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Taxonomic diversity of anaerobic glycerol dissimilation in the *Enterobacteriaceae*

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SUMMARY

A total of 1,123 strains representing 128 taxa in the *Enterobacteriaceae* (named species or subspecies and genomic species) were screened for the presence of glycerol dehydrogenases and 1,3-propanediol dehydrogenase. Only eight taxa, *Citrobacter freundii* sensu stricto, *C. youngae*, *C. braakii*, *C. werkmanii*, *Citrobacter* genomospecies 10 and 11, *Enterobacter gergoviae* and *Klebsiella pneumoniae* subsp. *pneumoniae* could grow fermentatively on glycerol and possessed both glycerol dehydrogenase type I (induced by glycerol and dihydroxyacetone) and 1,3-propanediol dehydrogenase which are typical enzymes of the anaerobic glycerol dissimilation pathway. Six other species, *C. koseri*, *E. aerogenes*, *E. intermedium*, *K. oxytoca*, *K. planticola* and *K. terrigena* could not grow fermentatively on glycerol and possessed a glycerol dehydrogenase type I but no 1,3-propanediol dehydrogenase. Other glycerol dehydrogenases types were found: type II (induced by glycerol and hydroxyacetone), type III (induced by glycerol only) and type IV (induced by hydroxyacetone only). They were widely distributed among the *Enterobacteriaceae*. Classification and identification may take advantage of tests exploring the dissimilation of glycerol.

Key-words: Taxonomy, *Enterobacteriaceae*, Glycerol; Anaerobiosis, Dihydroxyacetone, 1,3-Propanediol.

INTRODUCTION

Fermentative utilization of glycerol, a more reduced substrate than glucose, requires the disposal of the two extra hydrogen atoms. Glycerol catabolism has been examined extensively in bacteria, particularly in *Escherichia coli* and *Klebsiella pneumoniae* (Forage and Foster, 1979, 1982; Forage and Lin, 1982; Ruch *et al.*, 1974; Toraya *et al.*, 1976). *K. pneumoniae* differs from *E. coli*, *Salmonella typhimurium* and *Shigella flexneri* in having the ability to grow fermentatively on glycerol without an exogenous hydrogen acceptor (Lin, 1976). In *K. pneumoniae*, glycerol is fermented by a dismutation process involving two parallel pathways encoded by the *dha* regulon. Through the oxidative pathway, glycerol is dehydrogenated by an NAD^+-linked enzyme, a glycerol dehydrogenase type I (glyDH-I) (induced by glycerol and dihydroxyacetone) to dihydroxyacetone (DHA) which is then phosphorylated by an ATP-dependent kinase

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to dihydroxyacetone phosphate (DHAP). Through the parallel reductive pathway, glycerol is dehydrated by a B_{12}-dependent enzyme to form 3-hydroxypropionaldehyde which is then reduced to 1,3-propanediol (1,3-PD) by an NADH-linked oxidoreductase (1,3-PD-dehydrogenase, *i.e.* 1,3-PD-DH) thereby regenerating NAD^+ (fig. 1).

The occurrence of trimethylene glycol (1,3-PD), as a product of the fermentation of glycerol, was first observed in 1881 by Freund (1881). Then Werkman and Gillen (1932) proposed the generic name *Citrobacter* for trimethylene-glycol-producing enteric bacteria (Mickelson and Werkman, 1940; Toraya *et al.*, 1980; Werkman and Gillen, 1932). 1,3-PD is a significant end product of glycerol fermentation in other genera, *e.g. Bacillus* (Voisenet, 1914, 1918), *Clostridium* (Humphreys, 1924), *Klebsiella* (Abeles *et al.*, 1960; Lee and Abeles, 1963; Pawelkiewicz and Zagalak, 1965), *Ilyobacter polytropus* (Stieb and Schink, 1984), *Lactobacillus* (Smiley and Sobolov, 1962), *Pelobacter carbinolicus* (Schink, 1984) and *P. venetianus* (Schink and Stieb, 1983).

Recently, the anaerobic glycerol pathway was studied in a few *Enterobacteriaceae* strains, selected as representative of different behaviours in terms of glycerol dissimilation (Bouvet *et al.*, 1994). It was found that *K. oxytoca* lacked the glycerol dehydratase and possessed a low level of 1,3-PD-DH activity. *K. planticola* and *K. pneumoniae* subsp. *ozaenae* differed from *K. pneumoniae* subsp. *pneumoniae* and *K. oxytoca* in lacking the ability to grow on glycerol. *K. planticola* lacked both enzymes of the reductive pathway of glycerol fermentation and *K. ozaenae* possessed only gly-DH-I.

E. coli, which lacks the enzymes of the *dha* regulon, is unable to grow fermentatively on glycerol. An NAD^+-linked glycerol dehydrogenase type II (glyDH-II) (fig. 2) with a broad substrate specificity for diols has been described in *E. coli* (Jin *et al.*, 1983; St. Martin *et al.*, 1977; Tang *et al.*, 1979, 1982). The levels of this activity are markedly induced by oxidized substrates (*i.e.* aminoacetone, hydroxyacetone and dihydroxyacetone) and also by compounds involved in the pathway postulated for threonine catabolism/D-1-amino-2-propanol formation (Kelley and Dekker, 1985). The identity of gly-DH of *E. coli* with D-1-amino-2-propanol oxidoreductase has been reported and its participation in vitamin B_{12} biosynthesis suggested (Kelley and Dekker, 1985). GlyDHs from a few bacterial species, including *Hafnia alvei* and *"Salmonella enterica"*, were found to be distinct from *E. coli* glyDH. GlyDHs other than type I are unable to promote glycerol fermentation (Bouvet *et al.*, 1994). GlyDH type III of *H. alvei* is induced by glycerol and not by hydroxyacetone whereas glyDH type IV of *"Salmonella enterica"* is induced by hydroxyacetone and not by glycerol.

