JOURNAL OF BACTERIOLOGY, Nov. 1989, p. 6213–6217
0021-9193/89/116213-05$02.00/0

Vol. 171, No. 11

Anaerobic Fermentation Balance of *Escherichia coli* as Observed by In Vivo Nuclear Magnetic Resonance Spectroscopy

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Received 27 April 1989/Accepted 1 August 1989

Fermenting anaerobic cultures of *Escherichia coli* were observed by the nonintrusive technique of in vivo, whole-culture nuclear magnetic resonance. Fermentation balances were calculated for hexoses, pentoses, sugar alcohols, and sugar acids. Substrates more reduced than glucose yielded more of the highly reduced fermentation product ethanol, whereas more-oxidized substrates produced more of the less-reduced fermentation product acetate. These relationships were made more obvious by the introduction of *ldhA* mutations, which abolished lactate production, and Δ*frd* mutations, which eliminated succinate. When grown anaerobically on sugar alcohols such as sorbitol, *E. coli* produced ethanol in excess of the amount calculated by the standard fermentation pathways. Reducing equivalents must be recycled from formate to account for this excess of ethanol. In mutants deficient in hydrogenase (*hydB*), ethanol production from sorbitol was greatly decreased, implying that hydrogen gas released from formate by the formate-hydrogen lyase system may be partially recycled, in the wild type, to increase the yield of the highly reduced fermentation product ethanol.

The well-known laboratory organism *Escherichia coli* is a facultative anaerobe. In the absence of oxygen and alternative electron acceptors such as nitrate (11), *E. coli* ferments sugars and their derivatives to a mixture of acids and ethanol (D. P. Clark, FEMS Microbiol. Rev., in press). This has been referred to as the mixed-acid fermentation because a mixture of acetic, lactic, formic, and succinic acids is produced (8, 19). The ratio of these products has been measured by classical chemical methods for glucose fermentation under various culture conditions (7, 17, 20). Generally speaking, the overall proportion of lactic acid increases under acidic conditions, while the ratio of ethanol to acetate remains approximately 1:1 (1, 8). We have shown that the fermentative lactate dehydrogenase, which is responsible for lactic acid synthesis, is conjointly induced by acidic and anaerobic conditions (13). On the other hand, earlier reports that the nature of the buffer affects the fermentation product ratio (8, 20) were not confirmed (13). We believe that the difference in buffering strength and pK between various buffers may give the illusory appearance of such an effect. However, when these factors are allowed for, only the overall pH maintained is significant (13).

A factor which greatly affects the fermentation product ratio is the redox level of the substrate relative to glucose. Thus, sugar alcohols, which are more reduced than the corresponding hexoses, must yield a higher proportion of more-reduced fermentation products in order to achieve hydrogen balance (Clark, in press). As far as we know, no systematic survey has been done to relate the redox level of the fermentable substrate to the composition of the fermentation product. Here we report such a study based on the use of in vivo nuclear magnetic resonance (NMR) of anaerobic cultures (15, 16). The use of in vivo NMR avoids compromising anaerobic conditions by withdrawing samples, and it simultaneously analyzes all fermentation products. Inaccuracies due to sample manipulation and fractionation are avoided. We studied both wild-type and mutant *E. coli* K-12 by this technique. We used mutants defective in fumarate reductase (*frd*) and lactate dehydrogenase (*ldhA*) to eliminate succinate and lactate, respectively. The simplified fermentation mixtures in such mutants were related more obviously to the redox state of the substrate.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. All bacteria used were strains of *E. coli* K-12 (Table 1). Cultures for NMR were grown overnight in M9 medium (14) containing 0.4% carbon sources. They were then diluted 1:50 into fresh M9 medium containing 100 mM sugar or sugar derivative to be fermented. For this study we used the *ldhA39* mutation to eliminate lactic acid production (13). Production of succinic acid was eliminated by the introduction of a deletion mutation in the *frd* operon by P1 cotransduction with a nearby Tn*10* insertion. Hydrogenase activity was eliminated by using the *hydB* mutant DB12 provided by D. Boxer. Such mutants are defective in all of the hydrogenase isoenzymes of *E. coli* (18, 21).

