer (pH 7.2, 0.01 M) and distributed in 24-ml volumes into 50-ml glass jars equipped with air-tight diaphragm-lined screw caps through which a syringe needle could be introduced. Bacterial strains were subcultured onto blood agar plates consisting of Columbia blood agar base (GIBCO Laboratories, Madison, Wis.) with 5% defibrinated horse blood. These cultures were incubated for 48 h at 37°C under reduced oxygen tension and then suspended in buffer to a density equivalent to that of a McFarland turbidity standard of 10. A 1-ml sample of each suspension was then added to the 24 ml of D-asparagine solution. The jars were capped tightly and incubated in a water bath at 37°C for 16 h. The following controls were similarly incubated: (i) organism controls (1 ml of bacterial suspension of each test strain in 24 ml of buffer alone without D-asparagine) and (ii) amino acid control (25 ml of 0.001 M D-asparagine solution in buffer without bacterial suspension).

After the 16-h incubation period, the jars were removed from the water bath and placed on the bench for up to 1 h to equilibrate approximately to room temperature. Hydrochlo-
ric acid (1 M) was added to each bottle via the screw-cap diaphragm (to maintain ammonia in solution as ammonium ion). The AE used was model 95-10 (Orion Research Inc., Cambridge, Mass.); this was calibrated, maintained, and operated according to guidelines set forth in an accompanying instruction manual. To test for ammonia, the screw cap was removed, and the jar was placed on a magnetic stirrer. The AE was introduced into the jar together with a temperature compensation probe (series 700 “Thermiliner” temperature probe; Yellow Springs Instrument Co., Inc., Yellow Springs, Ohlo); both the AE and temperature compensation probe were connected to a millivolt meter (Accumet pH meter, model 815 MP; Fisher Scientific Co., Pittsburgh, Pa.). With the magnetic stirrer switched on, 0.25 ml of 10 M sodium hydroxide (which converts dissolved ammonium ion into ammonia gas) was added to the test jar, and the millivolt reading was recorded when it stabilized.

This reading was converted to ammonia concentration by using a standard curve, which had been determined before the test by using a series of ammonium chloride standards as described previously (3); in contrast to our previous study where 10 ammonium chloride standards (concentrations ranging from 10⁻⁶ to 10⁻² M) were used, the present study included the seven standards with the following ammonium chloride concentrations: 6 × 10⁻⁶, 1 × 10⁻⁵, 3 × 10⁻⁵, 5 × 10⁻⁵, 1 × 10⁻⁴, 1 × 10⁻³, and 1 × 10⁻². The ammonia concentration (AC) produced by each strain and control was recorded in AC units (ACU); 1 ACU is equal to 10⁻⁴ mol of ammonia per liter. The AE was thoroughly rinsed in distilled water after each individual test. All 212 strains and controls were tested for AC on two separate occasions.

Effect of inoculum size on ammonia production. The effect of inoculum size on ammonia production was evaluated by using the D-asparaginase-positive and -negative control strains MK87 and MK463, respectively. Each strain was tested in triplicate for ammonia production by using as the inoculum 0.5, 1.0, 2.0, and 4.0 ml of the bacterial suspension, which was added to the D-asparaginase solution to make up a final volume of 25 ml. Subsequently, the inoculum effect of various individual test strains was investigated by using inoculum sizes of 0.5 and 2.0 ml. The inoculum size ratio (IR) for the test strains was calculated as the ratio between the ACU generated with the 2.0-ml inoculum and that generated with the 0.5-ml inoculum.

Viable counts of inocula. To test the possibility that D-asparaginase-positive and -negative reactions were due to differences in viable counts between the positive and negative cultures, viable counts of four representative culture suspensions were performed in triplicate by the method of Miles et al. (6). The same suspensions were then tested for ammonia concentration. The cultures comprised one each of D-asparaginase-positive C. jejuni, D-asparaginase-negative C. jejuni, D-asparaginase-positive C. coli, and D-asparaginase-negative C. coli.

Influence of basal media on D-asparaginase activity. To test the influence of different basal media on the D-asparaginase activity, representative strains were tested with four different media. The strains used comprised one strain each of D-asparaginase-positive C. jejuni, D-asparaginase-negative C. jejuni, D-asparaginase-positive C. coli, and a weak-positive C. coli. The basal media used comprised Columbia agar (GIBCO), Mueller-Hinton agar (GIBCO), Diagnostic Sensitivity Test agar (Oxoid Ltd., Basingstoke, England), and Columbia agar (Difco Laboratories, Detroit, Mich.). All basal media were supplemented with 5% defibrinated horse blood.

