Anaerobic Growth of *Escherichia coli* on Glycerol by Importing Genes of the *dha* Regulon from *Klebsiella pneumoniae*

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(Received 5 October 1988; revised 19 January 1989; accepted 26 January 1989)

The *dha* regulon of *Klebsiella pneumoniae* specifying fermentative dissimilation of glycerol was mobilized by the broad-host-range plasmid RP4:mini Mu and introduced conjugatively into *Escherichia coli*. The recipient *E. coli* was enabled to grow anaerobically on glycerol without added hydrogen acceptors, although its cell yield was less than that of *K. pneumoniae*. The reduced cell yield was probably due to the lack of the coenzyme-B₁₂-dependent glycerol dehydratase of the *dha* system. This enzyme initiates the first step in an auxiliary pathway for disposal of the extra reducing equivalents from glycerol. The lack of this enzyme would also account for the absence of 1,3-propanediol (a hallmark fermentation product of glycerol) in the spent culture medium. In a control experiment, a large quantity of this compound was detected in a similar culture medium following the growth of *K. pneumoniae*. The other three known enzymes of the *dha* system, glycerol dehydrogenase, dihydroxyacetone kinase and 1,3-propanediol oxidoreductase, however, were synthesized at levels comparable to those found in *K. pneumoniae*. Regulation of the *dha* system in *E. coli* appeared to follow the same pattern as in *K. pneumoniae*: the three acquired enzymes were induced by glycerol, catabolite repressed by glucose, and glycerol dehydrogenase was post-translationally inactivated during the shift from anaerobic to aerobic growth. The means by which the *E. coli* recipient can achieve redox balance without formation of 1,3-propanediol during anaerobic growth on glycerol remains to be discovered.

INTRODUCTION

Klebsiella pneumoniae differs from the related enteric bacteria *Escherichia coli*, *Salmonella typhimurium* and *Shigella flexneri* in having the ability to grow fermentatively on glycerol without an exogenous hydrogen acceptor (Lin, 1976). The anaerobic growth of *K. pneumoniae* on glycerol, a compound with more reducing equivalents per carbon atom than glucose, depends on the cooperation of two separate pathways specified by the *dha* regulon (Fig. 1 and Lin, 1976). In the oxidative pathway, glycerol is dehydrogenated by an NAD⁺-linked enzyme (glycerol dehydrogenase; product of *dhaD*) to dihydroxyacetone, which is then phosphorylated and funnelled to glycolysis by an ATP-dependent enzyme (dihydroxyacetone kinase; product of *dhaK*). In the parallel reductive pathway, NAD⁺ is regenerated from the NADH. This is accomplished by the sequential actions of glycerol dehydratase (product of *dhaB*) and an NADH-linked oxidoreductase (product of *dhaT*). The end product, 1,3-propanediol, from this reductive pathway is irretrievably lost to the growth medium in quantities approaching one-half the moles of glycerol consumed.

Abbreviations: CAA, casein acid hydrolysate; PEP, phosphoenolpyruvate.

Fig. 1. Pathways for the fermentative dissimilation of glycerol specified by the *dha* regulon (broken lines) and the respiratory dissimilation of glycerol and glycerol 3-phosphate specified by the *glp* regulon (solid lines). Both systems are present in *K. pneumoniae* but only the *glp* system is present in *E. coli*. The *dha* regulon comprises the genes *dhaD* encoding the NAD⁺-dependent glycerol dehydrogenase, *dhaK* encoding the ATP-dependent dihydroxyacetone kinase, *dhaB* encoding the coenzyme B₁₂-dependent glycerol dehydratase, *dhaT* encoding the NAD⁺-dependent trimethylene glycol (1,3-propanediol) oxidoreductase, and *dhaR* encoding the specific repressor. DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; FDP, fructose 1,6-bisphosphate; G3P, glycerol 3-phosphate; FAD, flavin adenine dinucleotide.

