Substrate Utilization by Campylobacter jejuni and Campylobacter coli

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An attempt was made to elucidate in Campylobacter spp. some of the physiologic characteristics that are reflected in the kinetics of CO₂ formation from four ¹⁴C-labeled substrates. Campylobacter jejuni and C. coli were grown in a biphasic medium, and highly motile spiral cells were harvested at 12 h. Of the media evaluated for use in the metabolic tests, minimal essential medium without glutamine, diluted with an equal volume of potassium sodium phosphate buffer (pH 7.2), provided the greatest stability and least competition with the substrates to be tested. The cells were incubated with 0.02 M glutamate, glutamine, α-ketoglutarate, or formate, or with concentrations of these substrates ranging from 0.0032 to 0.125 M. All four substrates were metabolized very rapidly by both species. A feature of many of these reactions, particularly obvious with α-ketoglutarate, was an immediate burst of CO₂ production followed by CO₂ evolution at a more moderate rate. These diphasic kinetics of substrate utilization were not seen in comparable experiments with Escherichia coli grown and tested under identical conditions. With C. jejuni, CO₂ production from formate proceeded rapidly for the entire period of incubation. The rate of metabolism of glutamate, glutamine, and α-ketoglutarate by both species was greatly enhanced by increased substrate concentration. The approach to the study of the metabolism of campylobacters here described may be useful in detecting subtle changes in the physiology of cells as they are maintained past their logarithmic growth phase.

The nutritional and biochemical characteristics of campylobacters have been studied in great detail (8, 9, 19–22), but their isolation from the environment has not always been successful, even when epidemiological evidence strongly suggested that campylobacters were present (17). Smibert (21, 22) reviewed evidence that, while campylobacter cells in logarithmic growth phase are highly motile spirally curved rods, in old cultures they tend to become spherical or coccoid. These forms for the most part are not cultivable. This evidence has recently been confirmed by Merrell et al. (12) and Ng et al. (13). This phenomenon resembles one described by Colwell et al. (4) for Vibrio cholerae and other pathogens, which, when collected from the environment, often are not cultivable by standard techniques, although by certain tests they still appear to be viable.

To better understand the physiology of viable but not cultivable campylobacters (17), we need to develop additional information on the kinetics of utilization of some of their more rapidly metabolized substrates. In this study, we examined CO₂ formation from four such substrates by a strain of Campylobacter jejuni and a strain of C. coli. For both strains, there appeared to be, with some substrates, an initial burst of activity followed by a more moderate rate of metabolism. Furthermore, the kinetics of some of these reactions were greatly influenced by the concentration of the substrate. This work was done with highly motile cells known to be in logarithmic growth phase. These reactions may change as the cells are tested past this growth phase.

MATERIALS AND METHODS

Bacterial strains. C. jejuni ATCC 29428 was obtained from the American Type Culture Collection, Rockville, Md. C. coli WR, a human diarrheal isolate, was received from Walter Reed Army Hospital, Washington, D.C. The wild-type strain of Escherichia coli K-12 was obtained from our colleague, Michael E. Dobson.

Preparation of cells for metabolic experiments. Cells to be used in metabolic experiments were cultivated from frozen stock cultures. Following a passage on 5% sheep blood agar in an atmosphere of 5% O₂–10% CO₂–85% N₂, the cells were passaged twice at 37°C in biphasic medium (18). The incubation time was 12 h unless otherwise indicated. Cultures from the second passage were checked for morphology and motility, and only monomorphic cultures with highly motile spiral cells were used. The cells were harvested by centrifugation (17,500 × g for 15 min) and suspended in phosphate buffer or other diluent, as described below, to an optical density at 625 nm of 0.5 to 1.0 and diluted to the desired cell concentration for metabolic tests. The buffer consisted of 0.08 M monopotassium phosphate adjusted to pH 7.2 with NaOH. MgSO₄ was also added to a final concentration of 0.005 M. In most cases, the buffer was diluted prior to use with an equal volume of minimal essential medium (MEM) (Flow Laboratories, Inc., McLean, Va.) without glutamine.

The protein content of the cell preparations was determined by the Bio-Rad protein assay procedure (2), as described previously (23).

