J. Hyg., Camb. (1984), **93**, 189–196
Printed in Great Britain

Use of an ammonia electrode to study bacterial deamination of amino acids with special reference to D-asparagine breakdown by campylobacters

By M. A. KARMALI, A. WILLIAMS, P. C. FLEMING, C. KRISHNAN* and M. M. WOOD

Departments of Bacteriology and Biostatistics and the Research Institute, the Hospital for Sick Children, Toronto, Canada, M5G 1X8, and the Department of Microbiology, the University of Toronto

(*Received* 16 *February* 1984; *accepted* 29 *February* 1984)

SUMMARY

A method using an ammonia electrode is being developed for investigating the deamination of amino acids and amides by bacteria. Application of this method to *Campylobacter jejuni* and *C. coli* has led to the demonstration of D-asparaginase activity in some strains. This has allowed the subdivision of both species into D-asparaginase-positive and -negative biotypes. Even though the method is in the developmental stage, it was found to be generally reproducible and easy to perform. Areas for further improving the procedure have been identified. The ammonia electrode offers the theoretical possibility of investigating the breakdown of any amino acid by bacteria. It thus opens up a new and practical approach for separating species and strains, particularly in those bacterial groups that are difficult to subdivide by conventional means.

INTRODUCTION

Renewed interest in the genus *Campylobacter* has led to an ever-expanding number of species and subspecies within this group of organisms. At least 13 taxonomic groups are now known (Karmali & Skirrow, 1984) and additional ones are becoming recognized. A significant drawback in classifying campylobacters is the lack of a sufficient number of reliable phenotypic markers for differentiating species and strains (Véron & Chatelain, 1973). The most satisfactory markers have proven to be biochemical ones (Karmali & Skirrow, 1984). For example, the catalase test subdivides the genus into two broad groups – the catalase-positive, and catalase-negative campylobacters. Most human infections are caused by the former, and in particular the species *C. jejuni* and *C. coli*. The hippurate test (Harvey, 1980) separates *C. jejuni* (positive for hippurate hydrolysis) from the other catalase-positive species which are hippurate-negative (Skirrow & Benjamin, 1980). The ability of strains to produce hydrogen-sulphide in iron-metabisulphite-

* Present address: Department of Microbiology, University Hospital, P.O. Box 5339, Postal Station 'A', London, Ontario, Canada N6A 5A5.

containing media (Skirrow & Benjamin, 1980) has helped in separating *C. jejuni* into two biotypes, and is also a feature of the so-called 'NARTC' strains now classified as *C. laridis* (Benjamin *et al.* 1983). It has been suggested that *C. jejuni/C. coli* strains might be further subdivided on the basis of their ability to produce deoxyribonuclease (Hébert *et al.* 1982; Lior, 1983).

The availability of additional differential biochemical markers is hampered by the relative lack of reactivity of campylobacters in conventional biochemical tests. Since campylobacters use amino acids as primary nutritional sources (Smibert, 1974), one approach towards extending their biochemical profiles might be to investigate their amino acid breakdown patterns. However, practical methods for doing this in a clinical microbiological setting are not available for most amino acids. We are developing a technique using an ammonia electrode that makes it possible to study the deamination of potentially any amino acid by a variety of bacteria.

We describe here this method, and its application to the study of D-asparaginase activity in campylobacters.

MATERIALS AND METHODS

Ammonia electrode

The experiments were conducted using the Model 95-10 ammonia electrode (Orion Research Incorporated), which is designed to allow rapid and reliable measurements of dissolved ammonia in aqueous solutions. The apparatus required consists of the ammonia electrode attached to a digital pH/millivolt meter. The sample to be analysed is contained within a beaker placed on a magnetic stirrer. When the ammonia electrode is lowered into the test sample, the dissolved ammonia in the solution diffuses across a hydrophobic membrane at the base of the electrode, and interacts with the internal components of the electrode. This interaction leads to a change in potential which is expressed in millivolts on the digital readout of the pH/millivolt meter. The magnitude of the change in potential is proportional to the dissolved ammonia concentration.

When ammonia is dissolved in water, it reacts with hydrogen ion to form ammonium ion as follows: $NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-$. The relative amounts of ammonia and ammonium ion are determined by the solution pH. In acid solution, virtually all the dissolved ammonia is in the form of ammonium ion, whereas in alkaline solution, the predominant dissolved species is ammonia. There is a tendency for the dissolved ammonia in the sample to diffuse into the surrounding atmosphere at a rate that depends upon its partial pressure in solution, the partial pressure in the atmosphere (negligible), and the temperature. In order to minimize ammonia loss from the sample it is acidified with hydrochloric acid so that the dissolved species is ammonium ion. In the analytical procedure the ammonia electrode is lowered into the acidified sample. An excess of concentrated sodium hydroxide (10 M) is then added to the sample. This converts the dissolved ammonium ion into ammonia which diffuses across the hydrophobic membrane of the electrode, and leads to a change in potential.