Expression of the *dha* structural genes is controlled by a repressor (product of *dhaR*) which responds to dihydroxyacetone as the effector and by catabolite repression (Forage & Foster, 1982; Forage & Lin, 1982; Johnson *et al.*, 1984; Ruch *et al.*, 1974; Ruch & Lin, 1975). Control is also exerted post-translationally by the inactivation of glycerol dehydrogenase during transition from anaerobic to aerobic growth (Johnson *et al.*, 1985; Lin *et al.*, 1960; Ruch *et al.*, 1980). The enzyme is destroyed irreversibly during such shifts apparently to promote induction and exploitation of the *glp* system (Fig. 1 and Lin, 1976) which has a superior power for glycerol scavenging because the first metabolic step is an ATP-driven reaction (Forage & Foster, 1982; Hueting *et al.*, 1978; Neijssel *et al.*, 1975).

The system specified by the *glp* regulon, however, permits growth on glycerol only in the presence of an exogenous electron acceptor. The glycerol 3-phosphate formed from glycerol is converted to dihydroxyacetone phosphate by individual flavoproteins linked to various inorganic and organic oxidants including molecular oxygen, nitrate and fumarate (Lin, 1976).

When the *glp* regulon is highly expressed aerobically, strong repression of the *dha* regulon occurs (Forage & Lin, 1982; Lin *et al.*, 1960; Ruch & Lin, 1975). As a first step for studying the interaction of these two systems we transferred the *dha* regulon from *K. pneumoniae* to *E. coli*, whose *glp* regulon is already well characterized (Lin, 1987). This was accomplished by the conjugative transfer of the broad-host-range plasmid RP4:mini Mu, used as a vector.

METHODS

Chemicals. Glycerol was obtained from Fisher Scientific Co. Vitamin-free casein acid hydrolysate (CAA) was obtained from ICN Nutritional Biochemicals. Dihydroxyacetone, ampicillin, kanamycin sulphate, tetracycline and coenzyme B₁₂ were from Sigma. All other reagents used were commercial products of the highest grade available.

Table 1. *Bacterial strains and plasmids*

Strain	Derived from	Description*	Source or reference
<i>K. pneumoniae</i> KAY2026	2002	<i>arg gua</i> phage P1-sensitive	Sprenger & Lengeler (1984)
<i>K. pneumoniae</i> KAY2042	KAY2026	<i>dhaK arg gua</i>	This study
<i>K. pneumoniae</i> KAY2083	KAY2026	<i>his arg gua</i>	This study
<i>K. pneumoniae</i> KAY2089	KAY2083	<i>his arg gua</i> (pULB113)	This study
<i>K. pneumoniae</i> KAY2181(R' <i>dha</i> ⁺)	KAY2042	<i>dhaK arg gua</i> (R' <i>dha</i> ⁺)	This study
<i>E. coli</i> MXR(pULB113)	K-12	F ⁻ Δ (<i>lac-pro</i>) <i>galE recA1 thi</i> (pULB113)	van Gijsegem & Toussaint (1982)
<i>E. coli</i> MLM161		F ⁻ <i>glpK argH his ilv malA metB mtl rpsL</i>	Woodward & Charles (1982)
MLM161(R' <i>dha</i> ⁺)		Received R' <i>dha</i> ⁺ from KAY2181	This study
ECL707		F ⁻ <i>gld::</i> Δ Tn10 <i>glpK::</i> Δ Tn10 <i>ptsD::</i> Δ Tn10 <i>araD139 flbB</i> Δ (<i>lacU169</i>) <i>ptsF25 relA rpsL thi</i>	This study
ECL707(R' <i>dha</i> ⁺)		Received R' <i>dha</i> ⁺ from strain MLM161	This study

* Genetic symbols are used according to Bachmann (1983). Additional symbols: *ptsD*, PEP-dependent phosphotransferase system specific for DHA (Jin & Lin, 1984); *dha*, genes specifying the pathways for fermentative utilization of glycerol in *K. pneumoniae* (Forage & Lin, 1982); *dhaK*, the gene for ATP-dependent dihydroxyacetone kinase (Forage & Lin, 1982); *gld*, the gene for an NAD⁺-linked glycerol dehydrogenase found in *E. coli* (Tang *et al.*, 1979).

