Variability in Gas Production by Escherichia coli in Enrichment Media and Its Relationship to pH

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Variability in gas production in multiple subcultures of Escherichia coli was assessed in two selective enrichment media and in lactose peptone water. Considerable variability occurred with all media at 37 and 44°C. Addition of buffer increased gas production and decreased variability. The relationships between pH, growth, and gas production were complex. In buffered media, viable counts increased by 269 × 106 to 382 × 106/U of pH fall, whereas in unbuffered media, they increased by 9.45 × 104 to 30.37 × 106/U of pH fall. In buffered and unbuffered media, pH fell as gas production rose. However, variability in gas production among individual subcultures was not associated with changes in pH.

Standard methods for Escherichia coli enumeration use lactose-containing enrichment media together with a modified Eijkman elevated temperature test (1, 9). In several studies using the most-probable-number technique, not all tubes of enrichment media showing E. coli growth have shown gas production (2, 5, 8). These false-negative reactions may result from cultivation of environmentally damaged or anaerogenic strains of E. coli (4); thus, differences in E. coli strains have been thought to be responsible for variations in gas production. We show that this need not always be the case since multiple subcultures from individual strains exhibit variable gas production, particularly in enrichment media which are inadequately buffered.

MATERIALS AND METHODS

Organisms and growth media. The E. coli strains used were NCTC 123, NCTC 8623, and a fecal isolate.

Gas production by these E. coli strains was compared in lactose peptone water (LPW), lactose ricanolate broth (Oxoid) (LRB), and brilliant green bile broth (Difco) (BGBB). Buffered LRB and BGBB were prepared by adding 1.77 g of KH2PO4 and 15.2 g of K2HPO4 to 1 liter of medium.

Culture conditions. Media were dispensed in 4.5-ml portions into 7-ml Bijou bottles containing Durham tubes. Cells were washed from 37°C, 24-h slopes using 10 ml of nutrient broth (Oxoid). One loopful was used to inoculate each bottle of medium. Incubation was at 37 ± 0.25 or 44 ± 0.25°C.

Assessment of gas production. Gas production was categorized as follows: 0, no visible gas present; 1, the bubble did not fill the head of the tube and its breadth was less than the internal diameter of the tube; 2, the bubble just filled the head of the tube and its breadth was equal to the internal diameter of the tube; 3, the bubble filled the head and part of the tube, its breadth was the same as the internal diameter of the tube, and its height was greater than its breadth.

Analysis of pH. A model 290 pH meter (Pye Unicam) fitted with a combined microelectrode was used.

Viable counts. The contents of 10 bottles were bulked for each medium at each time interval. Quadruplicate viable counts were obtained from four plates of nutrient agar (Oxoid) incubated at 37°C.

RESULTS

Gas production in LPW, LRB, and BGBB. When a number of Bijou bottles containing LPW, LRB, or BGBB were inoculated with any of the three E. coli strains and incubated at 37 and 44°C, different amounts of gas were produced. This variability was assessed by counting the numbers of bottles having a gas category of 0, 1, 2, or 3. There was almost a wide spread of observations between the four gas categories.

The results obtained with NCTC 8623 are shown in the upper histograms of Fig. 1. The results obtained with NCTC 123 and the fecal isolate were similar.

Effect of buffer on gas production. Addition of buffer to the media considerably reduced the variability in gas production (Fig. 1, lower histograms). In buffered LPW and LRB, almost all of the bottles had a gas category of 3. The change was not so marked in buffered BGBB. The difference in the distribution of observations between the buffered and unbuffered media was highly significant for all three media (2 × 4x2 tests, P < 0.001).

Addition of buffer also improved the overall performance of the three media (Fig. 2). Bottle and gas scores increased faster when buffer was
present. By 24 h, the scores in buffered LPW and LRB had reached 100%, whereas in buffered BGBB, the bottle score had reached 95% and the gas score had reached 62%. By 48 h, these latter two scores reached 100 and 96%, respectively (Fig. 1).

Analysis of pH changes. The pH fell more rapidly and to a lower level in the unbuffered than in the buffered media, and at any given time, the pH in the unbuffered media was significantly lower (Student’s t tests) (Fig. 3). In LPW and LRB, the pH fell most rapidly during the first 12 h, but in BGBB, it decreased at a more constant rate during a 24-h period.

There was a clear correlation between the pH curves in Fig. 3 and the bottle and gas score curves in Fig. 2. When the bottle and gas scores rose rapidly (Fig. 2, LPW and LRB), the pH fell rapidly (Fig. 3, LPW and LRB), and when the bottle and gas scores rose more slowly (Fig. 2, BGBB), the pH fell more slowly (Fig. 3, BGBB).

However, for a given time and medium there was never any significant difference between the pH’s of different gas categories (F ratio tests on individual pH’s of 480 bottles having different gas categories).

Growth in buffered and unbuffered media. The results of growth in buffered and unbuffered media are shown in Table 1; conclusions are based on statistically significant effects (anovars). Counts at 12 and 24 h were much higher in buffered than in unbuffered media. Counts increased at 12 h and then decreased at 24 h (except for buffered BGBB, which increased for 24 h). The 24-h decrease was particularly marked in unbuffered LRB and BGBB, where the counts fell below the inoculum level.

Relationship between pH and growth. Figure 3 and Table 1 show that the pH fell more rapidly in unbuffered than in buffered media, whereas the viable counts increased more rapidly in buffered than in unbuffered media. In the presence of buffer, a decrease of one pH unit was
associated with increases in viable counts ranging from $269 \times 10^6$ (BGBB) to $382 \times 10^6$ (LPW), whereas in the absence of buffer, the increases were only $9.45 \times 10^6$ (LRB) to $30.37 \times 10^6$ (LPW). The difference between the buffered and unbuffered media was therefore about one to two orders of magnitude.

**DISCUSSION**

Gas production among identically prepared subcultures varied from gas category 0 (no visible gas) to gas category 3 (Fig. 1). This variability may be responsible for erroneous interpretation of the results of coliform multiple tube tests since there have been many reports of false-negative reactions (growth of *E. coli* without gas) in tubes during most-probable-number counts on water samples (2, 7). This has been interpreted as the chance cultivation of anaerogenic or environmentally damaged strains (5, 8). Our results show, however, that this need not be the case since variability arose among subcultures of known gas-producing strains. However, two of the three strains tested were culture collection types and not recent isolates from the water environment or feces. It is not known for certain how this may affect the interpretation of the frequency of non-gas-producing *E. coli* strains in the water environment.

Good gas production was obtained in buffered media with counts ranging from $370 \times 10^6$ to $941 \times 10^6$/ml; in unbuffered media, gas production was weak and variable, with counts of $14.5 \times 10^6$ to $75.3 \times 10^6$/ml (Fig. 1 and Table 1). These results can be compared with previous work (3) in which $40 \times 10^6$ to $391 \times 10^6$ coliform bacteria per ml were required to produce visible gas in lactose broth.

It has been known for some time that addition of buffer can increase gas production (6). However, our results show that inclusion of buffer

![Fig. 3. pH changes in unbuffered (V) and buffered (■) LPW, LRB, and BGBB at 44°C (E. coli NCTC 8623). Each point is the mean of 20 measurements, where each measurement was taken from a separate Bijou bottle. Vertical lines represent the standard deviation.](http://aem.asm.org/)
also decreases variability among subcultures. The virtual elimination of tubes with no visible gas (gas category 0) was especially important in this context (Fig. 1). These tubes represent a particular problem since, in the absence of further testing, they would be recorded as negative results in most-probable-number tests, thereby leading to underestimation of the coliform count.

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LITERATURE CITED