Calibration of the ammonia electrode

Before the electrode can be used to analyse test samples it requires to be calibrated using standard solutions of ammonium chloride. The choice of the concentrations for the standards depends upon the expected concentrations of ammonia in test samples. We have used a series of 10 ammonium chloride solutions with concentrations ranging from 10^{-6} to 10^{-2} M. Each standard is then analysed as follows: 50 ml of the freshly made standard solution is poured into a 100 ml beaker on a magnetic stirrer; the electrode is then lowered into the solution, after which 0·5 ml, 10 M-NaOH is added; this leads to the generation of any ammonia present, and a change in the millivolt reading of the pH meter. After the final millivolt reading had been recorded, the electrode is removed from the sample, rinsed with distilled water, and placed in a pH 4 buffer (sodium phosphate buffer 0·01 M) solution. The latter procedure re-equilibrates the inner components of the electrode. After all the standards have been analysed, a standard curve is plotted of ammonia concentration against millivolts. This allows the determination of the ammonia concentration in a test sample. It should be noted that all standards and test samples should be analysed at the same temperature.

Application of the ammonia electrode to study the deamination of D-*asparagine by campylobacters – general principles*

Our objective was to determine whether or not the ammonia produced from the bacterial deamination of D-asparagine could reliably be detected by means of the ammonia electrode. We used pre-sterilized standard 150 ml volume (6 oz) bottles equipped with airtight diaphragm-lined screw-caps through which a syringe needle could be introduced. Before sterilization the bottles were thoroughly washed and properly rinsed to ensure that there was no residual detergent that might contain traces of ammonia; residual ammonia could be minimized using acid-washed bottles, but this procedure was not routinely available to us.

The D-asparagine (Sigma Chemicals) was made up as a 0·01 % solution in sodium phosphate buffer (pH 7·2, 0·01 M); 50 ml aliquots of this solution were distributed into several screw-capped bottles.

The campylobacter strains to be tested were obtained as blood agar cultures 36 h old. The blood agar medium used consisted of Columbia blood agar base (Gibco Diagnostics) with 5 % horse blood. Bacteria from the blood agar cultures were suspended in sodium phosphate buffer (pH 7·2, 0·01 M). These suspensions were washed twice in the same buffer, and then resuspended to a turbidity equivalent to a McFarland no. 10 standard. A 1 ml volume of each suspension was inoculated into the 50 ml D-asparagine solution. A series of control bottles was included as follows: 50 ml of buffer alone (buffer control), 50 ml of amino acid solution alone (amino acid control), and 50 ml of buffer containing the bacterial suspension alone (organism control). The bottles were then removed from the water bath and allowed to stand on the bench for about 2 h until their temperature stabilized to that of the room. Prior to opening the screw caps, 1 M-HCl was added to each bottle via the diaphragm. The contents of each bottle were tested for ammonia as described. The millivolt end-point reading was obtained and converted to ammonia concentration with reference to the standard curve.

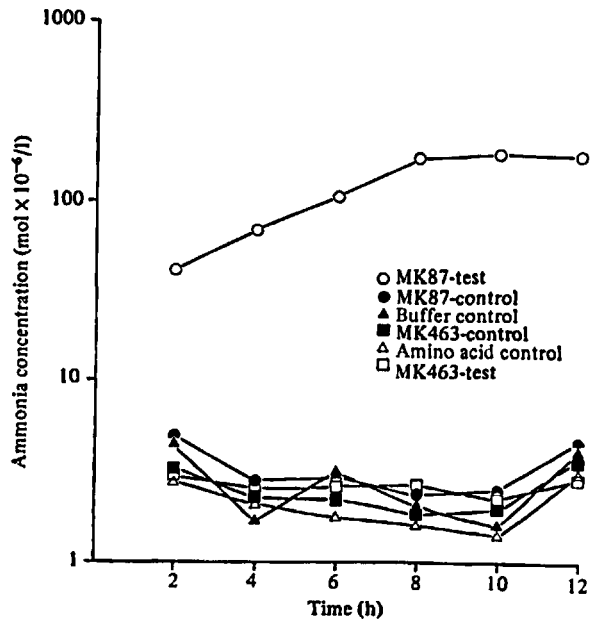

Fig. 1. Kinetics of ammonia production by a positive (MK 87) and negative (MK 463) control strain of *C. jejuni/C. coli*.