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. The plasmid pULB113 (*tra*⁺ *Mu3A*, Ap^r Km^r Tc^r), a derivative of the conjugative plasmid RP4::mini-Mu with a partial deletion in the mini-Mu (van Gijsegem & Toussaint, 1982), was used as a vector for transferring the *dha* genes from *K. pneumoniae* into *E. coli* ECL707. The recipient strain, defective in *glpK* encoding the ATP-dependent glycerol kinase (Cozzarelli & Lin, 1966), *gld* encoding the native NAD⁺-linked glycerol dehydrogenase with unknown physiological function (Jin *et al.*, 1983), and *ptsD* encoding the enzyme II for dihydroxyacetone of the phosphoenolpyruvate (PEP) dependent phosphotransferase system (Jin & Lin, 1984), was derived from *E. coli* MC4100 by a series of manipulations including the insertion of Tn10 into each of the three genes, followed by deletion of the transposon (Bochner *et al.*, 1980). The Tn10-insertion mutants were provided by R. J. Jin.

K. pneumoniae strains were verified by their auxotrophic requirement for arginine and guanine, their ability to grow on D-arabitol, ribitol, L-sorbose, cellobiose and sucrose as a carbon and energy source, and their formation of acetyl-methylcarbinol in the Voges-Proskauer test. *E. coli* strains were verified by their chemotactic motility on agar containing aspartate and serine and by negative results from the tests for *K. pneumoniae*. Where appropriate, the strains were also verified by their antibiotic resistance markers.

Aerobic cell growth in liquid culture took place in 300 ml side-arm flasks containing 20 ml of medium (Tanaka *et al.*, 1967) and agitated on a rotary shaker. For anaerobic growth 150 ml bottles were filled to the top with medium and slowly stirred magnetically. Culture density was monitored by reading in a Klett colorimeter (no. 42 filter). Anaerobic growth on agar was carried out in sealed jars containing an atmosphere of H₂ and CO₂ (GasPak Anaerobic System; BBL Microbiology Systems). Unless otherwise specified, growth experiments were carried out at 37 °C, glycerol was added to 30 mM, pyruvate to 30 mM, xylose to 20 mM, galactose to 20 mM, glucose at 10 mM, CAA at 0.5%, guanine at 40 µg ml⁻¹, amino acids at 20 µg ml⁻¹, thiamin at 1 µg ml⁻¹, ampicillin at 50 µg ml⁻¹, kanamycin at 25 µg ml⁻¹, and tetracycline at 20 µg ml⁻¹.

Matings. Cells of the donor and recipient strains were harvested from LB-medium during exponential growth, collected by centrifugation, washed with mineral medium (Tanaka *et al.*, 1967), and resuspended to about 10⁹ cells ml⁻¹ in the same medium. Samples (about 0.1 ml) of these suspensions were cross-streaked on the selective agar plates containing the appropriate antibiotics and incubated at 30 °C. Progenies of exconjugants (after 3–5 d) were purified once on the same selective agar and then on LB-agar. Phenotypic markers were checked on appropriate MacConkey indicator plates (supplemented with 1% of the appropriate carbohydrate) for fermentation patterns or on mineral agar plates for their guanine or amino acid requirements.

Enzyme assays. The assays for glycerol dehydrogenase (Ruch *et al.*, 1974), dihydroxyacetone kinase (Johnson *et al.*, 1984), 1,3-propanediol oxidoreductase (Forage & Foster, 1982), and glycerol dehydratase (Forage & Foster, 1982) were described previously. Protein concentrations were determined by the Lowry method, with bovine serum albumin as standard. All enzyme units are expressed in nmol min⁻¹ (mg protein)⁻¹ at 25 °C.

Aerobic inactivation of glycerol dehydrogenase. Cells grown anaerobically on glycerol, CAA and pyruvate were suspended at a density of about 80–100 Klett units in 200 ml 50 mM-potassium phosphate (pH 7.0) with or without glucose. The suspension was agitated at 37 °C in 2-litre flasks with baffles on a rotary shaker operated at 250 r.p.m. Samples of 25 ml were drawn at the indicated times and the cell extracts were assayed for glycerol dehydrogenase activity.