NMR. These experiments were modeled on the work of Ogino et al. (15, 16), who monitored the synthesis of fermentation products by performing in vivo NMR scans of whole cultures. *E. coli* cells were grown in M9 medium (pH 7.2) in the presence of 100 mM glucose as the sole carbon source. When cell density reached approximately 5×10^8/ml, the cells were collected by centrifugation at 7,000 rpm ($5,900 \times g$) for 2 min at 5°C. The cell pellets were suspended in M9 buffer and washed twice with the same buffer. The cells were finally suspended in M9 buffer (pH 7.2) containing 100 mM glucose at a cell density of 5×10^8/ml. A 0.9-ml sample of cell suspension was placed in a 5-mm-diameter NMR tube with 0.1 ml of D_2O. The suspension was then bubbled with argon gas to remove all oxygen. The cell suspension was incubated at 37°C for 4 h, after which proton NMR spectra were measured with a Varian VXR-500 spectrometer operating at 500 MHz. The parameters used were the following: pulse width, 14.5 μs; delay time, 5 s; and 50 acquisitions per spectrum. The water peak was suppressed by irradiation at high power during the preacquisition delay time. The field was locked onto the solvent D_2O, and internal H_2O was used as a reference peak (4.65 ppm). Dimethyl sulfone (20 mM) was used as an internal standard (3.12 ppm)

TABLE 1. Bacterial strains

Strain	Relevant characteristics	Source reference
LCB320	*thr leu thi lacY tonA rpsL*	M. C. Pascal
FMJ112	*ldhA* of LCB320	F. Mat-Jan et al. (13)
DC1025	Δ*frd* of LCB320	P1 (DW12) × LCB320
DC1026	Δ*frd* of FMJ112	P1 (DW12) × FMJ112
DC861	*mel supF*	D. P. Clark
W1485	Wild type	B. Bachmann
P4X	Hfr P4X *metB relA*	D. Boxer
DB12	*hydB1028* of P4X	D. Boxer
MC4100	*araD* Δ(*argF-lac*) *relA ptsF rbsR rpsL thiA*	M. Casabadan
DW12	Δ*frd-102 zjd*::Tn*10*	R. Gunsalus

for quantitation of unknowns. From the integration of peak heights, the concentration of unknowns was calculated. Proton chemical shifts are reported in parts per million downfield from tetramethylsilane. Authentic samples of fermentation products were dissolved in the same M9 buffer in order to assign the NMR signals of the metabolites. The metabolites shown in the figures are (from low to high field) succinate, pyruvate, acetate, lactate, and ethanol. A formate peak (not shown) was also identified at 8.39 ppm. To assess the accuracy of these experiments, we ran three separate experiments in which LCB320 (wild type) fermented glucose. The standard deviation for the proportion of individual fermentation products was calculated to be 6.5%.

During the standard 4-h incubation there was little cell growth. For example, the cell density of the wild type increased 10 to 20% in typical experiments. The proportion of glucose consumed after 4 h ranged from 5 to 10% of the initial concentration, depending on the strain and conditions. Thus, excess substrate was still available at the time of assay.

RESULTS

Redox balance and fermentation. The fermentation of glucose or another hexose could be balanced by lactate alone or by a 50:50 mixture of ethanol plus acetate (Fig. 1 and Table 2). Sugar alcohols should give more ethanol, the most reduced of these products, whereas hexonic and hexuronic acids should give successively more acetic acid. Theoretical balances are given in Table 2 and illustrated in Fig. 1. We therefore tested the series of substrates sorbitol, glucose, gluconate, and glucuronate against LCB320 (wild type in its fermentation response) and a series of derivatives carrying

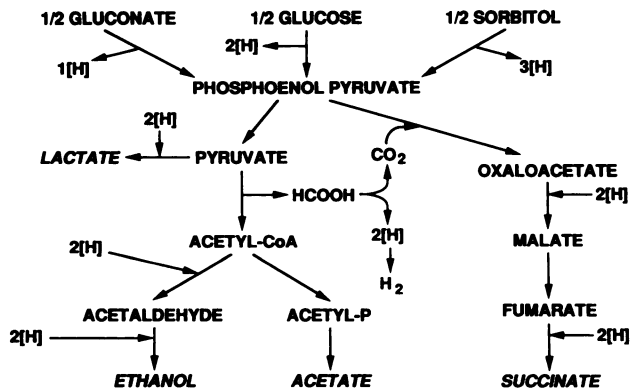

FIG. 1. Fermentation pathways of *E. coli*.