Kinetics and ammonia production

During our preliminary studies, we were able to identify strains that were clearly positive, and strains that were clearly negative for ammonia production. Two of these strains, MK 87 and MK 463, were chosen for detailed kinetic studies. For this, multiple bottles containing D-asparagine were prepared and inoculated with identical concentrations of either culture MK 87 or MK 463. All test bottles and appropriate controls were incubated at 37 °C. Three bottles of each test sample and organism control were removed at 2 h intervals over a 12 h period and tested for ammonia production. The mean value for each of these triplicates was calculated and recorded.

Reproducibility studies

Strains MK 87, MK 463 and appropriate controls were tested in replicates of 20 after incubating at 37 °C for 16 h, and the results were analysed to determine the degree of scatter between individual values.

Determination of the distribution of D-*asparagine activity of* C. jejuni *and* C. coli

A total of 164 strains (88 *C. jejuni* and 76 *C. coli*) were tested in several batches for D-asparaginase activity. Eighty-three of these strains were tested on at least two occasions.

For each batch the positive (MK 87) and negative (MK 463) control strains were always run in parallel, and the Ammonia Electrode was calibrated with freshly made standards.

Table 1. *Variation in recorded values of ammonia production from* D-*asparagine using* 20 *separate samples of strains* MK 463 *and* MK 87

	Organism control (C)	Test sample (T)	Ratio (R) T:C	$\text{Log}_{10} R$ (LR)
		Strain MK 463		
Range of recorded values for ammonia*	1·32–2·94	2·79–4·39	0·94–2·58	−0·03–0·41
Mean ± S.D.	2·15 ± 0·41	3·52 ± 0·42	1·69 ± 0·36	0·22 ± 0·1
		Strain MK 87		
Range of recorded values for ammonia	1·69–2·66	459·14–560·8	191·3–300·25	2·28–2·41
Mean ± S.D.	2·19 ± 0·22	507·14 ± 32·29	233·14 ± 24·82	2·37 ± 0·05

* Ammonia concentration in moles $\times 10^{-6}$/l.

RESULTS

Kinetics of ammonia production

Kinetic studies of ammonia production using strains MK 87 and MK 463 were performed on three separate occasions. Representative results are shown in Fig. 1. A comparison of the kinetics of the buffer control and the amino acid control shows that D-asparagine was stable and did not undergo spontaneous deamination under the conditions of the test. Easily detectable levels of ammonia were generated by strain MK 87, but not by strain MK 463. Ammonia production from D-asparagine by MK 87 was already evident after 2 h of incubation, rose steadily until about 8 h of incubation, and thereafter remained constant.

Calibration of the electrode and recording of results

Calibration of the ammonia electrode on different occasions consistently produced an accurate straight-line graph when a semi-logarithmic plot was used with the millivolt reading on the linear scale, and the ammonia concentration on the logarithmic scale. When 20 replicates of test and control samples of MK 87 and MK 463 were examined using the same standard curve, variation in the recorded values was observed (Table 1). Up to three-fold differences in values were observed between different samples of the same organism. In contrast, the differences in both mean and individual values between the D-asparaginase-positive (MK 87) and -negative (MK 463) strains were 100-fold in magnitude. When individual strains and appropriate controls were tested on different days using a different standard curve, additional variability was noted; sometimes both test and control samples read 'high' and at other times they read 'low'. In order to overcome this the results were recorded as the ratio between the ammonia concentration in the test sample and that in the organism control, this ratio being referred to as the 'R' value. The logarithm ($\log_{10}$) of the ratio, 'LR', value was also recorded (Table 1).

Distribution of D-*asparaginase activity in* C. jejuni *and* C. coli

The 'R' and 'LR' values were obtained for 164 strains of *C. jejuni* and *C. coli*; 'LR' values were plotted. The distribution of the LR values (Fig. 2) shows a left component with a distinct mode of 0·130 and an irregular right component. For

https://doi.org/10.1017/S0022172400064706 Published online by Cambridge University Press