Detection of 1,3-propanediol in fermentation media. Cells grown anaerobically on glycerol, CAA and pyruvate were removed from the media by centrifugation, and 100 ml samples of the supernatant fractions were concentrated by rotary evaporation. (Since 1,3-propanediol has a boiling point of 210–212 °C, no special precaution was taken to avoid the loss of the other volatile products.) The pellet was redissolved in 30 ml methanol, and anhydrous sodium sulphate was added to remove the residual water. The sample was filtered through Whatman no. 1 paper, and again dried by rotary evaporation. The residual oil was redissolved in 2 ml methanol. Insoluble material was pelleted by spinning in a Brinkmann microfuge for 10 min. The supernatant fraction was analysed for 1,3-propanediol by gas chromatography-mass spectroscopy on a Finnegan/MAT 4500 instrument. Gas chromatography was performed on a 30 m fused silica capillary column (0.25 mm i.d.) with 0.25 µm film thickness (Durabond 5, G & W Scientific). Injector temperature was 220 °C. Two microlitres of sample were injected; the temperature was maintained at 50 °C for 1 min, and then increased at 20 °C min⁻¹ to 270 °C. All peaks that eluted were fragmented by electron impact ionization at 70 eV and fragments of 25–350 atomic mass units were determined. The mass spectra of eluted peaks were compared with spectra from the National Bureau of Standards.

RESULTS

Transfer of the dha genes from K. pneumoniae to E. coli

The plasmid pULB113 (Km^rTc^r) in *E. coli* MXR (*galE*) was transferred to *K. pneumoniae* KAY2083 in a heterospecific cross. An exconjugant, *K. pneumoniae* KAY2089(pULB113), was selected on minimal agar containing galactose (to eliminate the donors), kanamycin and tetracycline (to eliminate unmated recipients). A pULB113 plasmid that had mobilized the *dha*⁺ genes (hereafter referred to as R'*dha*⁺) from the chromosome of *K. pneumoniae* KAY2089 (*his*) was transferred to *K. pneumoniae* KAY2042 (*dhaK*) by conjugation and anaerobic selection on minimal agar containing glycerol, kanamycin and tetracycline, but not histidine. *K. pneumoniae* KAY2181 [*dhaK* (R'*dha*⁺)] was thus isolated.

The plasmid R'*dha*⁺ was introduced from *K. pneumoniae* KAY2181 into *E. coli* MLM161 (*glpK rpsL*) by heterospecific conjugation and anaerobic selection on minimal agar containing glycerol (as carbon and energy source), streptomycin (to eliminate the donors), kanamycin and tetracycline (to eliminate unmated recipients). Colonies arising from exconjugants were purified by streaking on the same agar and were confirmed as *E. coli* by their metabolic characteristics (see Methods).

In wild-type *E. coli* cells there are several enzymes known to act on metabolites of the dihydroxyacetone pathway. An ATP-dependent kinase, encoded by *glpK* at min 88, converts glycerol to glycerol 3-phosphate (for a review see Lin, 1976). An NAD⁺-linked dehydrogenase of uncertain physiological function (Campbell *et al.*, 1978; Dekker & Swain, 1968; Jin *et al.*, 1983; Kelley & Dekker, 1984, 1985; St Martin *et al.*, 1977; Tang *et al.*, 1979, 1982*a, b*), encoded by *gld* at min 89 (G. Sprenger & E. C. C. Lin, unpublished data), converts glycerol to dihydroxyacetone. The enzyme II of the PEP-dependent phosphotransferase specific for dihydroxyacetone (Jin & Lin, 1984; Jin *et al.*, 1983), encoded by *ptsD* at about min 26 between *dadA* and *hemA* (R. Z. Jin & E. C. C. Lin, unpublished data), converts dihydroxyacetone to dihydroxyacetone phosphate. To avoid possible confusion of the activities of these enzymes with those of the *dha* system, we transferred again the plasmid from *E. coli* MLM161 to *E. coli* ECL707 (*glpK gld ptsD*). The exconjugant ECL707(R'*dha*⁺) was selected for anaerobic growth on minimal agar containing glycerol (40 mM) and tetracycline.

R'*dha*⁺ was unstable in the *E. coli* host in the absence of selective pressure to maintain the plasmid. After overnight growth on LB broth, 40% of the cells gave glycerol-negative colonies (on MacConkey-glycerol agar) and became sensitive to ampicillin, kanamycin or tetracycline. For this reason, strains harbouring R'*dha*⁺ were routinely maintained on glycerol-agar with the three antibiotics and were purified by recloning before use in physiological experiments.

Table 2. Anaerobic growth on glycerol or D-xylose as sole source of carbon and energy

Strain	Growth in liquid medium (Klett units)*			Colony size (mm) on agar medium with 20 mM-glycerol†
	Basal medium	+ 30 mM-Glycerol	+ 20 mM-Xylose	
<i>E. coli</i> ECL707	96	86	170	0.5
<i>E. coli</i> ECL707(R' <i>dha</i> ⁺)	63	140	170	2.5
<i>K. pneumoniae</i> KAY2026	103	180	200	3.5

* The basal medium contained CAA, pyruvate and guanine. The cultures were inoculated to give 20 Klett units and incubated for 44 h.