Metabolic experiments. L-[U-¹⁴C]glutamic acid, L-[U-¹⁴C]glutamine, L-[U-¹⁴C]-α-ketoglutaric acid, and L-[¹⁴C]formic acid were obtained from New England Nuclear Corp., Boston, Mass. These compounds were diluted with their unlabeled counterparts to provide 0.05 μCi per tube and a 0.02 M concentration unless otherwise indicated. The formation of CO₂ was determined, as previously described (23), with tubes (16 by 100 mm) containing final volumes of 0.4 ml. The tubes were closed with rubber stoppers from which were suspended plastic cups containing filter paper wicks. The tubes were shaken in a water bath at 37°C. The reactions were stopped by injecting 0.15 ml of Hyamine hydroxide (New England Nuclear) into each cup and 0.1 ml
Table 1. Influence of the suspending medium on CO₂ production from 0.02 M glutamate by C. jejuni

<table>
<thead>
<tr>
<th>Suspensing medium</th>
<th>Amt of CO₂ (μmol/mg of protein) after incubation at 37°C for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 h</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>6.2</td>
</tr>
<tr>
<td>Brain heart infusion broth containing 1% yeast extract</td>
<td>2.1</td>
</tr>
<tr>
<td>Phosphate buffer + MEM vitamins</td>
<td>7.4</td>
</tr>
<tr>
<td>Phosphate buffer + MEM vitamins and essential and nonessential amino acids</td>
<td>6.7</td>
</tr>
<tr>
<td>MEM diluted 1:2 with phosphate buffer</td>
<td>9.8</td>
</tr>
</tbody>
</table>

* Protein was used at 16 μg per tube.
* The medium was diluted 1:8 with phosphate buffer.
* No glutamine.

The experiment illustrated in Table 1 was done with cells harvested from biphasic medium at 12 h. The influence of the time of harvest on the kinetics of glutamate catabolism by C. jejuni is shown in Fig. 1. For this experiment, the cells were grown in shaken flasks containing brain heart infusion broth supplemented with 1% yeast extract, rather than in a stationary biphasic medium. Moderate differences in kinetics were obtained for cells harvested at 8, 12, and 24 h, but specific activity was greatly reduced by 36 h and was almost negligible by 48 h. Plate counts (Fig. 1, inset) indicated that, by 48 h, the number of culturable bacteria in the suspension had greatly declined. Since the highest activity was obtained with the 12-h harvest, this time interval was used for cell harvests in subsequent experiments.

The optimal cell density and the effect of washing the cells with phosphate buffer or MEM (1:2) prior to testing were investigated next. The best results were obtained when the density of the cell suspensions was adjusted spectrophotometrically to provide no more than 0.08 mg of cell protein per tube. Comparable results were obtained with washed and unwashed cells, suggesting that chemical contamination from medium constituents was not great enough in

### Results

Table 1 illustrates some of the experiments designed to determine which diluent was the most appropriate for kinetic studies of substrate utilization by campylobacters. These tests were done with C. jejuni cells and with glutamate as the substrate. When the cells were suspended in unsupplemented phosphate buffer, activity was relatively low and the rate decreased with time. When a rich medium was used as the diluent (brain heart infusion broth with 1% yeast extract [Difco Laboratories, Detroit, Mich.]), activity was very low, most probably because of competing constituents in the medium, even though the medium was diluted 1:8 with phosphate buffer. There was an increase in activity between 1 and 2 h, possibly because of competing metabolite depletion or campylobacter multiplication. Since niacin is required for the growth of campylobacters and other vitamins are stimulatory (21), the phosphate buffer was enriched with the vitamin constituents of MEM to determine whether the vitamins would stabilize the cells without competing with glutamate metabolism. The rate of CO₂ formation was improved over that obtained with unsupplemented phosphate buffer, and variation among replicate experiments was reduced. On the other hand, when phosphate buffer was enriched with MEM vitamins plus MEM essential amino acids without glutamine and the nonessential amino acids, there was evidence of competition at 0.5 and 1 h but increased activity at 2 h. Somewhat similar results were obtained when nonessential amino acids were omitted (data not shown). When MEM without glutamine was diluted with phosphate buffer, the most satisfactory results in terms of activity and linearity were obtained with a 1:2 dilution (Table 1). In the experiments described below, MEM diluted 1:2 with phosphate buffer was used.

![Graph showing CO₂ formation from glutamate by C. jejuni](image-url)
Formate was 0.02 M. With the appropriate concentration became reaction were required. of concentrations was facilitated comparative entirely satisfactory glycerol prior 0.4 arithmetic methods. were the same. At lower acetic acid was used to stop the release chemically when the cells were frozen. Although this procedure not subtracted from the other values in Fig. 2, to better illustrate the initial burst of activity. The possibility that this activity was due to free enzymes released from the cells was checked by quickly removing the cells from the diluent by centrifugation and filtration. No activity was detected in the cell-free diluent. The initial burst of activity was usually followed, at 5 to 15 min, by a second, less rapid, rate of activity. The two-rate kinetics were particularly obvious with α-ketoglutarate as the substrate. Considerable variation was encountered in repeated experiments with α-ketoglutarate, although the two-rate kinetics usually prevailed (data not shown). This variability was possibly due to the instability of this compound or to the presence of trace amounts of 13C-labeled impurities or both. With C. jejuni cells incubated with formate, the initial burst of activity continued at a virtually undiminished rate for all 3 h of incubation. This was demonstrated only in an experiment with a low cell concentration that did not exhaust the substrate.