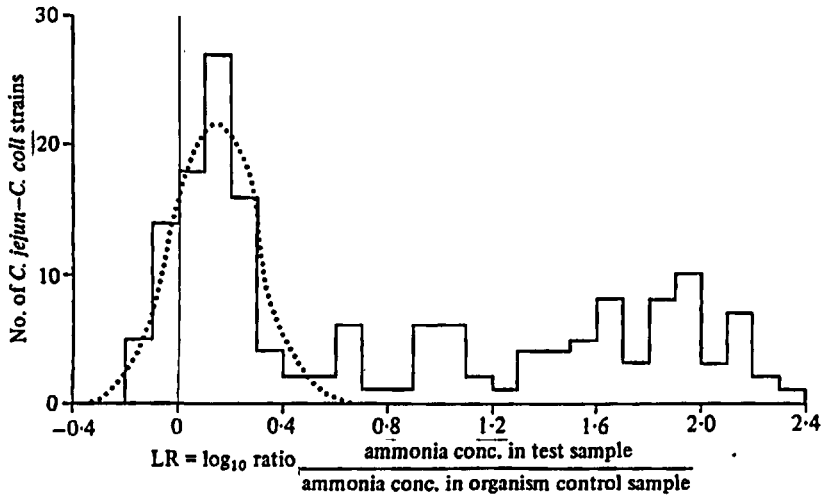

Fig. 2. Distribution of D-asparaginase activity among 164 strains of *C. jejuni/C. coli*. ——, Observed results; ....., fitted normal distribution curve.

Table 2. *Distribution of* D-*asparaginase activity in* C. jejuni *and* C. coli

		Number (%) of strains		
LR* value	D-asparaginase	*C. jejuni/C. coli*	*C. jejuni*	*C. coli*
<0·6	Negative	88 (53·7)	74 (84·1)	14 (18·4)
0·6–1·2	Weak-positive	23 (14·0)	6 (6·8)	17 (22·4)
>1·2	Positive	53 (32·3)	8 (9·1)	45 (59·2)
	Total (%)	164 (100)	88 (100)	76 (100)

* LR value = Log_{10} ratio of $\frac{\text{ammonia concentration in test sample}}{\text{ammonia concentration in organism control}}$.

the n low values ($n = 83, 84, \ldots, 97$) the median and the mode are equal. By iteration through these values of n it was found that symmetry was at maximum for $n = 89$. The distribution of the low 89 values (mean 0·129, S.D. 0·161) fits a normal distribution ($\chi^2 = 10{\cdot}20$ on 8 degrees of freedom, $P < 0{\cdot}75$). Assuming this normal distribution for the low values, then the probability is 0·05, 0·01 and 0·001 that LR values greater than 0·40, 0·51, and 0·67, respectively, are negative for D-asparaginase. The high values were irregularly distributed and were not characterized mathematically. Without such characterization it is not possible to assign a probability that a value is positive for D-asparaginase, and hence the relative probability that a specific value is negative or positive.

On the basis of the above data, a practical system was created whereby LR values less than 0·6 were considered to be negative, values greater than 1·2 to be positive, and values lying between 0·6 and 1·2 to be weakly-positive for D-asparaginase. The relative distributions of these reactions for *C. jejuni* and *C. coli* are shown in Table 2. It can be seen that this allows both species to be subdivided into two biotypes.

https://doi.org/10.1017/S0022172400064706 Published online by Cambridge University Press

Reproducibility of results

When the positive control (MK 87) and negative (MK 463) control strains were tested 20 times, each strain consistently gave the appropriate positive (LR > 1·2) or negative (LR < 0·6) reaction (Table 1). When 30 strains that were positive on the first occasion were tested a second time, 27 remained positive, two gave a weak-positive and one gave a negative reaction. A second test on 30 negative strains showed that 27 remained negative and three became weak-positive. Repeat testing of the 23 weak-positive strains produced a weak-positive reaction in 12, a negative reaction in five, and a positive reaction in six.

DISCUSSION

The ammonia electrode has been used for detecting ammonia in various biological, industrial, or environmental samples such as urine, plasma, beer, wines, soil and water (*Analytical Methods Guide*, 9th ed., Orion Research Inc., Cambridge, Massachusetts, U.S.A., 1978). In this preliminary report we have shown that it can also be used in the clinical microbiological setting for testing deamination of amino acids or amides by bacteria. Amino acids or amides that are suitable for use in this procedure are those that do not undergo spontaneous deamination under the conditions of the test.

We have demonstrated D-asparagine breakdown by campylobacters. About 54 % of the 164 strains of *C. jejuni–C. coli* studied were clearly negative for D-asparaginase, 32 % were clearly positive, and a further 14 % gave a weak-positive reaction. D-asparaginase activity occurred more frequently in *C. coli* than in *C. jejuni*. Both the latter species could be subdivided into D-asparaginase-positive and -negative biotypes. The incorporation of the D-asparaginase test into the existing biotyping schemes will result in a greater number of biotypes, and this is likely to be of value in epidemiological studies. The investigation of D-asparaginase in other *Campylobacter* species, as well as the use of additional amino acids, is expected to further clarify the classification of campylobacter.