† The medium was supplemented with trace quantities of arginine and guanine. The average colony size on two individual plates was determined after 4 d incubation.

Table 3. Anaerobic synthesis of the *dha* enzymes in *K. pneumoniae* and *E. coli* hosts with or without R'*dha*

Enzyme	Growth on*	Specific activity of <i>dha</i> -encoded enzymes					
		<i>K. pneumoniae</i> KAY2026		<i>E. coli</i> ECL707		<i>E. coli</i> ECL707(R' <i>dha</i>)	
		Xylose	Glycerol	Xylose	Glycerol	Xylose	Glycerol
Glycerol dehydrogenase		360	1100	<3	<3	20	1500
1,3-Propanediol dehydrogenase		60	390	<3	<3	7	520
Dihydroxyacetone kinase		30	110	<2	<2	21	130
Glycerol dehydratase		340	900	<50	<50	<50	<50

* All growth media contained xylose, CAA, pyruvate and guanine.

Anaerobic growth on glycerol

In a medium containing glycerol, CAA and pyruvate as carbon and energy sources, *E. coli* ECL707(R'*dha*⁺) showed a higher anaerobic growth yield than *E. coli* ECL707, although still not as high a yield as *K. pneumoniae* KAY2026 (Table 2). A control growth experiment showed that the presence of this plasmid in strain ECL707 did not alter the anaerobic growth yield when glycerol was substituted by xylose. On simple agar medium with glycerol as added carbon and energy source (supplemented with trace quantities of arginine and guanine for growth comparison with strain KAY2026), strain ECL707(R'*dha*⁺) yielded larger colonies than strain ECL707 following anaerobic incubation, although even larger colonies were observed with KAY2026.

As expected, because of the inability of strain ECL707 to produce the enzyme II of the PEP-dependent phosphotransferase specific for dihydroxyacetone (*ptsD*), it was unable to grow on this compound. Strain ECL707(R'*dha*⁺), however, was able to grow on dihydroxyacetone, presumably because of its ability to produce the ATP-dependent kinase (data not shown).

Levels and regulation of the *dha* enzyme activities in anaerobically grown cells

To see if the ability of the *E. coli* cells to use glycerol anaerobically was associated with the *dha* genes, extracts of cells grown in the presence or absence of glycerol were compared for the enzyme activities. As shown in Table 3, cells of *E. coli* ECL707 did not contain significant levels of any of the four enzymes whether grown on xylose (low catabolite repression) or on xylose plus glycerol, whereas cells of *E. coli* ECL707(R'*dha*⁺) contained induced activity levels of glycerol dehydrogenase, dihydroxyacetone kinase and 1,3-propanediol oxidoreductase comparable to those found in *K. pneumoniae* KAY2026. No significant glycerol dehydratase activity, however, was detected in either *E. coli* strain. No activity of this enzyme was found even when coenzyme B₁₂ or Co²⁺ was added to the growth medium (results not shown). As in *K. pneumoniae*, the *dha* enzymes were subject to catabolite repression in *E. coli* ECL707(R'*dha*⁺). The specific activities

Fig. 2. Mass spectra of fermentation end product isolated from the culture medium of *K. pneumoniae* (top) and a comparison spectrum of 1,3-propanediol from the National Bureau of Standards (bottom). The numbers 28, 31, and 58 refer to the mass of the fragments from 1,3-propanediol.

of glycerol dehydrogenase and propanediol oxidoreductase were repressed about twofold when glucose was added to the anaerobic growth medium. The presence of 3 mM-cAMP reversed the glucose effect (data not shown).