Figure 2 also illustrates differences in the kinetics of substrate utilization between the two species. C. jejuni utilized glutamine more rapidly than glutamate and formate more rapidly than α-ketoglutarate. The reverse was true of C. coli. Whether these results reflected species or strain differences is not known.

It appeared of interest to determine whether another bacterium unrelated to Campylobacter would show the same diphasic kinetics and high rate of CO₂ production if grown

unwashed cells to compete with the substrates tested. Unwashed cells were used in most of the subsequent experiments. Attempts were also made to use cells that had been subjected to controlled-rate freezing and stored at −100°C in a liquid-nitrogen chest. The cells were harvested from logarithmic growth phase and suspended in small volumes of 0.2 or 0.4 M sucrose, 15% glycerol, or a combination of sucrose and glycerol prior to being frozen. Although this procedure was entirely satisfactory for Legionella spp. and greatly facilitated comparative studies (23), it proved to be unsuitable for campylobacters. Only a small percentage of the activity was retained by cells that had been frozen.

It was also necessary to determine the most satisfactory method for terminating the reaction of cells incubated with formate. Both 25% trichloroacetic acid and undiluted acetic acid added to stop the reaction caused the chemical release of some CO₂ from formate. However, when due precautions were taken not to exceed 0.1 ml as the volume of acid injected in each tube and not to exceed 30 min for secondary incubation (to trap CO₂ to the Hyamine), chemical release of CO₂ was not excessive. With 25% trichloroacetic acid or undiluted acetic acid, it was estimated that about 0.7 and 0.1%, respectively, of the total CO₂ from formate was released chemically when the concentration of formate was 0.02 M. With the appropriate corrections, the results obtained by either method were the same. At lower concentrations of formate, proportionally larger corrections were required. Undiluted acetic acid was used to stop the reaction with formate in subsequent experiments.

Figure 2 illustrates the kinetics of CO₂ production by C. jejuni and C. coli from four substrates. The substrate concentration was 0.02 M, and the conditions for the experiments were as described above. A feature common to many of these reactions was the very rapid release of CO₂ during the first few minutes. In fact, a significant amount of CO₂ was released by the time defined as zero. Time zero, by our definition, was preceded by the time required to fill and stopper the tubes in ice-water plus a 2-min period of thermal equilibration at 37°C. The values obtained at time zero were not subtracted from the other values in Fig. 2, to better illustrate the initial burst of activity. The possibility that this activity was due to free enzymes released from the cells was checked by quickly removing the cells from the diluent by centrifugation and filtration. No activity was detected in the cell-free diluent. The initial burst of activity was usually followed, at 5 to 15 min, by a second, less rapid, rate of activity. The two-rate kinetics were particularly obvious with α-ketoglutarate as the substrate. Considerable variation was encountered in repeated experiments with α-ketoglutarate, although the two-rate kinetics usually prevailed (data not shown). This variability was possibly due to the instability of this compound or to the presence of trace amounts of 13C-labeled impurities or both. With C. jejuni cells incubated with formate, the initial burst of activity continued at a virtually undiminished rate for all 3 h of incubation. This was demonstrated only in an experiment with a low cell concentration that did not exhaust the substrate.

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It appeared of interest to determine whether another bacterium unrelated to Campylobacter would show the same diphasic kinetics and high rate of CO₂ production if grown
and tested under identical conditions. Such an experiment was done with the K-12 strain of *E. coli* (Fig. 3). *E. coli*, in contrast to campylobacters, metabolizes glucose, a constituent of MEM, and is capable of replication in such a medium. The kinetics of CO₂ production from the four substrates were quite different from those of the campylobacters. CO₂ production from glutamate and glutamine was low for 1 h and increased during the next 2 h. This increase can be attributed to exhaustion of glucose, which undoubtedly was utilized preferentially, or to cell replication, or both. α-Ketoglutarate and formate were metabolized more rapidly than the other two substrates, but even for these substrates, the rates were generally lower than those obtained with the campylobacters. Although it cannot be excluded that the rates during the first 30 min were somewhat higher than during the following 30 min, the obvious diphasic kinetics of campylobacter metabolism were not seen.

**FIG. 3.** Experiments comparable to those shown in Fig. 2 carried out with *E. coli* K-12. *E. coli* was grown in the medium used for campylobacters, except that, because of more rapid growth, the cells were harvested after 6 instead of 12 h of incubation. Bacterial protein content was approximately 30 to 40 μg per tube.