TABLE 2. Reducing equivalents

Molecule	Formula	No. of H per C6[a]
Substrates		
Sorbitol	$C_6H_{14}O_6$	+6
Glucose	$C_6H_{12}O_6$	+4
Gluconate	$C_6H_{12}O_7$	+2
Glucuronate	$C_6H_{10}O_7$	0
Products		
Ethanol	CH_3CH_2OH	−8
Lactate	$CH_3CHOHCOOH$	−4
Acetate	CH_3COOH	0
Succinate	CH_2COOH | CH_2COOH	−8

[a] +, Produced during glycolysis; −, consumed during fermentation.

the *ldhA39* mutation or an *frd* deletion or both. The parent, LCB320, fermented glucose to a mixture of ethanol, acetate, lactate, and succinate (Fig. 2). A peak due to formate was also found downfield at 8.39 ppm (not shown in Fig. 2). Introduction of the *ldhA* mutation abolished lactic acid production, as seen in strain FMJ112. However, the ratio of ethanol to acetate and the proportion of succinate are little affected for most substrates. Introduction of an *frd* deletion into LCB320 gave strain DC1025 and resulted in the disappearance of succinic acid, except when glucose was the carbon source. The alternative pathway(s) to succinate has been discussed by Creaghan and Guest (5). When both *ldhA* and Δ*frd* were present, as in DC1026, both lactate and succinate were absent. The decrease of ethanol relative to acetate as the growth substrate becomes increasingly oxidized can be most clearly seen in DC1026, in which the only other product is formate (Table 3).

Other substrates. In addition to the growth substrates listed in Table 3, we surveyed several other substrates, but only for the wild type and the *ldhA* mutant. We found that pentose sugars (e.g., arabinose) gave fermentation mixtures very similar to those of glucose (data not shown). When deoxysugars such as fucose were used, no ethanol or lactate was produced. However, in addition to acetate and succinate, a fermentation product not found with other carbon sources was observed (data not shown). This is presumably L-1,2-propanediol (2, 3). *E. coli* can grow on pyruvate anaerobically. Since no NADH is produced during glycolysis with this substrate, it is not necessary to get rid of reducing equivalents by making reduced fermentation products. As might be expected, no ethanol was produced from pyruvate, and only small amounts of lactate and succinate were formed. By far the major product was acetate (data not shown).

We have also tested *adhE* mutations, which result in a lack of the fermentative alcohol dehydrogenase, in several genetic backgrounds (4, 12). Although such mutants cannot grow anaerobically on hexoses, pentoses, or sugar alcohols, they do produce fermentation products when incubated anaerobically with these substrates. As expected, no ethanol is produced, and the fermentation product mixture is hence "unbalanced." Apart from this, the fermentation product mixtures are very similar to those seen with the isogenic adh^+ parents (data not shown).

Hydrogen recycling. The results given above can all be accommodated by the standard fermentation pathways (Fig. 1 and Table 2) except for one anomalous observation. The excessive proportion of ethanol produced from sorbitol can

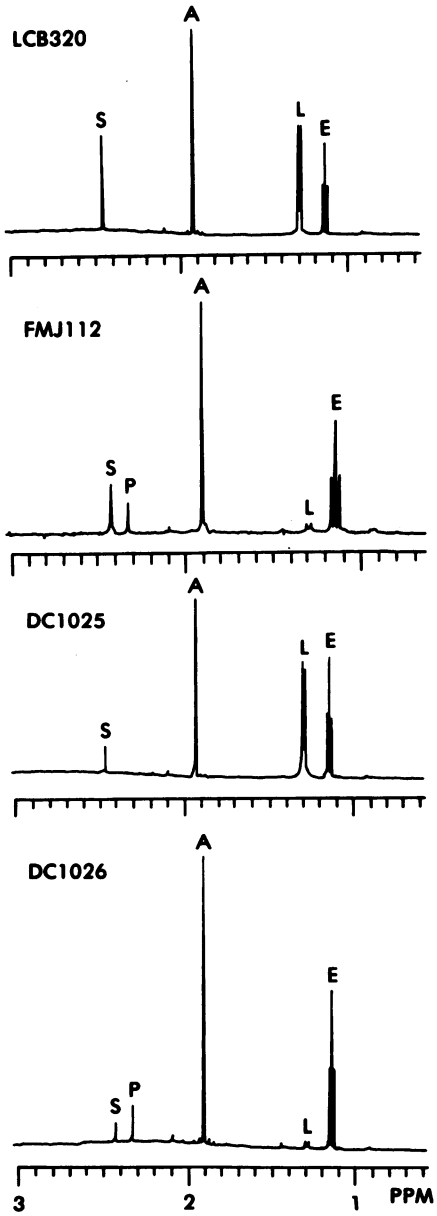

FIG. 2. NMR scans of glucose fermentation. Cultures were incubated anaerobically in minimal medium with glucose. From top to bottom the strains are LCB320 (wild type), FMJ112 (*ldhA*), DC1025 (Δ*frd*), and DC1026 (*ldhA* Δ*frd*). The fermentation products are ethanol (E), lactate (L), acetate (A), pyruvate (P), and succinate (S).