No excretion of 1,3-propanediol by E. coli ECL707(R'dha⁺) during anaerobic growth on glycerol

Lack of glycerol dehydratase activity in the extract of heterologous cells might be due to any of several reasons: the absence of the *dhaB* gene in R'*dha*⁺, the failure of phenotypic expression of *dhaB*, or the lability of the enzyme synthesized in a foreign cytoplasm. To test whether the enzyme activity was present *in vivo*, we compared the culture media of strains KAY2026, ECL707 and ECL707(R'*dha*⁺) following anaerobic growth in a medium containing glycerol, CAA and pyruvate. If any active enzyme was synthesized, 1,3-propanediol should be excreted. Analysis of the culture medium of strain KAY2026 by gas chromatography revealed a prominent peak that eluted at 212 °C. Its mass spectrum matched well with a standard spectrum given by 1,3-propanediol (Fig. 2). No such peak was detected in media from cultures of *E. coli* ECL707 with or without the R'*dha*⁺ plasmid, although acetic acid was detected as a prominent product (data not shown). Since the culture broth was concentrated by rotary evaporation, volatile alcohols including ethanol would not be detected in our analysis of fermentation products.

Oxidative inactivation of glycerol dehydrogenase

Glycerol dehydrogenase of *K. pneumoniae* induced anaerobically is inactivated during aerobiosis in the presence of a carbon and energy source (Lin *et al.*, 1960; Ruch *et al.*, 1980). It was suggested that the enzyme was attacked by oxygen radicals generated from the aerobic metabolism (Johnson *et al.*, 1985). When cells of *E. coli* ECL707(R'*dha*⁺) were grown anaerobically in a glycerol-CAA-pyruvate medium and subsequently incubated in mineral medium with vigorous aeration for 3 h, about 50% of the total glycerol dehydrogenase activity in the culture disappeared. In the presence of glucose, about 73% of the activity was lost (data not shown). Cells of *K. pneumoniae* KAY2026 and *E. coli* ECL707(R'*dha*⁺) grown on glycerol and CAA under vigorous aeration lacked detectable levels of glycerol dehydrogenase activity (< 10 nmol min⁻¹ mg⁻¹).

DISCUSSION

Although many of the *Enterobacteriaceae* can grow readily on glycerol as the sole carbon and energy source by respiratory pathways (i.e. with molecular oxygen, nitrate, fumarate, and so on as exogenous electron acceptor), none are known to be able to do so by fermentative pathways except *Klebsiella* (Lin, 1976). The results described here showed that, with the possible exception of *dhaB*, all the known genes of the *dha* regulon of *K. pneumoniae* were mobilized by the plasmid RP4:mini Mu and transferred to *E. coli*. In the heterospecific host, the *dha* regulon was controlled by induction, indicating that the regulator gene was transferred together with the structural genes. As in *K. pneumoniae*, the *dha* regulon was subject to cAMP control and glycerol dehydrogenase was post-translationally inactivated during adaptation to aerobiosis.

The successful transfer of three of the four known structural genes together with the regulator gene *dhaR* would suggest that all the genes of the regulon are clustered. This notion is consistent with a mutational study which indicated that *dhaB*, whose product was not detected in *E. coli* ECL707(R'*dha*⁺), belonged to the same operon containing at least two of the other structural genes. Thus, among a number of Tn5 insertion mutants of *K. pneumoniae* (strain NCIB 418) analysed, two independent mutants simultaneously lost the activities of glycerol dehydratase, glycerol dehydrogenase and 1,3-propanediol oxidoreductase, although not that of dihydroxyacetone kinase (R. G. Forage & E. C. C. Lin, unpublished data).

If all the *dha* genes were indeed closely linked, then the lack of the dehydratase activity in extracts of the *E. coli* cells harbouring the R'*dha*⁺ plasmid would be due either to the exclusion of *dhaB* because it is at one end of the gene cluster, or to inability of the gene to effect the synthesis of an active B₁₂-dependent dehydratase in a foreign cytoplasm. To address this question, we are in the process of cloning *in vitro* a segment of the *K. pneumoniae* chromosome that encompasses the entire *dha* regulon, for physical mapping and for comparing the ability of *dhaB* to direct the synthesis of its product in homologous and heterologous cytoplasm. Another question to be answered is how the *E. coli* recipient disposes of the extra reducing equivalents from glycerol without the production of 1,3-propanediol. A more detailed analysis of the glycerol fermentation products will be necessary.

We thank Paul Lyne of the Wisconsin State Laboratory of Hygiene for assistance with the GC/MS analysis. G. A. Sprenger was supported by a grant of the Deutscher Akademischer Austauschdienst, Sonderprogramm Gentechnologie. This work was supported by Public Health Service grant 5-RO1-GM11983 from the National Institute of General Medical Sciences.

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