**FIG. 4.** CO₂ formation by *C. jejuni* and *C. coli* from four substrates used at different concentrations as shown. Each symbol represents CO₂ evolution measured after 1 h of incubation. The values obtained after thermoequilibration (0 h) were subtracted. The procedures were otherwise identical to those described for Fig. 1 and 2. Note that both abscissa and ordinate depict geometric progressions.
Figure 4 illustrates CO₂ production by the two species of *Campylobacter* when the concentrations of substrate varied over a wide range (from 0.0032 to 0.125 M). The values shown represent the activities during the first hour, following subtraction of the time zero values. Somewhat similar trends were seen when the activities were measured after 7 h of incubation (not shown). Three of the substrates (glutamate, glutamine, and α-ketoglutarate) were utilized at substantially higher rates as the concentrations were increased. This suggests that these substrates were metabolized through multiple pathways and that saturation of all the enzymes involved was not achieved under the conditions of these experiments. This was not true of formate, which was utilized at approximately the same rate irrespective of concentration. As indicated above, when formate was tested at the lower concentrations, large corrections were required, and some of the results were not sufficiently accurate to be shown. Even with these limitations, the difference between the metabolism of formate and that of the other three substrates examined under these conditions is quite obvious.

**DISCUSSION**

The buffer used to suspend cells in our metabolic experiments was in part based on the results of Hänninen (7) and Doyle and Roman (5), which indicated that most *Campylobacter* strains are stabilized by a medium of moderate electrolyte content. To further stabilize the cells, it was necessary to select a medium that would not unduly compete with the substrates to be tested. MEM without glutamine, diluted 1:2 with 0.08 M phosphate buffer, appeared to fulfill this requirement.

The four substrates used in this study are rapidly metabolized by campylobacters. Our results are widely supported by oxygen uptake, electron transport, enzymatic, and nutritional studies reported by other investigators. Alexander (1) found that glutamate and α-ketoglutarate serve as excellent energy sources when added to a basal medium for the growth of *V. fetus*. Kiggins and Plaistrider (10) demonstrated in manometric experiments that glutamic acid and glutamine are actively oxidized, glutamate more rapidly than glutamine, by whole cells and crude cell extracts of *V. fetus*. α-Ketoglutarate is oxidized well by cell extracts, but only slightly or not at all by whole cells. Lecce (11) studied electron transport by a large number of *V. fetus* strains and found that formate and α-ketoglutarate are excellent electron donors. However, growth of the vibrios is inhibited when formate concentrations of 0.5% are added to the medium.

Recent work has focused on the importance of formate as a substrate for *C. sputorum* (14–16) and for *C. jejuni* and other species (3, 6, 9). Hoffman and Goodman (9) studied the respiratory activities of membrane vesicles of *C. jejuni* and observed that the activities with formate and hydrogen were 50- to 100-fold higher than with some of the other substrates, such as succinate, lactate, malate, or NADH. These results were consistent with their observations that the dehydrogenases of hydrogen and formate were located on the periplasmic face of the cytoplasmic membrane, while the other enzymes were inside the cytoplasmic membrane. These authors concluded that formate and hydrogen probably serve as major energy sources for growth. Goodman and Hoffman (6) developed a rapid test for hydrogenase activity by using the redox dye benzyl violen and found that by this test, *C. jejuni* and *C. coli* were positive, while the other *Campylobacter* spp. were negative. When formate was tested with the same dye, the test was positive with all species.

The advantages of studying the metabolic activities of campylobacters by the method described in this paper are flexibility and sensitivity. A large number of substrates can be used singly or in combination with unlabeled substrates (23). With due caution, this method can be applied to formate as the substrate.

The rapid metabolism of formate, especially by *C. jejuni*, can best be interpreted by assuming that this compound is utilized in the periplasmic spaces, as observed by Hoffman and Goodman (9), and that transport across the cytoplasmic membrane is not required. The same may be true of the initial metabolic burst of activity found with α-ketoglutarate. This reaction presumably produced succinate, which then required transport for further utilization, and this is reflected in the more moderate, secondary rate of activity.

It is difficult to interpret the significance of the differences in the rate of substrate utilization by the two species or the differences encountered with both species in rates of metabolism as the substrate concentrations were increased. Such an interpretation would require the extension of this work to other strains and the elucidation of the carbon flux between pathways involved in the reactions described. It is tempting to speculate, however, that this approach to the study of the metabolism of campylobacters may provide sufficient detail to permit us to evaluate subtle changes in metabolism as the organisms are maintained beyond their logarithmic growth phase or under natural conditions that are encountered in the environment.

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**LITERATURE CITED**