Formic Hydrogenlyase Induction as a Basis for the Eijkman Fecal Coliform Concept

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Many *Enterobacter* species recovered from a relatively unpolluted freshwater stream had the capacity to produce gas from glucose, lactose, and formate at 44.5 C. Composition of the evolved gas was shown to be CO_2 and H_2, which suggested that the enzyme system formic hydrogenlyase functions as the basis for the Eijkman fecal coliform concept. It is postulated that there are at least two different and distinct biochemical types of *Enterobacter* in the natural aquatic environment: one that lacks an active formic hydrogenlyase at 44.5 C and is associated with the intestinal tract of man and certain animals, and another which possesses the active enzyme at the elevated temperature and is found in soil or on vegetation free from fecal pollution.

The coliform group of enteric bacteria is a heterogeneous collection of microorganisms which have been defined as "all the aerobic and facultative anaerobic, gram-negative, nonspore-forming rod-shaped bacteria which ferment lactose with gas within 48 hr at 35 C" (1). Although it is current practice to consider the presence of coliform bacteria in natural waters as an indication of fecal contamination, it is obvious that organisms from environments other than the fecal material of man and certain animals will conform to the above definition.

There have been numerous attempts to differentiate between a "fecal" and a "non-fecal" coliform, and the most successful of these are those of the elevated temperature of incubation variety (6). These procedures are primarily modifications based upon the Eijkman (4) observation that members of the *Escherichia* group, as described by Parr (17, 18), could produce gas in a glucose medium at 46 C, whereas *Enterobacter* (*Aerobacter*) could not. Perry and Hajna (19) improved upon the Eijkman technique by substituting lactose for glucose, adding bile salts and a buffering system to the medium, and lowering the incubation temperature to 44.5 C. Utilizing modifications of this basic fecal coliform procedure, Geldreich et al. (7), and more recently Mishra et al. (15), have observed a positive correlation of gas production at the elevated incubation temperature with Parr's *Escherichia* group of coliforms. More significantly, however, Mishra et al. found that more than 52% of the *Enterobacter* types recovered also gave positive elevated temperature reactions.

The basic mechanism by which gas is produced by *Escherichia* and not by *Enterobacter* at elevated temperatures is not clearly understood, nor is it known with certainty which *Enterobacter* strains from aquatic or terrestrial environments are capable of producing gas at these temperatures. The present investigation was undertaken to determine which particular gas-producing enzyme system in *Enterobacter* is affected by the incubation at 44.5 C and to test the basic concept of the Eijkman procedure with known coliform strains and organisms recovered from the aquatic environment.

MATERIALS AND METHODS

Selection of organisms. Enteric bacteria employed in this study were obtained both from the American Type Culture Collection (Rockville, Md.) and by recovery from the North Oconee River in Clarke County, Ga. River water samples were collected by techniques suggested by the American Public Health Association (1) and cultured on M-Endo-MF Broth (BBL) by the use of the membrane filter procedure. After incubation at 44.5 ± 0.1 C for 16 to 18 hr, all colonies were counted that conformed to the "enteric" designation (lactose fermentation) and to the "coliform" designation (lactose fermentation with the production of green, metallic sheen). Organisms confroming to either the "enteric" or "coliform" designation were further differentiated by their ability to produce gas at 44.5 C in MR-VP medium (BBL), and by indole, methyl red, Voges-Proskauer, citrate (IMViC) classification (8). The taxonomic classification of the isolates was confirmed by techniques suggested by Edwards and Ewing (3), and the nomenclature used in the remainder of this study follows that proposed by Ewing (5), which substitutes the generic

name *Enterobacter* for *Aerobacter*. After these pre-liminary tests were completed, all organisms, including the stock cultures of *Escherichia coli* (ATCC 11775) and *Enterobacter aerogenes* (ATCC 12658), were maintained on Nutrient Agar (Difco) slants in the refrigerator for later use.

Gas production by Enterobacter. Each strain recovered was inoculated into a duplicate series of Durham fermentation tubes containing 1% concentrations of glucose (Difco), lactose (Difco), or sodium formate (Fisher Scientific Co., Pittsburgh, Pa.) to qualitate gas production. The basal medium was Nutrient Broth (Difco), and each tube was pre-incubated to equilibrate the medium to the desired experimental temperature. One set of fermentation tubes was incubated at 30.0 C and the other at 44.5 C, and all tubes were observed for collected gas in the inverted vials at 24 and 48 hr. After incubation, 1 ml of 20% KOH solution was added to each culture tube containing gas to absorb any evolved CO_2, and, after 30 min of incubation at room temperature, the inverted vial was removed from the culture tube and the open end was placed in a flame to determine if H_2 was present.