Inducibility of D-asparaginase activity. Representative cultures were incubated on Columbia agar (Gibco) supplemented with 5% defibrinated horse blood and in parallel on the same medium containing D-asparagine (final concentration, 0.001 M) and tested for D-asparaginase activity. The strains used comprised one strain each of D-asparaginase-positive C. jejuni, D-asparaginase-negative C. jejuni, D-asparaginase-positive C. coli, D-asparaginase-negative C. coli, and a weak-positive C. coli.

RESULTS

Distribution of ACs of test strains and controls. The ACs of the amino acid control (tested on 30 separate occasions) ranged from 0.41 to 1.37 ACU (mean 0.73 ± 0.20 ACU). The ACs of the 212 organism controls ranged from 0.44 to 2.0 ACU (mean 0.93 ± 0.24 ACU). The test ACs of these organisms ranged from 0.60 to 46.0. For the purposes of analysis, the distribution of the ACs was categorized into 10 groups of concentration ranges as follows: 0 to 1.99, 2.0 to 2.99, 3.0 to 3.99, 4.0 to 4.99, . . . , 9.0 to 9.99, and ≥10 ACU. The distribution of the test ACs (of both C. jejuni and C. coli) into these various categories is shown in Fig. 1. Strains having ACs in the 0- to 1.99-ACU range were considered negative since this range incorporated the organism control values, whereas ACs of ≥10 ACU were considered positive for D-asparagine breakdown; strains in the range of 2.0 to 9.99 ACU were provisionally assigned an intermediate status pending further characterization of the positive and negative groups with the inoculum test.

Inoculum size effect. The effect of inoculum size on the amount of ammonia produced by the control strains is shown in Fig. 2. A marked inoculum effect was seen with the positive control strain (MK87), but not with either the negative control strain (MK463) or the organism controls.

The IR of 50 strains randomly selected from 97 which had an AC of <2.0 ACU ranged from 1.41 to 2.83 (mean, 2.12 ± 0.32; median, 2.10). The IRs of 54 strains randomly selected from 77 with ACs of ≥10 ACU ranged from 3.30 to 4.12 (mean, 3.67 ± 0.21; median, 3.66). Both sets of values were normally distributed in a bimodal fashion with no overlap; the difference between the two groups was highly significant (P < 0.001). Based on these results the following guidelines were formulated for negative and positive IRs: a strain was considered to be negative for inoculum size effect if its IR was ≤2.76 (mean negative IR ± 2 standard deviations); it was considered to be positive if its IR was ≥3.25 (mean
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Characterization of strains provisionally assigned an intermediate status. All strains that were provisionally assigned an intermediate status (i.e., strains with ACs in the range of 2.0 to 9.99) were tested for the inoculum size effect by using the guidelines indicated above for interpreting the IRs. All strains with ACs between 5.0 and 9.99 ACU (18 strains) gave a positive inoculum effect and were thus considered positive for D-asparaginase breakdown. All 13 strains in the 2.0- to 2.99-ACU range gave negative IRs and were therefore considered negative for D-asparaginase. The seven strains in the 3.0- to 4.99-ACU range gave variable IRs (Table 1) and were thus assigned, provisionally, a "weak-positive" status.

Definition of the breakpoint. Strains with ACs of <3.0 and ≥5.0 ACU were clearly associated with negative and positive inoculum responses, respectively; consequently, these values were chosen as the cutoff points for the test. The seven weak-positive strains with ACs in the range 3.01 to 5.0 ACU were considered positive if they gave a positive IR, negative if they gave a negative IR, and unclassifiable (or variable) if they had a borderline IR.

Reproducibility of results and variability in ACs of individual test strains and organism controls. The reproducibility of the IR values was tested using the guidelines indicated above (Table 1). All 205 strains considered to be positive or negative on the first test retained their original status on repeat testing. Of the seven strains (3.3% of total) in the weak-positive group, two retained a positive status (≥5.0 ACU or weak-positive with positive IR), and two retained a negative status (<3.0 ACU or weak-positive with negative IR); of the three strains that were unclassifiable on the first occasion, one remained unclassifiable, one became negative, and the other became positive. Overall, 209 (98.6%) retained the same positive or negative status on repeat testing using both the AC and IR criteria.

The variability in ACs of individual strains (test and organism control) is shown in Table 2. Organism control values on repeat testing were very similar to those obtained on the first test; the AC values of individual strains varied by a mean of only 0.26 ACU on the two separate occasions. Test strains with values in the negative AC range (0 to 2.99) on the first occasion remained in the same range on repeat testing; the ACs of individual strains in this range varied by a mean of 0.47 ACU on the two separate occasions. A progressive increase in variability from first and second test values was seen with increasing ACs in the positive range.

The reproducibility of the inoculum test was assessed by repeating it on 12 randomly selected strains that previously gave a positive IR and 12 that gave a negative IR. All 24 strains fell into the same positive or negative range of IR on repeat testing.