be accounted for only by the recycling of hydrogen released from formate by the formate-hydrogen lyase system (8; Clark, in press). We therefore compared the hydrogenase-defective mutant DB12 with its parent, P4X. The *hyd* mutation in this mutant is pleiotropic and abolishes all of the hydrogenase isoenzymes of *E. coli* (18, 21). When the *hyd* mutant was grown on sorbitol, the ratio of ethanol to acetate was greatly reduced relative to that in the parental strain, P4X (Fig. 3 and Table 4). The production of succinate, another highly reduced product, was also decreased substantially in the *hyd* mutant (Table 4). However, the presence of the *hyd* mutation had little effect when cultures were grown on glucose. This is not surprising, since the original over-

TABLE 3. Fermentation balances[a]

Strain	ETOH	LCT	SUC	FMT
LCB320 wild type				
Sorbitol	605	49	79	114
Glucose	116	222	52	37
Gluconate	72	107	58	17
Glucuronate	18	16	0	18
Maltose	111	0	182	23
FMJ112 *ldhA*				
Sorbitol	746	0	63	126
Glucose	111	0	54	35
Gluconate	33	0	68	20
Glucuronate	18	0	50	11
Maltose	80	0	91	9
DC1025 Δ*frd*				
Sorbitol	884	59	0	138
Glucose	210	353	23	52
Gluconate	58	79	0	23
Glucuronate	33	31	0	10
Maltose	97	42	0	18
DC1026 *ldhA* Δ*frd*				
Sorbitol	585	0	0	104
Glucose	94	0	8	34
Gluconate	58	0	0	35
Glucuronate	21	0	0	15
Maltose	144	0	0	33

[a] Fermentation balances are set relative to a value of 100 for acetate, which is a major product under all the conditions tested here. ETOH, Ethanol; LCT, lactate; SUC, succinate; FMT, formate.

production of ethanol was observed only with sugar alcohols.

DISCUSSION

The key issue in fermentation is the recycling of reduced NADH to regenerate the oxidized form, NAD^+, so that glycolysis may continue (8; Clark, in press). Since the amount of NADH to be recycled varies with the nature of the substrate, the composition of the fermentation product mixture must vary as well. Hexoses such as glucose or fructose produce 2 NADH per C_6 compound upon conversion to pyruvate, whereas hexitols (e.g., sorbitol or mannitol) produce 3 NADH per C_6 (Fig. 1). Conversely, the more-oxidized sugar derivatives such as hexonic and hexuronic acids yield less than 2 NADH per C_6 (Fig. 1 and Table 2). To achieve a proper fermentation balance, it is necessary to match the NADH produced with the NADH consumed by

TABLE 4. Hydrogenase and excess ethanol[a]

Product	Amt produced with the following substrate:			
	Sorbitol		Glucose	
	P4X	DB12	P4X	DB12
Acetate	100	100	100	100
Ethanol	650	379	102	131
Lactate	0	23	185	240
Succinate	210	45	85	92
Formate	94	97	26	44
E + S[b]	860	426	187	222

[a] Strains P4X wild type and DB12 *hydB* were incubated anaerobically with sorbitol or glucose as growth substrate. Amounts of fermentation products are given relative to the amount for acetate, which was set at 100.

[b] E + S, Sum of ethanol plus succinate, relatively the two most reduced fermentation products.