The purposes of this study were to propose tests for screening for bacterial glyDHs and 1,3-PD-DH and to study the distribution of glyDHs and 1,3-PD-DH among enterobacterial species.

MATERIALS AND METHODS

Bacterial strains

A total of 1,123 strains representing 128 taxa (named species or subspecies and genomospecies) in the *Enterobacteriaceae* were studied. Type strains and strains used as reference in DNA-relatedness studies were included.

Reagents

1,3-PD and hydroxyacetone were from Aldrich (Strasbourg, France). All other biochemicals were obtained from Sigma Chemicals Co. (St. Louis, MO) and were reagent grade.

DHA = dihydroxyacetone.
DHAP = DHA phosphate.
glyDH = glycerol dehydrogenase.
LB = Luria-Bertani (medium).
1,3-PD = 1,3-propanediol.
1,3-PD-DH = 1,3-PD dehydrogenase.
TCS = tryptocasein soy (agar).

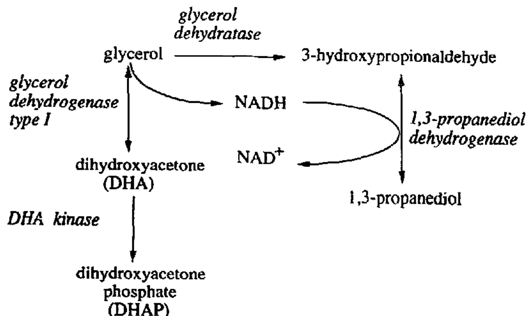

Fig. 1. Pathway for anaerobic glycerol dissimilation in *K. pneumoniae* subsp. *pneumoniae*. In the absence of any electron acceptor, the *dha* regulon is expressed.

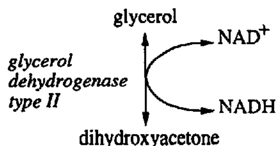

Fig. 2. NAD^+-linked glyDH type II of *E. coli*.

Growth conditions

Bacteria were grown overnight at 30°C in shaken Luria broth (LB). Collection strains or strains cultivated on carbohydrates such as glucose or lactose were subcultured twice in LB medium before performing tests.

For the detection of glyDH, bacteria were grown overnight at 30°C on tryptocasein soy agar plates (TCS, Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) supplemented with 1% glycerol or 70 mM hydroxyacetone both sterilized by filtration. Cultures grown on TCS-glycerol were incubated in an anaerobic jar with catalyst, H_2+CO_2 generators (GasPak, BBL Becton Dickinson Microbiology systems, Cockeysville, MD) and methylene blue indicator strip. Cultures grown on TCS-hydroxyacetone were incubated aerobically.

For the detection of 1,3-PD-DH, bacteria were grown overnight at 30°C on TCS agar plates supplemented with 10 mM DHA, 10 µg per liter cobalt chloride, 100 µM $MnSO_4$ and 1 µM coenzyme B_{12} (CoB_{12}). Cultures grown on this medium were incubated anaerobically as described above. Filter-sterilized solutions of DHA, CoB_{12}, $CoCl_2$ and $MnSO_4$ can be stored frozen at −20°C.

GlyDH test

Bacterial growth from TCS-glycerol or TCS-hydroxyacetone agar plates was collected and suspended in reaction buffer to an absorbance (at 600 nm) of about 0.6. The reaction buffer contained 0.1 M K_2CO_3 and 30 mM $(NH_4)_2SO_4$, and was adjusted to pH 9.0 with HCl or KOH. It was stored in a well-stoppered bottle at 4°C for up to one month. Bacterial suspensions from TCS-glycerol or TCS-hydroxyacetone agar plates were immediately dispensed (150 µl) into 96-well microtitre plates (Dynatech AG, Denkerdorf, Germany). Then, 30-µl portions of a reagent (NAD^+, 210 mg; glycerol, 600 µl; nitro-blue tetrazolium, 42 mg; phenazine methosulphate, 2 mg; distilled water to 10 ml) (kept frozen at −20°C in the dark) were added to the wells

and the plates were gently shaken and incubated in the dark at room temperature. The plates were examined for the development of a purple color within 15 to 30 min. The colour in the uninoculated control medium remained unchanged (yellow). *Serratia odorifera* (Grimont) 10-73^T and *E. coli* CNS 75-88 (French Salmonella Reference Center) were used as negative and positive control strains, respectively.

1,3-PD-DH test

The 1,3-PD-DH test was the same as the test for glyDH except that the bacterial growth from TCS-DHA was collected and suspended in reaction buffer (as described above); 1,3-PD was used in place of glycerol in the reagent. A control medium contained the same ingredients except 1,3-PD. The control strains were *E. coli* CNS 75-88 (negative) and *K. pneumoniae* subsp. *pneumoniae* CIP 8291^T (positive).