Even though the method we have described is currently at a developmental stage, we found it generally reliable, reproducible and easy to perform. Most strains tested could be satisfactorily classified as positive or negative for D-asparaginase. A small group of strains was identified that gave a weak-positive reaction. This group probably represents an area of some overlap between the positive and negative strains, and additional work is needed to clarify its status. The statistical analysis of our data was limited by the fact that only the low ('negative') values fit a normal distribution. The investigation of a much larger number of strains should allow statistical methods to be applied to the high and intermediate values. It is expected that this will lead to a better characterization of the weak-positive group, and a more accurate definition of the breakpoint between positive and negative.

Studies on the kinetics of ammonia production from D-asparagine by the positive control strain (MK 87) showed that significant levels of ammonia were present by 2 h of incubation. Most strains tested in the present study were investigated for ammonia production after overnight incubation. Further studies are required

for optimizing the incubation period, as well as the organism and substrate concentrations.

The method may be significantly improved by accurate standardization of the temperature at which the readings are made. The instruction manual for the Ammonia Electrode states that a 1° (Celsius) difference in temperature will lead to a 2% measurement error. This problem may potentially be overcome by using a temperature-compensation probe that automatically records values corrected to a reference temperature. Such a device is currently being evaluated. Additional improvement should result from the routine use of acid-washed glassware, since this would reduce any residual ammonia-containing detergent in the incubation bottles.

A wide range of amino acid deaminases are known to occur in bacteria (Gottschalk, 1979), but only a few are used for taxonomic purposes. These include phenylalanine deaminase and L-tryptophan indole-lyase (basis for the well-known indole test). Both the latter are useful for separating species and strains particularly among the Enterobacteriaceae (Cowan & Steel, 1974). Satisfactory methods for the routine investigation of other bacterial deaminases are not available. The ammonia electrode offers the theoretical possibility of investigating the breakdown of any amino acid by a variety of different bacteria. Such deamination reactions might be useful taxonomically for a variety of bacterial groups other than *Campylobacter*; these include *Legionella*, *Neisseria* and *Haemophilus*. In this context, the ammonia electrode may find an increasing number of potentially useful applications in the clinical microbiological setting.

This work was supported by an Ontario Ministry of Health Grant, number 00679. M. A. Karmali is an Ontario Ministry of Health Career Scientist.

REFERENCES

BENJAMIN, J., LEAPER, S., OWEN, R. J. & SKIRROW, M. B. (1983). Description of *Campylobacter laridis*, a new species comprising the nalidixic acid resistant thermophilic *Campylobacter* (NARTC) group. *Current Microbiology* **8**, 231–238.

COWAN, S. T. & STEEL, K. J. (1974). *Manual for the Identification of Medical Bacteria*, 2nd ed. Cambridge University Press.

GOTTSCHALK, G. (1979). *Bacterial Metabolism*. New York: Springer-Verlag.

HARVEY, S. M. (1980). Hippurate hydrolysis by *Campylobacter fetus*. *Journal of Clinical Microbiology* **11**, 435–437.

HEBERT, G. A., HOLLIS, D. G., WEAVER, R. E., LAMBERT, M. A., BLASER, M. J. & MOSS, C. W. (1982). Thirty years of campylobacters: biochemical characteristics and a biotyping proposal for *Campylobacter jejuni*. *Journal of Clinical Microbiology* **15**, 1065–1073.

KARMALI, M. A. & SKIRROW, M. B. (1984). Taxonomy of the genus *Campylobacter*. In *Campylobacter Infection in Man and Animals* (ed. J. P Butzler), pp. 1–20. Boca Raton, Florida: CRC Press Inc.

LIOR, H. (1983). A new extended biotyping scheme for *Campylobacter jejuni*, *Campylobacter coli* and '*Campylobacter laridis*' (NARTC). In *campylobacter*, vol. II (ed. A. D. Pearson *et al.*), p. 42. London: PHLS.

SKIRROW, M. B. & BENJAMIN, J. (1980). Differentiation of enteropathogenic campylobacter. *Journal of Clinical Pathology* **33**, 1122.

SMIBERT, R. M. (1974). *Campylobacter*. In *Bergey's Manual of Determinative Bacteriology*, 8th ed. (ed. R. E. Buchanan and N. E. Gibbons), pp. 207–212. Baltimore: Williams and Wilkins.

VERON, M. & CHATELAIN, R. (1973). Taxonomic study of the genus *Campylobacter* Sebald and Véron and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Véron. *International Journal of Systematic Bacteriology* **23**, 122–134.