Formic hydrogenlyase assay. Pure culture cell suspensions of the ATCC *Escherichia* and *Enterobacter* strains and selected strains of aquatic enteric bacteria were prepared by inoculating each into flasks containing 200 ml of sterile, prewarmed Trypticase Soy Broth (BBL). These cultures were grown aerobically with vigorous shaking to avoid inducing the lyase system. Growth temperature was either 30.0 or 44.5 C, depending on desired experiment, and cells were harvested after 18 hr of incubation. The cells were collected by centrifugation and washed three times in 0.1 M phosphate buffer, pH 6.8, and suspended in buffer to give an optical density (OD) of 0.2 at 540 nm with a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.) colorimeter-spectrophotometer. Dry weights of cells were obtained by comparing the OD with a standard curve, which was prepared with organisms washed with deionized water and then dried overnight at 105 C.

The activity of induced formic hydrogenlyase was determined in a Warburg respirometer (Aminco) by the technique of Quist and Stokes (21). Duplicate vessels contained 2.0 ml of a washed cell suspenstion in the main compartment, and 0.2 ml of a 20% KOH solution in the center well for the absorption of CO_2. Substrates for induction included in the side-arms were 60 μmoles of sodium formate (0.3 ml), 20 μmoles of glucose (0.2 ml), and 0.1 ml of a 10% concentration of yeast extract (Difco). To ensure that the enzyme system was being induced in the Warburg vessels and was not preformed in the original culture flasks, 0.03 mg of chloramphenicol (0.3 ml) was added to one of the Warburg vessels in each series to inhibit de novo protein synthesis. The remaining flask received 0.3 ml of 0.1 M phosphate buffer (pH 6.8). The gas phase in each experiment was N_2, and the bath temperature was at either 30.0 or 44.5 C, depending on the particular experiment. Each vessel was incubated with shaking until a constant rate of H_2 evolution was reached, and results were expressed in $Q(H_2)$ values, as microliters of H_2 evolved per hour per milligram of dry cells. There was virtually no endogenous H_2 production and no H_2 formed in control vessels containing only cells and sodium formate.

RESULTS

A total of 629 isolates were recovered from the Oconee River over a 1-year period and were broadly categorized into either the "coliform" or "enteric" groups on the basis of their cultural reactions on Endo medium. These organisms were further differentiated into *Escherichia*, *Enterobacter*, and intermediate groups by the IMViC procedure and for gas production in glucose at 44.5 C (Table 1). Geldreich (6) reported that enteric bacterial strains producing gas at the elevated temperature and having $++--$, $-+--$, or $+---$ IMViC reactions may be considered to be *Escherichia* species and of fecal

TABLE 1. *Gas production by enteric bacteria recovered from river water on M-Endo MF medium at 44.5 C*

Parr's IMViC[a] group	Coliform isolates[b]			Enteric isolates[c]			Total isolates		
	Organisms recovered	Gas[d]	Per cent (group total)	Organisms recovered	Gas	Per cent (group total)	Organisms recovered	Gas	Per cent (group total)
Escherichia group ($++--$, $+---$, $-+--$)	226	221	97.8	86	77	89.5	312	298	95.5
Enterobacter group ($--++$, $--+-$, $---+$)	130	23	17.7	28	15	53.6	158	38	24.0
Intermediate group	86	44	51.2	73	66	90.4	159	110	69.2

[a] Indole, methyl red, Voges-Proskauer, citrate.
[b] Organisms exhibiting lactase utilization and a metallic, green sheen.
[c] Organisms exhibiting lactose utilization.
[d] Production of gas in MR-VP medium at 44.5 C.

TABLE 2. *Gas production from glucose, lactose, and formate at 30.0 and 44.5 C by aquatic forms of Enterobacter aerogenes conforming to the "coliform" designation*[a]

Organism	No. of strains	Source	Gas production from					
			Glucose		Lactose		Formate	
			30.0 C	44.5 C	30.0 C	44.5 C	30.0 C	44.5 C
Enterobacter aerogenes								
− − + +	1	ATCC 12658	+	−	+	−	+	−
− − + +	12	Oconee R.	+	+	+	+	+	+
− − + −	3	Oconee R.	+	+	+	+	+	+
− − − +	8	Oconee R.	+	+	+	+	+	+
Escherichia coli	1	ATCC 11775	+	+	+	+	+	+

[a] These organisms produced gas in MR-VP medium at 44.5 C.

origin, whereas − − + +, − − + −, or − − − + strains lacking the ability to produce gas at 44.5 C are *Enterobacter* and indicative of soil or vegetative origin.