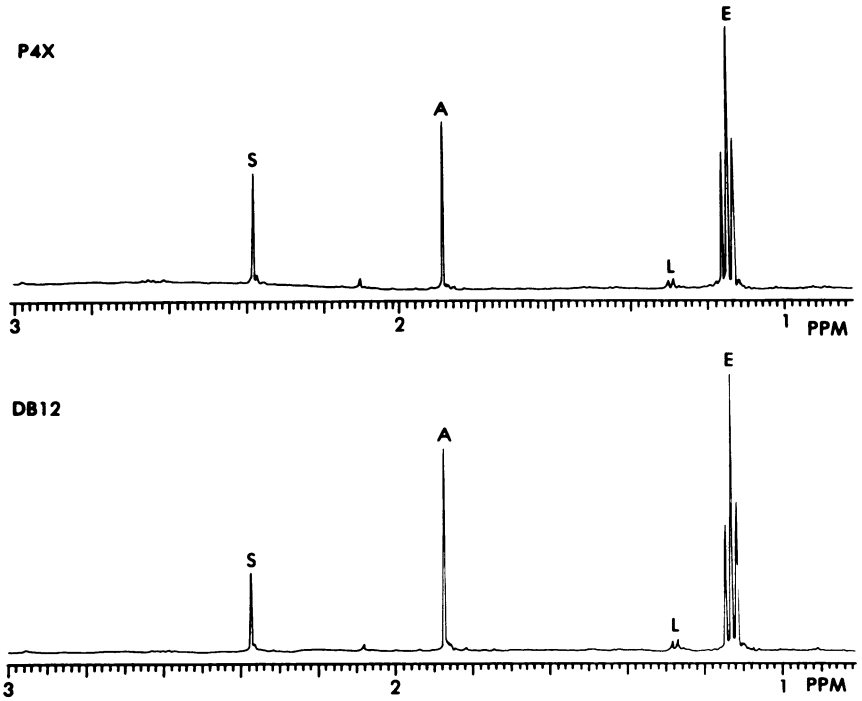

FIG. 3. Effect of hydrogenase mutation. Strains P4X (wild type) and DB12 (*hydB*) were incubated anaerobically with sorbitol. The abbreviations are as in Fig. 2.

excretion of fermentation products (Clark, in press). In practice, *E. coli* uses a mixture of ethanol, lactate, and acetate, all of which consume different amounts of H per C_6 (Table 2). By varying the proportions of these it is possible to match the substrate, thus achieving redox balance (Fig. 1). Table 2 lists the reducing equivalents produced and consumed by various substrates and fermentation products.

Since it is possible to balance most fermentations by mixing ethanol and acetate in appropriate proportions, the production of lactate is unnecessary. As expected, *ldh* mutants which lack lactate dehydrogenase show no anaerobic growth defects (13; Clark, in press). Production of ethanol is necessary only for substrates more reduced than glucuronate. Hence, *adhE* mutants, which lack alcohol dehydrogenase, cannot grow anaerobically on sorbitol, glucose, or gluconate but will ferment glucuronate (6, 10). Mutants defective in phosphotransacetylase (*pta*) cannot produce acetic acid and cannot grow anaerobically on any of these substrates (6, 9).

Eliminating both acetate and ethanol production by mutations in both *adhE* and *pta* restores the ability to ferment glucose, a function which is defective in either mutant singly (4, 10). Such *pta adhE* double mutants rely on lactate as the major fermentation product and are restricted to growth on sugars themselves. They are unable to use either sugar alcohol or acid derivatives, since they can cope with neither more nor fewer reducing equivalents than 4 H per C_6 (10).

The results of our NMR measurements agree with the known pathways (Fig. 1) and the calculated redox balances shown in Table 2. Elimination of lactate or succinate or both makes the redox balance simpler to observe. The proportion of lactic acid relative to the concentration of ethanol plus acetate depends on medium acidification (13) and is much higher when glucose is the carbon source rather than maltose or xylose, which acidify the culture medium much less. One anomalous observation was the overproduction of ethanol from sorbitol as growth substrate. This could be accounted for only if extra reducing equivalents were transferred from formic acid to ethanol synthesis. In accord with this idea, elimination of hydrogenase activity greatly decreased the production of ethanol from sorbitol. In addition, the amount of succinate, another relatively reduced molecule, was also decreased. The overall proportion of succinate is much less than that of ethanol. However, the relative decrease in succinate production caused by the *hydB* mutation was in fact greater than that seen for ethanol. Presumably hydrogen recycled by hydrogenase can be readily used by the membrane-bound fumarate reductase for the production of succinate. One remaining anomaly is that strains deleted for fumarate reductase still produce 15 to 40% of the normal level of succinate, but only when growing on glucose. Possible alternative pathways for succinate generation have been discussed by Creaghan and Guest (5). However, no conclusive evidence presently exists for or against these alternatives, namely, the glyoxylate cycle, the reverse operation of succinate dehydrogenase, or a route via succinate semialdehyde.

ACKNOWLEDGMENT

This research was supported by a grant to David Clark from the U.S. Department of Energy, Office of Basic Energy Sciences (contract DE-FG02-88ER13941).