Determination of glycerol content in culture media (PAS test)

Fifteen-ml sterile, disposable tubes (Falcon, Becton Dickinson and Company, NJ) were filled to the top with nutrient broth (Difco, Detroit, MI) containing 15 mM glycerol and were then inoculated with 30 µl of subculture in LB medium and incubated overnight at 30°C. Residual glycerol in culture media was determined using the test kit "Periodic acid-Schiff" (PAS, Sigma). One ml of culture was centrifuged at 2,000 *g*. To 190 µl distilled water were added 10 µl of the culture supernatant and successively 50-µl portions of periodic acid and 100-µl portions of Schiff reagent. Tubes were gently shaken between each addition. The tubes were examined for the development of a purple colour within 5 to 10 min at room temperature. The control strains were *E. coli* CNS 75-88 (positive; purple colour developed) and *K. pneumoniae* subsp. *pneumoniae* CIP 8291^T (negative; no colour developed).

Enzyme electrophoresis

Electrophoresis under non-denaturing conditions on polyacrylamide gels with a discontinuous buffer system was performed as described previously. Dehydrogenase activities were visualized by specific detection (Bouvet *et al.*, 1994).

Fermentative utilization of glycerol

Fifteen ml sterile, disposable tubes (Falcon) were filled to the top with membrane-filtered mineral medium adjusted to pH 7.0 containing 0.034 M NaH_2PO_4, 0.064 M K_2HPO_4, 0.02 M $(NH_4)_2SO_4$, 3×10^{-4} M $MgSO_4$, 10^{-6} M $FeSO_4$, 10^{-6} M $ZnCl_2$, 10^{-5} M $CaCl_2$, 30 mM glycerol and casein hydrolysate to a final concentration of 0.03%. The medium was then inoculated with 30 µl of culture in LB medium and incubated overnight at 30°C. The control strains which we used were *E. coli* CNS 75-88 (no growth) and *K. pneumoniae* subsp. *pneumoniae* CIP 8291^T (growth).

RESULTS AND DISCUSSION

Definition of dehydrogenase types

A glyDH-I was defined by (i) a positive glyDH test with bacterial suspension from TCS-glycerol, (ii) a negative PAS test (no colour developed with PAS reagent in spent culture media) and (iii) electrophoretic mobility (Rf) between 0.40 to 0.60 in a non-denaturing gel. Strains that produced glyDH-I only were not able to grow anaerobically in mineral medium with glycerol as carbon source (Bouvet *et al.*, 1994).

A 1,3-PD-DH was defined by a positive 1,3-PD-DH test and electrophoretic mobility between 0.14 to 0.16 in a non-denaturing gel. A 1,3-PD DH activity was always found associated with glyDH I activity. Bacteria producing both glyDH I and 1,3-PD DH were able to grow fermentatively in mineral medium with glycerol as carbon source.

Other glyDH types (II, III and IV) were defined by (i) a positive glyDH test with bacterial suspension from TCS-glycerol and/or TCS-hydroxyacetone, (ii) a positive PAS test (purple color developed with PAS reagent in spent culture media) and (iii) an electrophoretic mobility between 0.19 to 0.25 in a non-denaturing gel (Bouvet *et al.*, 1994). A glyDH-II gave a positive glyDH test with bacterial suspension from TCS-glycerol and TCS-hydroxyacetone. A glyDH-III gave a positive glyDH test only with bacterial suspension from TCS-glycerol. A glyDH-IV gave a positive glyDH test only with bacterial suspension from TCS-hydroxyacetone. GlyDHs other than type I were unable to promote anaerobic glycerol dissimilation (Bouvet *et al.*, 1994). It

is not possible to detect simultaneously a glyDH-II or -III and a glyDH-I. GlyDH-II and -III should be identified only in non-denaturing gel by specific detection.

Bacteria possessing glyDH-I only

A total of 38 strains (table I) belonging to *Citrobacter koseri (C. diversus), Enterobacter aerogenes, E. intermedium, K. oxytoca, K. planticola* and *K. terrigena* possessed a glyDH-I (major glyDH). Of the 38 strains studied, 12 were checked for electrophoretic mobilities of glyDHs in non-denaturing gel. An atypical behaviour was observed for *K. pneumoniae* subsp. *ozaenae*. This species possessed glyDH-I (as described previously (Bouvet *et al.*, 1994)) and was found unable to consume glycerol from culture medium, so the test for determination of glycerol content in culture media was negative.

Bacteria possessing both glyDH-I and 1,3-PD-DH

A total of 163 strains (table I) belonging to *C. freundii, C. youngae, C. braakii, C. werkmanii, Citrobacter* genomospecies 10 and 11 (Brenner *et al.*, 1993), *E. gergoviae, K. pneumoniae* subsp. *pneumoniae* and glycerol positive strains of *"Salmonella enterica"* subsp. *enterica* serovar Senftenberg and Derby, one strain of *C. farmeri*, one strain of *E. cloacae* group 4, one strain of *Leclercia adecarboxylata* and one strain of *E. agglomerans* group XII produced both glyDH-I and 1,3-PD-DH. All 163 strains were able to grow anaerobically in mineral medium with glycerol as carbon source. The presence of both glyDH-I and 1,3-PD-DH in 35 of these strains was checked in non-denaturing gel by specific detection of dehydrogenase activity.