Further information on intrastain and intracontrol variability in values was obtained by analyzing the AC and IR data pertaining to the amino acid control and the positive (MK87) and negative (MK463) test controls (Table 3). The range and mean values of the organism controls of both strains MK87 and MK463 showed striking similarities to those obtained for all 212 test strains on two separate occasions. Both strains consistently retained their respective positive or negative status when tested on 30 separate occasions. Similarly, their IRs were also consistently positive or negative.

Distribution of D-asparaginase-positive and -negative biotypes within species. The distribution of D-asparaginase activity in C. jejuni and C. coli was as follows: for C. jejuni, 15 (12.7%) D-asparaginase-positive isolates and 103 (87.3%) D-asparaginase-negative isolates; for C. coli, 81 (86.2%) D-asparaginase-positive isolates, 9 (9.5%) D-asparaginase-negative isolates, and 5 (5.3%) variable isolates. A variable category has been added for five initially weak-positive C. coli strains (indicated in Table 1) which in practice, when

TABLE 1. Reproducibility of the AE method in separating Campylobacter strains into D-asparaginase-positive and -negative groups

<table>
<thead>
<tr>
<th>Classification on first test</th>
<th>No. of strains</th>
<th>No. of strains in each category on repeat testing/final status (+, −, or v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>95</td>
<td>92 (+) 3 (+) 3 (+)</td>
</tr>
<tr>
<td>Weak positive (+ IR)</td>
<td>2</td>
<td>1 (+) 1 (+) 1 (v)</td>
</tr>
<tr>
<td>Weak positive (± IR)</td>
<td>3</td>
<td>1 (v) 1 (v) 1 (v)</td>
</tr>
<tr>
<td>Negative</td>
<td>110</td>
<td>110 (−)</td>
</tr>
</tbody>
</table>

a AC, ≥5.0 ACU.
b AC, 3.0 to 4.99 ACU; + IR, ≥3.25; − IR, ≥2.76; ± (borderline) IR, 2.77 to 3.24.
c AC, <3.0 ACU.
d A group of C. coli strains originating from pigs in Germany (from V. Stich-Groß, all belonging to Penner serotype 48 (1), could not be properly classified because on several repeat tests they gave either a weak-positive or negative reaction. These strains were classified as variable (v).
tested on several occasions, usually gave ACs in the 3.0- to 4.99-ACU (weak-positive) range, but had variable IRs.

**Viable counts of inocula.** The D-asparaginase-positive and -negative C. jejuni and C. coli cultures all gave equivalent mean viable counts ranging from $1.6 \times 10^8$ to $2.2 \times 10^9$ organisms per ml.

**Influence of basal media on D-asparaginase activity.** The D-asparaginase-positive strains of C. jejuni and C. coli both gave clear-cut positive ACs, the D-asparaginase-negative strains of C. jejuni and C. coli both gave clearly negative ACs, and the weak-positive C. coli strain gave a weak-positive AC after growth on the four different media.

**Inducibility of D-asparaginase activity.** The D-asparaginase-negative and -positive strains of C. jejuni and C. coli and the weak-positive C. coli strain all maintained the same status after growth on Columbia blood agar and the same medium supplemented with D-asparaginase.

**DISCUSSION**

The AE method described here was found to be simple and reliable for separating *Campylobacter* strains on the basis of D-asparaginase activity. Specific developmental problems reported in an earlier preliminary method (3) were overcome; notably (i) a defined breakpoint was established, and (ii) the reproducibility of the method was improved for separating D-asparaginase-positive and -negative subtypes.

The definition of an effective breakpoint for separating positive and negative values became possible by observing that strains with high ACs gave a significant inoculum effect (Fig. 2), whereas those with low ACs did not. The presence or absence of an inoculum effect allowed strains giving intermediate ACs to be classified into positive or negative for D-asparaginase. The guidelines, based on the results of this report, that are currently used for classifying strains are as follows: ACs of less than 3.0 ACU are considered negative, whereas those $\geq 5.0$ ACU are considered positive for D-asparagine breakdown; strains with values in the 3.0- to 5.0-ACU range are classified as positive when they give a positive inoculum ratio $\geq 3.25$ and negative when they give a negative one $\leq 2.76$. These criteria allowed 97.6% of the strains to be reproducibly classified into positive or negative for D-asparaginase (Table 1). It was of interest that the five strains which could not be reliably classified all came from the same source (porcine isolates of *C. coli* from Sticht Gröh, West Germany), and all belonged to the same Penner serotype (type 48) (7; unpublished data). It is likely that these strains represent a homogeneous group whose status may become clearer with the use of additional amino acids in the modified AE method.