The results of these experiments (Table 1) show that 70.9% of all organisms recovered produced gas, which proved to be a mixture of H_2 and CO_2, but less than 50% of the isolates were *Escherichia* species. Of all *Enterobacter*, 24% also had the capacity for gas production. Much the same correlation pattern of gas production at 44.5 C with IMViC types was observed when only the "coliform" isolates were considered. Of significant importance is the observation that 17.7% of the *Enterobacter* species in the coliform group produced gas from glucose.

Formate and lactose were observed to serve satisfactorily as substrates for gas production by those aquatic *Enterobacter* species which could produce gas at 44.5 C from glucose. The aquatic strains and *E. coli* could produce gas from the three substrates at both 30.0 and 44.5 C, whereas the ATCC *E. aerogenes* culture could not at the higher temperature. These data indicate that the evolved CO_2 and H_2 result from an active formic hydrogenlyase and that this particular enzyme system can be functional in *Enterobacter* at the temperature specified for the Eijkman procedure (Table 2).

Data from the Warburg experiments (Fig. 1) suggest that formic hydrogenlyase is synthesized by both the *E. coli* and *E. aerogenes* stock cultures at 30.0 C and by *E. coli* at 44.5 C. The induction time for formic hydrogenlyase synthesis by the stock cultures was about 30 min (Fig. 1) in each case, but, when coliform organisms recovered from the aquatic environment were used in similar experiments, times of induction were variable and ranged from 30 min to approximately 2 hr (Fig. 2). None of the aquatic or-
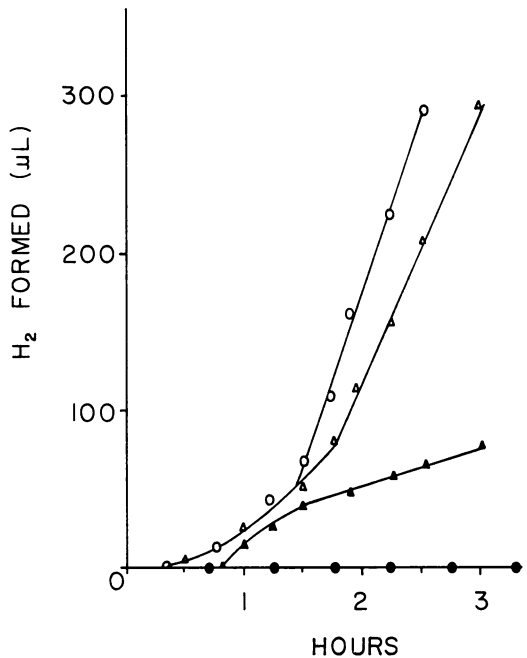

FIG. 1. *Formic hydrogenlyase synthesis by Escherichia coli ATCC 11775 at 30.0 C* (△), *44.5 C* (▲), *and Enterobacter aerogenes at 30.0 C* (○), *44.5 C* (●).

ganisms used in these experiments could be biochemically grouped with the *Escherichia* species, but all organisms used, with the exception of one (+ − + +), synthesized formic hydrogenlyase. These included an organism selected from the 23 gas-producing *E. aerogenes* strains. Evidence for formic hydrogenlyase induction was further demonstrated by the lack of enzymatic activity in control experiments, in which chloramphenicol

was added to the substrates for induction. These data indicate that the formic hydrogenlyase system was induced and not simply an activation of the system which might have been present.
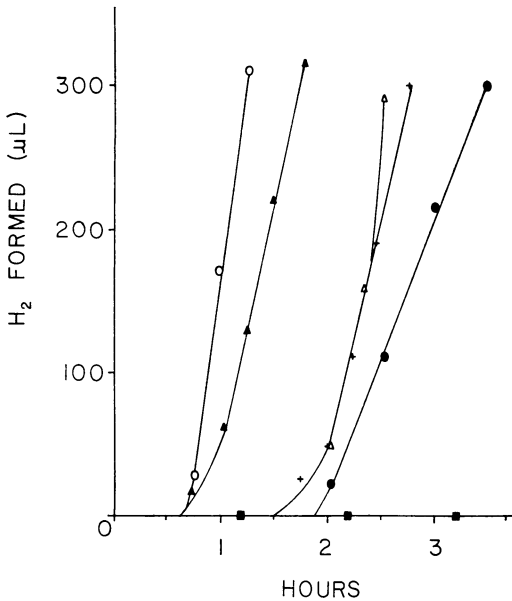

FIG. 2. *Formic hydrogenlyase synthesis at 44.5 C by various aquatic enteric bacteria. Symbols used refer to the indole, methyl red, Voges-Proskauer, citrate classification of the bacteria:* ▲, − − + −; +, − + + −; ○, + + + −; ■, + − + +; △, − − + +; ●, − + − +.