The 1,3-PD-DH test did not permit detection of the low 1,3-PD-DH activity of *K. oxytoca* (as described previously) (Bouvet *et al.*, 1994) even when CoB_{12} was added to the growth medium. Atypical behaviour was observed for 2 strains of *E. agglomerans* group II, one strain of *Yokenella regensburgei* and all strains of *Proteus mirabilis*, *P. vulgaris* and *P. penneri* which gave a positive test for 1,3-PD-DH even in control medium (without substrate). These did not produce glyDH-I, were unable to grow fermentatively in mineral medium with glycerol as sole carbon source, and did not produce 1,3-PD (results not shown).

Bacteria possessing glyDH-II, -III or -IV

A total of 422 strains (table I) possessed glyDHs type II, III or IV. Of these studied, 152 were checked for electrophoretic mobilities of glyDH in a non-denaturing gel. Of the 422 strains studied, 146 possessed a glyDH type II induced by glycerol and by hydroxyacetone, 179 possessed a glyDH type III induced only by glycerol, 97 possessed a glyDH type IV induced by HA and not by glycerol.

An atypical behaviour was observed for *H. alvei* which possessed a glyDH type III as described previously (Bouvet *et al.*, 1994). *H. alvei* showed a higher glyDH activity than *E. coli*. For this reason, although this enzyme was not induced by hydroxyacetone, it was occasionally possible to detect a low residual glyDH activity with bacterial suspension from TCS-HA.

Validity of glyDH and 1,3-PD-DH tests

These tests were designed in light of recently acquired knowledge (Bouvet *et al.*, 1994). Coenzyme B_{12}, $CoCl_2$ and $MnCl_2$ should be present in the culture medium, since glycerol dehydratase and 1,3-PD-DH of some bacteria *(e.g. Klebsiella)* require this cofactor and ions. For the 1,3-PD-DH test, prior induction by growth on a DHA medium and not on a glycerol medium should be done. Although DHA and glycerol induce the *dha* regulon, DHA cannot be oxidized by glyDH-I (Forage and Lin, 1982).

The presence of the oxidative and reductive pathways of the *dha* regulon allow the cells to grow fermentatively on glycerol. The first two enzymes of the *dha* regulon (glyDH-I and DHA kinase) represent the oxidative pathways and the

Table I. Distribution of glyDH and 1,3-PD-DH among enterobacterial species.

Taxon	PAS test (*)	glyDH induced by: glycerol	glyDH induced by: HA (**)	Glycerol fermentation	1,3-PD-DH	glyDH type
Budvicia aquatica	+ (6/6)	– (0/6)	NG	– (0/6)	– (0/6)	none
Buttiauxella agrestis	+ (5/5)	– (0/5)	+ (3/3)	– (0/5)	– (0/5)	IV
Cedecea davisae	+ (5/5)	– (0/5)	– (0/2)	– (0/5)	– (0/5)	none
Cedecea lapagei	+ (5/5)	– (0/5)	– (0/2)	– (0/5)	– (0/5)	none
Citrobacter amalonaticus	+ (8/8)	– (0/8)	+ (3/3)	– (0/8)	– (0/8)	IV
Citrobacter braakii	d (1/16)	d (15/16)	+ (3/3)	d (13/16)	d (13/16)	I+ and II
Citrobacter farmeri	d (13/14)	d (1/14)	+ (3/3)	d (1/14)	d (1/14)	(I+) and IV
Citrobacter freundii	– (0/5)	+ (5/5)	+ (3/3)	+ (5/5)	+ (5/5)	I+ and II
Citrobacter koseri	– (0/12)	+ (12/12)	+ (3/3)	– (0/12)	– (0/12)	I and II
Citrobacter sedlakii	+ (4/4)	d (3/4)	+ (3/3)	– (0/4)	– (0/4)	II
Citrobacter werkmanii	– (0/4)	+ (4/4)	+ (3/3)	+ (4/4)	+ (4/4)	I+ and II
Citrobacter youngae	– (0/7)	+ (7/7)	+ (3/3)	+ (7/7)	+ (7/7)	I+ and II
Citrobacter group 9	+ (3/3)	– (0/3)	– (0/3)	– (0/3)	– (0/3)	none
Citrobacter group 10	– (0/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	I+ and II
Citrobacter group 11	– (0/3)	+ (3/3)	+ (2/2)	+ (3/3)	+ (3/3)	I+ and II
Edwardsiella hoshinae	+ (5/5)	– (0/5)	– (0/2)	– (0/5)	– (0/5)	none
Edwardsiella ictaluri	+ (3/3)	– (0/3)	NG	– (0/3)	– (0/3)	none
Edwardsiella tarda	+ (5/5)	– (0/5)	– (0/2)	– (0/5)	– (0/5)	none
Enterobacter aerogenes	– (0/4)	+ (4/4)	+ (3/3)	– (0/4)	– (0/4)	I and II
Enterobacter agglomerans complex						
Pantoea agglomerans senso stricto	+ (5/5)	– (0/5)	– (0/5)	– (0/5)	– (0/5)	none
Group I	+ (2/2)	– (0/2)	– (0/2)	– (0/2)	– (0/2)	none
Group II	+ (8/8)	d (1/8)	– (0/3)	– (0/8)	– (0/8)	(III)
Group III (*Pantoea dispersa*)	+ (3/3)	– (0/3)	–	– (0/3)	– (0/3)	none
Group IV	+ (4/4)	– (0/4)	–	– (0/4)	– (0/4)	none
Group V	+ (8/8)	d (1/8)	– (0/2)	– (0/8)	– (0/8)	(III)
Group VI (*Pantea ananas*)	+ (3/3)	+ (3/3)	–	– (0/3)	– (0/3)	III
Group VII	+ (8/8)	+ (8/8)	– (0/3)	– (0/8)	– (0/8)	III
Group VIII	+ (2/2)	– (0/2)	–	– (0/2)	– (0/2)	none
Group IX	+ (4/4)	– (0/4)	–	– (0/4)	– (0/4)	none
Group X strain 1599-71	+	–	–	–	–	none
Group XI	+ (6/6)	d (1/6)	– (0/2)	– (0/6)	– (0/6)	(III)
Group XII	d (8/9)	d (1/9)	+ (9/9)	d (1/9)	d (1/9)	(I+) and IV
Enterobacter asburiae	+ (4/4)	– (0/4)	+ (4/4)	– (0/4)	– (0/4)	IV
Enterobacter amnigenus	+ (4/4)	+ (4/4)	– (0/4)	– (0/4)	– (0/4)	III
Enterobacter cancerogenus	+ (6/6)	– (0/6)	– (0/3)	– (0/6)	– (0/6)	none
Enterobacter cloacae group 1 (***)	+ (4/4)	+ (4/4)	+ (2/2)	– (0/4)	– (0/4)	II