There was variability in the ACs of individual strains and organism controls tested on two separate occasions. The interstrain and intercontrol variability (Table 2) was paralleled by the intrastrain and intracontrol variability shown in Table 3. This is probably a reflection of (i) the inherent limits of accuracy of this method and (ii) minor variations in culture conditions and inoculum size of cultures tested on separate occasions. However, despite this variability, the ACs of test strains always fell into the appropriate positive or negative range, indicating that the method is reliable for separating strains into D-asparaginase-positive and -negative subtypes. It was shown that positive and negative ACs were not a reflection of the viable count of the inoculum.

As reported in our earlier study (3), there was a striking difference in the distribution of D-asparaginase activity in the two species studied. A majority of *C. jejuni* isolates (87.3%) were negative for D-asparaginase, whereas a majority of *C. coli* isolates (86.2%) were positive. The taxonomic implications of this remain to be established, as does the value of the D-asparaginase reaction in biotyping *C. jejuni* and *C. coli*.

The AE method involves the incubation of a bacterial culture in a single substrate (an approach that is being increasingly exploited in clinical microbiology) and is independent of specific growth constituents and conditions. In the absence of growth, substrate breakdown must be due to preformed enzyme. In a few representative cultures that were tested, enzyme activity was not inducible. However, complex basal media were used for growing test cultures, and such media (containing a variety of undefined peptones, peptides, and amino acids) may not be optimal for testing inducibility of specific enzymes. Of practical importance, our findings indicated that the type of complex basal medium used did not influence the D-asparaginase reaction.

The AE method offers the possibility of investigating the breakdown of any amino acid or amide by bacteria and provides a practical approach for separating species and strains not only in the genus *Campylobacter*, but also in

### TABLE 2. Variability in AC produced by individual test strains and controls when tested on two separate occasions

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of strains</th>
<th>First test</th>
<th></th>
<th>Second test</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
<td>Mean ± SD</td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Controls</td>
<td>212</td>
<td>0.44–2.0</td>
<td>0.93 ± 0.24</td>
<td>0.41–2.1</td>
<td>1.0 ± 0.26</td>
</tr>
<tr>
<td>Negative (0 to 2.99 ACU)</td>
<td>110</td>
<td>0.60–2.90</td>
<td>1.37 ± 0.48</td>
<td>0.84–2.70</td>
<td>1.48 ± 0.37</td>
</tr>
<tr>
<td>Weak positive (3.0 to 4.99 ACU)</td>
<td>7</td>
<td>3.0–4.70</td>
<td>3.80 ± 0.57</td>
<td>1.25–6.80</td>
<td>3.41 ± 1.68</td>
</tr>
<tr>
<td>Positive (5.0 to 9.99 ACU)</td>
<td>18</td>
<td>5.0–8.60</td>
<td>6.44 ± 1.21</td>
<td>4.4–10.50</td>
<td>6.63 ± 1.84</td>
</tr>
<tr>
<td>Positive (≥10 ACU)</td>
<td>77</td>
<td>10.0–46.0</td>
<td>21.93 ± 8.85</td>
<td>5.4–38.5</td>
<td>20.63 ± 7.49</td>
</tr>
</tbody>
</table>

### TABLE 3. Variations in ACs and IRs

<table>
<thead>
<tr>
<th>Prepn</th>
<th>ACU</th>
<th>IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>D-Asparagine</td>
<td>0.41–1.37</td>
<td>0.73 ± 0.20</td>
</tr>
<tr>
<td>MK463</td>
<td>1.89–2.40</td>
<td>2.18 ± 0.17</td>
</tr>
<tr>
<td>Control</td>
<td>0.66–1.85</td>
<td>1.04 ± 0.27</td>
</tr>
<tr>
<td>Test strain</td>
<td>1.45–2.40</td>
<td>1.80 ± 0.20</td>
</tr>
<tr>
<td>MK87</td>
<td>3.31–4.0</td>
<td>3.61 ± 0.17</td>
</tr>
<tr>
<td>Control</td>
<td>0.48–1.85</td>
<td>0.99 ± 0.30</td>
</tr>
<tr>
<td>Test strain</td>
<td>1.30–4.30</td>
<td>23.5 ± 7.0</td>
</tr>
</tbody>
</table>

* ACs were determined on 30 occasions; IRs were determined on 12 occasions.

* Amino acid solution alone without organism.

* Organism in buffer.

* Organism in amino acid solution.
other bacteria that are difficult to subdivide by conventional means. The basic equipment and reagents, which consist of a pH meter, an ammonia electrode, screw-cap bottles, and amino acids, are inexpensive and widely available. The method could therefore be easily established in any reference laboratory. If further studies show the method to be useful in subdividing other difficult-to-classify bacterial groups (e.g., Legionella, Bordetella, Neisseria, etc.), it could also find a useful place in the routine clinical laboratory.

ACKNOWLEDGMENTS

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LITERATURE CITED