TABLE 3. *Formic hydrogenlyase activity of selected coliform bacteria*

Organism	Source	Assay temp	Activity[a]
		C	
Escherichia coli + + − −[b]	ATCC 11775	30.0	2,360
		44.5	360
Enterobacter aerogenes − − + +	ATCC 12658	30.0	2,680
		44.5	0
− − + +	Oconee R.	44.5	2,860
− − + −	Oconee R.	44.5	2,500
Intermediate group + + + −	Oconee R.	44.5	3,300
− + + −	Oconee R.	44.5	2,250
− + − +	Oconee R.	44.5	2,260
+ − + +	Oconee R.	44.5	0

[a] Expressed in microliters of H_2 per hour per milligram (dry weight) of cells.

[b] Parr (17, 18) IMViC grouping.

Table 3 shows specific activity of the synthesis of formic hydrogenlyase by using data from the Warburg experiments. Known stock cultures of *E. coli* produced approximately 10% of the activity at the elevated temperature, whereas *E. aerogenes* failed to synthesize the enzyme. However, at 44.5 C, the aquatic strains of *E. aerogenes* were capable of synthesizing formic hydrogenlyase at a rate comparable to the stock culture at the lower temperature. In general, the intermediate forms were quite active at 44.5 C and formed formic hydrogenlyase at a rate equivalent to that of the stock cultures at 30.0 C.

DISCUSSION

Gray and Gest (9) have postulated that the hydrogenlyase system present in *Enterobacteriaceae* contains two distinct enzymes, a hydrogenase and a formate dehydrogenase. This system is inducible and anaerobic, with carbon dioxide and molecular hydrogen as reaction end products. Both of the ATCC cultures used in this investigation had the capacity to induce formic hydrogenlyase at 30.0 C, but *E. aerogenes* was unable to synthesize the enzyme system at 44.5 C. These results are consistent with those of Quist and Stokes (21). The recovery of biochemically sound strains of *E. aerogenes* from the aquatic environment which have an inducible formic hydrogenlyase system at 44.5 C is not surprising, since biochemical, morphological, and serological variations in enteric bacteria as laboratory phenomena are well known (3, 10). Reports of such phenomena occurring under natural conditions are rare, although Velaudapillai (22) and Hendricks and Morrison (11) have suggested that exchange of genetic material between bacteria can occur in various natural environments to give rise to biochemically aberrant organisms.

Explanations for the presence of a high temperature-insensitive hydrogenlyase system in some of the aquatic strains of *E. aerogenes* are difficult. It is entirely possible that the wild type, as represented by the ATCC culture, cannot synthesize the enzyme system or synthesizes a portion that is inactivated at elevated temperature and that the selective pressures of the aquatic environment allow a particular mutant population to survive. This hypothesis is consistent with observations of Peterson and Gunderson (20) and Morita and Burton (16). It is also reasonable to suggest that a formic hydrogenlyase system active at an elevated temperature may be irreversibly lost upon prolonged storage as are virulence factors of certain pathogenic organisms.

Considerable evidence has recently been discovered which indicates that the positive correlation between gas production at an elevated temperature of incubation and the presence of fecal

coliforms (E. coli) may be restricted to environments that are grossly contaminated by feces of man and certain warm-blooded animals (7, 15). In this particular environment, it is obvious that the gas-producing coliforms are indicative of fecal pollution, since E. coli assumes dominant proportions among those organisms capable of synthesizing an active formic hydrogenlyase. However, in environments where fecal contamination and numbers of E. coli are minimal, other gas-producing enteric bacteria, which are not fecal coliforms, can reach significant proportions to alter the statistical fecal coliform relationship. The results of this study substantiate the latter hypothesis, in which significant numbers of Enterobacter species (24%) from relatively clean river water had the capacity to produce H_2 and CO_2 at 44.5 C. Although Enterobacter species may be recovered from the feces of man and animals (14), these organisms are not considered to be indicators of fecal pollution, but rather they are associated with soil and vegetation. The present data become significant when one considers the increasing reports (2, 12, 13) of growth and multiplication of the E. coli-E. aerogenes group of coliforms in natural waters. It is entirely possible that water of questionable quality may be needlessly rejected if differentiation between fecal and nonfecal coliforms is made solely on the basis of an elevated-emperature fecal coliform test.