Taxon	PAS test (*)	glyDH induced by: glycerol	glyDH induced by: HA (**)	Glycerol fermentation	1,3-PD-DH	glyDH type
Enterobacter cloacae group 2	+ (6/6)	+ (6/6)	+ (2/2)	– (0/6)	– (0/6)	II
Enterobacter cloacae group 3	+ (5/5)	+ (5/5)	+ (2/2)	– (0/5)	– (0/5)	II
Enterobacter cloacae group 4	d (2/3)	+ (3/3)	+ (3/3)	d (1/3)	d (1/3)	(I+) and II
Enterobacter cloacae group 5	+ (3/3)	– (0/3)	+ (3/3)	– (0/3)	– (0/3)	IV
Enterobacter cloacae group 6	+ (2/2)	+ (2/2)	+ (2/2)	– (0/2)	– (0/2)	II
Enterobacter gergoviae	– (0/5)	+ (5/5)	+ (4/4)	+ (5/5)	+ (5/5)	I+ and II
Enterobacter hormaechei	+ (5/5)	+ (5/5)	– (0/5)	– (0/5)	– (0/5)	III
Enterobacter intermedium	– (0/5)	+ (5/5)	– (0/5)	– (0/5)	– (0/5)	I and III
Enterobacter nimipressuralis	+ (6/6)	+ (6/6)	+ (3/3)	– (0/6)	– (0/6)	II
Enterobacter pyrinus	+ (7/7)	+ (7/7)	– (0/7)	– (0/7)	– (0/7)	III
Enterobacter sakazakii	+ (5/5)	– (0/5)	– (0/2)	– (0/5)	– (0/5)	none
Erwinia amylovora 78-1	+	–	–	–	–	none
Erwinia carnegieana EC-186	+	+	+	–	–	II
Erwinia carotovora 26	+	–	–	–	–	none
Erwinia chrysanthemi SR 80	+	–	–	–	–	none
Erwinia herbicola E20	+	–	–	–	–	none
Erwinia mallotivora 2851	+	–	–	–	–	none
Erwinia nigrifluens 104	+	–	–	–	–	none
Erwinia quercina 102	+	–	–	–	–	none
Erwinia rhapontici 106	+	+	+	–	–	II
Erwinia rubrifaciens 105	+	–	–	–	–	none
Escherichia blattae 9005-74	+	+	–	–	–	III
Escherichia coli genomic group						
Prototrophs	+ (151/151)	+ (148/151)	+ (29/29)	– (0/151)	– (0/151)	(IV) II
Shigella boydii	+ (74/74)	– (0/42)	d (33/74)	– (0/42)	– (0/42)	(IV)
Shigella dysenteriae	+	– (0/27)	d (11/30)	– (0/27)	– (0/27)	(IV)
Shigella flexneri	+	– (0/33)	d (3/56)	– (0/33)	– (0/33)	(IV)
Shigella sonnei	+	– (0/29)	– (0/53)	– (0/29)	– (0/29)	none
Escherichia fergusonii	+ (13/13)	+ (13/13)	+ (13/13)	– (0/5)	– (0/5)	II
Escherichia hermannii	+ (26/26)	– (0/26)	– (0/26)	– (0/5)	– (0/5)	none
Escherichia vulneris	+ (11/11)	– (0/11)	– (0/11)	– (0/5)	– (0/5)	none
Ewingella americana	+ (4/4)	– (0/4)	– (0/2)	– (0/4)	– (0/4)	none
Hafnia alvei	+ (12/12)	+ (12/12)	d (2/6)	– (0/12)	– (0/12)	III
Klebsiella oxytoca	– (0/8)	+ (8/8)	+ (3/3)	– (0/8)	– (0/8)	I and II
Klebsiella planticola	– (0/5)	+ (5/5)	+ (3/3)	– (0/5)	– (0/5)	I and II
Klebsiella pneumoniae subsp. *pneumoniae*	d (14/152)	d (138/152)	+ (6/6)	d (112/152)	d (112/152)	I+ and II
Klebsiella pneumoniae subsp. *ozaenae*	+ (18/18)	d (16/18)	+ (15/15)	– (0/18)	– (0/18)	(IV or II)