LITERATURE CITED

1. **Blackwood, A. C., A. C. Neish, and G. A. Ledingham.** 1956. Dissimilation of glucose at controlled pH values by pigmented and non-pigmented strains of *Escherichia coli*. J. Bacteriol. **72**:497–499.
2. **Boronat, A., and J. Aguilar.** 1981. Metabolism of L-fucose and L-rhamnose in *Escherichia coli*. Differences in induction of propanediol oxidoreductase. J. Bacteriol. **147**:181–185.
3. **Chen, Y. M., E. C. C. Lin, J. Ros, and J. Aguilar.** 1983. Use of

operon fusions to examine the regulation of the L-1,2-propanediol oxidoreductase gene of the fucose system in *Escherichia coli* K-12. J. Gen. Microbiol. **129**:3355–3362.
4. **Clark, D. P., P. R. Cunningham, S. G. Reams, F. Mat-Jan, R. Mohammedkhani, and C. R. Williams.** 1988. Mutants of *Escherichia coli* defective in acid fermentation. Appl. Biochem. Biotechnol. **17**:163–173.
5. **Creaghan, I. T., and J. R. Guest.** 1978. Succinate dehydrogenase dependent nutritional requirement for succinate in mutants of *Escherichia coli* K12. J. Gen. Microbiol. **107**:1–13.
6. **Cunningham, P. R., and D. P. Clark.** 1986. The use of suicide substrates to select mutants of *Escherichia coli* lacking enzymes of alcohol fermentation. Mol. Gen. Genet. **205**:487–493.
7. **Dawes, E. A., and S. M. Foster.** 1956. The formation of ethanol in *Escherichia coli*. Biochim. Biophys. Acta **22**:253–265.
8. **Gottschalk, G.** 1985. Bacterial metabolism, 2nd ed. Springer-Verlag, New York.
9. **Guest, J. R.** 1979. Anaerobic growth of *Escherichia coli* K12 with fumarate as terminal electron acceptor. Genetic studies with menaquinone and fluoroacetate-resistant mutants. J. Gen. Microbiol. **115**:259–271.
10. **Gupta, S., and D. P. Clark.** 1989. *Escherichia coli* derivatives lacking both alcohol dehydrogenase and phosphotransacetylase grow anaerobically by lactate fermentation. J. Bacteriol. **171**: 3650–3655.
11. **Ingledew, W. J., and R. K. Poole.** 1984. The respiratory pathways of *Escherichia coli*. Microbiol. Rev. **48**:222–271.
12. **Lorowitz, W., and D. Clark.** 1982. *Escherichia coli* mutants with a temperature-sensitive alcohol dehydrogenase. J. Bacteriol. **152**:935–938.
13. **Mat-Jan, F., K. Y. Alam, and D. P. Clark.** 1989. Mutants of *Escherichia coli* deficient in the fermentative lactate dehydrogenase. J. Bacteriol. **171**:342–348.
14. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Ogino, T., Y. Arata, and S. Fujiwara.** 1980. Proton correlation nuclear magnetic resonance study of metabolic regulation and pyruvate transport in anaerobic *Escherichia coli* cells. Biochemistry **19**:3684–3691.
16. **Ogino, T., Y. Arata, S. Fujiwara, H. Shoun, and T. Beppu.** 1978. Proton correlation nuclear magnetic resonance study of anaerobic metabolism of *Escherichia coli*. Biochemistry **17**:4742–4745.
17. **Paege, L. M., and M. Gibbs.** 1961. Anaerobic dissimilation of glucose–C14 by *Escherichia coli*. J. Bacteriol. **81**:107–110.
18. **Sawers, R. G., S. P. Ballantine, and D. H. Boxer.** 1985. Differential expression of hydrogenase isoenzymes in *Escherichia coli* K-12: evidence for a third isoenzyme. J. Bacteriol. **164**:1324–1331.
19. **Sokatch, J. R.** 1969. Bacterial physiology and metabolism. Academic Press, Inc. (London), Ltd., London.
20. **Stokes, J. L.** 1949. Fermentation of glucose by suspensions of *Escherichia coli*. J. Bacteriol. **57**:147–158.
21. **Waugh, R., and D. H. Boxer.** 1986. Pleiotropic hydrogenase mutants of *Escherichia coli* K-12: growth in the presence of nickel can restore hydrogenase activity. Biochimie **68**:157–166.