ACKNOWLEDGMENTS

LITERATURE CITED

1. American Public Health Association. 1965. Standard methods for the examination of water and waste water, including bottom sediment and sludges, 12th ed. American Public Health Association, Inc., New York.
2. Boyd, W. L., and W. Boyd. 1962. Viability of thermophiles and coliform bacteria in arctic soils and water. Can. J. Microbiol. 8:189–192.
3. Edwards, P. R., and W. H. Ewing. 1962. Identification of Enterobacteriaceae, 2nd ed. Burgess Publishing Co., Minneapolis.
4. Eijkman, C. 1904. Die Gärungsprobe bei 46° als Hilfsmittel bei der Trinkwasseruntersuchung. Zentralbl. Bakteriol. Parasitenk, Infektionskr. Hyg. Abt. Orig. 34:742–748.
5. Ewing, W. H. 1963. An outline of nomenclature for the family Enterobacteriaceae. Intern. Bull. Bacteriol. Nomencl. Taxon. 12:95–110.
6. Geldreich, E. E. 1966. Sanitary significance of fecal coliforms in the environment. Federal Water Pollution Control Administration, U.S. Department of the Interior, Washington, D.C.
7. Geldreich, E. E., C. B. Huff, R. H. Bordner, P. W. Kabler, and H. F. Clark. 1962. The faecal coli-aerogenes flora of soils from various geographical areas. J. Appl. Bacteriol. 25:87–93.
8. Geldreich, E. E., B. A. Kenner, and P. W. Kabler. 1964. Occurrence of coliforms, fecal coliforms, and streptococci in vegetation and insects. Appl. Microbiol. 12:63–69.
9. Gray, C. T., and H. Gest. 1965. Biological formation of molecular hydrogen. Science 148:186–192.
10. Hayes, W. 1968. The genetics of bacteria and their viruses, 2nd ed. John Wiley and Sons, Inc., New York.
11. Hendricks, C. W., and S. M. Morrison. 1967. Strain alteration in enteric bacteria isolated from river water. Can. J. Microbiol. 13:271–277.
12. Hendricks, C. W., and S. M. Morrison. 1967. Multiplication and growth of selected enteric bacteria in clear mountain stream water. Water Res. 1:567–576.
13. Kusnezow, S. I. 1959. Die Rolle der Mikroorganism in Stoffkeislauf der Seen. [Transl. from Russian by A. Pochmann]. Veb. Deutscher Verlag der Wissenschaften, Berlin.
14. Lofton, C. B., S. M. Morrison, and P. D. Leiby. 1962. The Enterobacteriaceae of some Colorado small mammals and birds, and their possible role in gastroenteritis in man and domestic animals. Zoonoses Res. 1:277–293.
15. Mishra, R. P., S. R. Joshi, and P. V. R. C. Panicker. 1968. An evaluation of the standard biochemical and elevated temperature tests for differentiating faecal and non faecal coliforms. Water Res. 2:575–585.
16. Morita, R. Y., and S. D. Burton. 1963. Influence of moderate temperature on growth and malic dehydrogenase activity of a marine psychrophile. J. Bacteriol. 86:1025–1029.
17. Parr, L. W. 1938. The occurrences and succession of coliform organisms in human feces. Amer. J. Hyg. 27:67–87.
18. Parr, L. W. 1938. Coliform intermediates in human feces. J. Bacteriol. 36:1–15.
19. Perry, C. A., and A. A. Hajna. 1944. Further evaluation of EC Medium for the isolation of coliform bacteria and Escherichia coli. Amer. J. Public Health Nat. Health 34:735–748.
20. Peterson, A. C., and M. F. Gunderson. 1960. Some characteristics of proteolytic enzymes from Pseudomonas fluorescens. Appl. Microbiol. 8:98–104.
21. Quist, R. G., and J. L. Stokes. 1969. Temperature range for formic hydrogenlyase induction and activity in psychrophilic and mesophilic bacteria. Antonie van Leeuwenhoek J. Microbiol. Serol. 35:1–8.
22. Velaudapillai, T. 1961. Of the naming of salmonellas, is there no end? Intern. Bull. Bacteriol. Nomencl. Taxon. 11:1–4.