Taxon	PAS test (*)		glyDH induced by: glycerol		glyDH induced by: HA (**)		Glycerol fermentation		1,3-PD-DH		glyDH type
Klebsiella pneumoniae subsp. *rhinoscleromatis*	+	(20/20)	d	(8/20)	+	(12/12)	–	(0/19)	–	(0/19)	(IV or II)
Kluyvera terrigena	–	(0/4)	+	(4/4)	+	(3/3)	–	(0/4)	–	(0/4)	I and II
Kluyvera ascorbata	+	(7/7)	+	(7/7)	–	(0/5)	–	(0/7)	–	(0/7)	III
Kluyvera cryocrescens	+	(7/7)	+	(7/7)	–	(0/3)	–	(0/7)	–	(0/7)	III
Leclercia adecarboxylata	d	(6/7)	d	(4/7)	+	(4/4)	d	(1/7)	d	(1/7)	(I+), II
Leminorella grimontii	+	(4/4)	+	(4/4)	–	(0/2)	–	(0/4)	–	(0/4)	III
Leminorella richardii 978-82	+		+		–		–		–		III
Leminorella sp. strain 3346-72	+		+		–		–		–		III
Moellerella wisconsensis	+	(5/5)	+	(5/5)	–	(0/1)	–	(0/5)	–	(0/5)	III
Morganella morganii	+	(5/5)	+	(5/5)	+	(4/4)	–	(0/5)	–	(0/5)	II
Obesumbacterium proteus 4302-74	+		–		–		–		–		none
Pragia fontium	+	(5/5)	–	(0/5)	–	(0/2)	–	(0/5)	–	(0/5)	none
Proteus mirabilis	+	(5/5)	+	(5/5)	+	(3/3)	–	(0/5)	–	(0/5)	II
Proteus myxofaciens 19692	+		–		–		–		–		none
Proteus penneri	+	(5/5)	+	(5/5)	+	(5/5)	–	(0/5)	–	(0/5)	II
Proteus vulgaris	+	(4/4)	+	(4/4)	+	(3/3)	–	(0/4)	–	(0/4)	II
Providencia alcalifaciens	+	(14/14)	d	(6/14)	–	(0/14)	–	(0/14)	–	(0/14)	(III)
Providencia heimbachae	+	(5/5)	+	(5/5)	–	(0/3)	–	(0/5)	–	(0/5)	III
Providencia rettgeri	+	(6/6)	+	(6/6)	–	(0/3)	–	(0/6)	–	(0/6)	III
Providencia rustigianii	+	(5/5)	+	(5/5)	–	(0/4)	–	(0/5)	–	(0/5)	III
Providencia stuartii	+	(4/4)	+	(4/4)	–	(0/2)	–	(0/4)	–	(0/4)	III
Rahnella aquatilis	+	(5/5)	+	(5/5)	+	(3/3)	–	(0/5)	–	(0/5)	II
"*Salmonella enterica*" subsp. *enterica*	+	(13/13)	–	(0/13)	+	(13/13)	–	(0/13)	–	(0/13)	IV
serovar Senftenberg (glycerol$^+$)	–	(0/5)	+	(5/5)	+	(3/3)	+	(5/5)	+	(5/5)	I+ and II
serovar Derby (glycerol$^+$)	–	(0/2)	+	(2/2)	+	(2/2)	+	(2/2)	+	(2/2)	I+ and II
"*Salmonella enterica*" subsp. *salamae*	+	(2/2)	–	(0/2)	+	(2/2)	–	(0/2)	–	(0/2)	IV
"*Salmonella enterica*" subsp. *arizonae*	+	(3/3)	–	(0/3)	+	(3/3)	–	(0/3)	–	(0/3)	IV
"*Salmonella enterica*" subsp. *diarizonae*	+	(3/3)	–	(0/3)	+	(3/3)	–	(0/3)	–	(0/3)	IV
"*Salmonella enterica*" subsp. *houtenae*	+	(3/3)	–	(0/3)	+	(3/3)	–	(0/3)	–	(0/3)	IV
"*Salmonella enterica*" subsp. *indica* 1240	+		–		+		–		–		IV
Salmonella bongori	+	(3/3)	–	(0/3)	+	(3/3)	–	(0/3)	–	(0/3)	IV
Serratia entomophila	+	(6/6)	–	(0/6)	–	(0/2)	–	(0/6)	–	(0/6)	none
Serratia ficaria	+	(5/5)	–	(0/5)	–	(0/2)	–	(0/5)	–	(0/5)	none
Serratia fonticola	+	(6/6)	+	(6/6)	–	(0/3)	–	(0/6)	–	(0/6)	III
Serratia grimesii	+	(12/12)	+	(12/12)	–	(0/3)	–	(0/12)	–	(0/12)	III
Serratia liquefaciens	+	(8/8)	+	(8/8)	–	(0/3)	–	(0/8)	–	(0/8)	III
Serratia marcescens	+	(7/7)	+	(7/7)	–	(0/3)	–	(0/7)	–	(0/7)	III

Taxon	PAS test (*)	glyDH induced by: glycerol	HA (**)	Glycerol fermentation	1,3-PD-DH	glyDH type
Serratia odorifera	+ (5/5)	– (0/5)	– (0/2)	– (0/5)	– (0/5)	none
Serratia plymuthica	+ (6/6)	– (0/6)	– (0/3)	– (0/6)	– (0/6)	none
Serratia proteamaculans	+ (24/24)	+ (24/24)	– (0/2)	– (0/24)	– (0/24)	III
Serratia rubidaea	+ (4/4)	– (0/4)	– (0/2)	– (0/4)	– (0/4)	none
Trabulsiella guamensis	+ (2/2)	– (0/2)	–	– (0/2)	– (0/2)	none
Xenorhabdus luminescens	+ (6/6)	– (0/6)	NG	– (0/6)	– (0/6)	none
Xenorhabdus nematophilus	+ (3/3)	– (0/3)	NG	– (0/3)	– (0/3)	none
Xenorhabdus sp.	+ (4/4)	– (0/4)	NG	– (0/4)	– (0/4)	none
Yersinia aldovae	+ (12/12)	d (5/12)	– (0/4)	– (0/12)	– (0/12)	(III)
Yersinia bercovieri	+ (3/3)	– (0/3)	– (0/2)	– (0/3)	– (0/3)	none
Yersinia enterocolitica	+ (8/8)	+ (8/8)	– (0/3)	– (0/8)	– (0/8)	III
Yersinia frederiksenii	+ (5/5)	+ (5/5)	– (0/3)	– (0/5)	– (0/5)	III
Yersinia intermedia	+ (5/5)	– (0/5)	– (0/3)	– (0/5)	– (0/5)	none
Yersinia kristensenii	+ (5/5)	+ (4/5)	– (0/3)	– (0/5)	– (0/5)	III
Yersinia mollaretii	+ (3/3)	d (1/3)	– (0/2)	– (0/3)	– (0/3)	(III)
Yersinia pseudotuberculosis	+ (6/6)	– (0/6)	– (0/2)	– (0/6)	– (0/6)	none
Yersinia rohdei	+ (6/6)	+ (6/6)	– (0/3)	– (0/6)	– (0/6)	III
Yersinia ruckeri	+ (5/5)	d (1/5)	– (0/3)	– (0/5)	– (0/5)	(III)
Yokenella regensburgei	+ (6/6)	– (0/6)	– (0/2)	– (0/6)	– (0/6)	none

(*) Determination of glycerol content in culture media; (**) HA=hydroxyacetone.

+=all strains positive; –=all strains negative; d=different reactions. When more than one strain was tested, the numbers in parentheses indicate the number of positive strains/number of strains tested. NG=no growth. I+strains which possess both glyDH type I and 1,3-PD-DH; I=strains which possess glyDH type I only. The glyDH type of different reactions is indicated in parentheses.

(***) See Grimont and Grimont, 1992.

latter two (glycerol dehydratase and 1,3-PD DH) represent the reductive pathway of glycerol fermentation. After growth of *K. pneumoniae* subsp. *pneumoniae* on glycerol, the dominant byproducts are 1,3-PD, ethanol, formate and, to a lesser extent, acetate (Bouvet *et al.*, 1994).

In this study, all eight species which possess both glyDH-I and 1,3-PD-DH were able to grow fermentatively in mineral medium with glycerol as carbon source. Therefore, these species possess the other two enzymes of the *dha* regulon (DHA kinase and glycerol dehydratase). The pathway of glycerol fermentation has been extensively investigated in *"Aerobacter aerogenes"* ATCC 8724 (synonym of *K. oxytoca*, previously classified as *"Aerobacter aerogenes"*, *K. pneumoniae* and *E. aerogenes*) (Brenner *et al.*, 1977; Jain *et al.*, 1974). The slower utilization of glycerol in *K. oxytoca* was due to a low production of 1,3-PD. *K. oxytoca* lacks the glycerol dehydratase and possesses a low 1,3-PD-DH activity (Bouvet *et al.*, 1994) which can not be detected in our test.

Five species which possess glyDH-I but not 1,3-PD-DH were not able to grow fermentatively on glycerol. They had the same behaviour as *K. planticola* (as described previously (Bouvet *et al.*, 1994)) which was found to possess both enzymes of the oxidative pathway (glyDH-I and DHA kinase) and lacks both enzymes of the reductive pathway. In these five species, the presence of the oxidative pathway allowed them to rapidly utilize glycerol in culture media as shown by the PAS test. Peptones in the medium, by their own pathway, regenerate the NAD needed by the oxidative pathway without 1,3-PD production.

K. pneumoniae subsp. *ozaenae* lacked both the enzyme of the reductive pathway and the dihydroxyacetone kinase, and possessed glyDH-I (as described previously) (Bouvet *et al.*, 1994). Despite the presence of the glyDH-I, *K. ozaenae*, as shown by the PAS test, cannot utilize glycerol in culture media.

GlyDHs types II, III and IV do not support growth on glycerol, and high concentration of glycerol is detected by the PAS reagent in spent culture media.

This glyDH test might not give the same results and seems more restrictive than the conventional biochemical reaction using acid production from glycerol. Peptone present in fermentation media permitted anaerobic growth on glycerol without 1,3-PD production.

Taxonomic interest of tests exploring anaerobic glycerol dissimilation

The results of the glyDH test and the 1,3-PD-DH test given by 128 taxa (named species or subspecies and unnamed genomic species) in the *Enterobacteriaceae* are shown in table I.

The following organisms were negative in all tests of the anaerobic glycerol pathway: members of genera *Budvicia*, *Cedecea* (two species), *Edwardsiella* (three species), and *Xenorhabdus* (two species), *Citrobacter* genomospecies 9, *Pantoea agglomerans* sensu stricto, *Enterobacter agglomerans* genomospecies I, II, III, IV, V, VIII, IX and X, *Enterobacter sakazakii*, *Enterobacter cancerogenus (=Enterobacter taylorae)*, *Erwinia amylovora*, *Erwinia carotovora*, *Erwinia chrysanthemi*, *Erwinia herbicola*, *Erwinia mallotivora*, *Erwinia nigrifluens*, *Erwinia quercina*, *Erwinia rubrifaciens*, *Shigella sonnei*, *Escherichia hermannii*, *Escherichia vulneris*, *Ewingella americana*, *Obesumbacterium proteus*, *Pragia fontium*, *Proteus myxofaciens*, *Serratia entomophila*, *Serratia ficaria*, *Serratia odorifera*, *Serratia plymuthica*, *Serratia rubidaea*, *Trabulsiella guamensis*, *Xenorhabdus luminescens*, *Xenorhabdus nematophilus*, *Yersinia bercovieri*, *Yersinia intermedia*, *Yersinia pseudotuberculosis* and *Yokenella regensburgei*. Either these organisms lack these pathways or the corresponding dehydrogenases have requirements for unknown cofactors or conditions not met by our tests.

Only eight species, *Citrobacter freundii*, *Citrobacter youngae*, *Citrobacter braakii* (13 positive strains/16 strains tested), *Citrobacter werkmanii*, *Citrobacter* genomospecies 10 and 11, *Enterobacter gergoviae*, *Klebsiella pneumoniae* subsp. *pneumoniae* (112 positive strains/152 strains tested) and the rare glycerol-positive strains of *"Salmonella enterica* subsp. *enterica"* serovar Senftenberg and Derby, one strain of *Citrobacter*

farmeri, *Enterobacter cloacae* group 4 (Grimont and Grimont, 1992), *Enterobacter agglomerans* group XII and of *Leclercia adecarboxylata*, could grow fermentatively on glycerol and possessed both glyDH type I and 1,3-PD-DH which are typical enzymes of the anaerobic glycerol dissimilation pathway.

Six other species, *Citrobacter koseri*, *Enterobacter aerogenes*, *Enterobacter intermedium*, *Klebsiella oxytoca*, *Klebsiella planticola* and *Klebsiella terrigena* could not grow fermentatively on glycerol and possessed a glycerol dehydrogenase type I but no 1,3-PD-DH. *Klebsiella pneumoniae* subsp. *ozaenae* was unique in having glyDH-I without DHA kinase.

Other glycerol dehydrogenase types were found: type II (induced by glycerol and hydroxyacetone), type III (induced by glycerol only) and type IV (induced by hydroxyacetone only) were widely distributed among the *Enterobacteriaceae*. They are not responsible for anaerobic glycerol pathway and their physiological roles are not clear.

Table I provides new characters for differentiating species in the genera *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella* and *Serratia*. In some cases, the study of anaerobic glycerol dissimilation may have epidemiological interest. Different reactions were observed for *Klebsiella pneumoniae* subsp. *pneumoniae* and *Shigella*; more strains need to be studied before any epidemiological application of this test can be proposed.

The tests currently used for the identification of bacteria were often empirically designed and do not take advantage of the large diversity of metabolic pathways. This work is part of a continuing effort (Bouvet and Grimont, 1987, 1989) to design new identification tests with a strong biochemical basis.

Diversité du métabolisme anaérobie du glycérol chez les *Enterobacteriaceae*: applications taxonomiques

La présence de glycérol-déshydrogénases et de 1,3-propanediol-déshydrogénase a été recherchée chez 1.123 souches appartenant aux 128 genomospecies de la famille des *Enterobacteriaceae*. Seuls huit espèces, *Citrobacter freundii* sensu stricto, *C. youngae*, *C. braakii*, *C. werkmanii*, *Citrobacter* genomospecies 10 and 11, *Enterobacter gergoviae* et *Klebsiella pneumoniae* subsp. *pneumoniae* peuvent fermenter le glycérol et possèdent une glycérol-déshydrogénase de type I (induite par le glycérol et la dihydroxyacétone) et une 1,3-propanediol-déshydrogénase, deux enzymes essentielles au métabolisme anaérobie du glycérol. Une activité glycérol-déshydrogénase de type I a été détectée chez six autres espèces: *C. koseri*, *E. aerogenes*, *E. intermedium*, *K. oxytoca*, *K. planticola* et *K. terrigena*. Ces dernières sont dépourvues d'activité 1,3-propanediol-déshydrogénase et ne fermentent pas le glycérol. D'autres types de glycérol-déshydrogénases ont été identifiés: de type II (induite par le glycérol et l'hydroxyacétone), de type III (induite uniquement par le glycérol) et de type IV (induite uniquement par l'hydroxyacétone). Ces dernières sont très répandues au sein de la famille des *Enterobacteriaceae*. Les tests proposés, permettent d'explorer le métabolisme anaérobie du glycérol, pourront être une aide au diagnostic et à la classification des bactéries.

Mots-clés: Taxonomie, *Enterobacteriaceae*, Glycérol; Anaérobiose, Dihydroxyacétone, 1,3-Propanediol.

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