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Taxonomic Investigations on Expressed and Cryptic Phospho-β-glucosidases in *Enterobacteriaceae*

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In the *Enterobacteriaceae*, β-glucosides are catabolized by a complex system formed of three permeases, with partly overlapping substrate specificities, and two hydrolytic enzymes, phospho-β-glucosidase A and B, which hydrolyze only phosphorylated β-glucosides. Some *Enterobacteriaceae* such as *Klebsiella-Aerobacter* (*Enterobacter*) possess the complete system; others possess only parts of it or may have a cryptic phospho-β-glucosidase activity without permease activity. A screening test applied to strains belonging to several genera of *Enterobacteriaceae* showed that strains of *Citrobacter*, *Hafnia*, and *Serratia* exhibit a degree of similarity in phospho-β-glucosidase activity and inducibility which could be useful in their taxonomic characterization; others, such as *Aerobacter aerogenes*, *Erwinia*, and *Proteus vulgaris*, are more heterologous. Owing to the presence of inducible phospho-β-glucosidases A and B in *Citrobacter*, the fermentation of β-methyl glucoside and the fermentation of arbutin in mixture with cellobiose could be of diagnostic value in the differentiation of *Citrobacter* from *Salmonella*. Wild-type strains of *Escherichia coli*, *Shigella*, and *Salmonella* are phenotypically similar in their inability to catabolize β-glucosides, the presence of constitutive P-β-glucosidase A, and the lack of β-glucoside permeases I and II. Their β-glucoside-fermenting mutants show, however, a phospho-β-glucosidase and β-glucoside permease activity which is characteristic for mutants from each genus. The differences in the phenotype of the mutants reflect probable differences in the presence of cryptic genes in the wild-type strains and could be of evolutionary significance.

Enterobacteriaceae are characterized by marked differences in the utilization of β-glucosides (2, 4). Wild-type strains of the *Klebsiella-Aerobacter* (*Enterobacter*) group ferment β-methyl glucoside, aromatic β-glucosides such as arbutin and salicin, and also cellobiose. Most *Citrobacter* wild-type strains utilize cellobiose but show a weak or not detectable utilization of aromatic β-glucosides, whereas β-glucoside-positive strains of *Proteus vulgaris* ferment salicin and arbutin but not cellobiose. Previous investigations of one of us (14) showed that wild-type strains of *Escherichia coli* and *Salmonella* are β-glucoside-negative, but can be differentiated by the phenotype of their β-glucoside-fermenting mutants. Mutants of various *E. coli* strains ferment arbutin and salicin but not cellobiose (10, 14). Mutants of *Salmonella* ferment cellobiose but not arbutin and salicin, although a weak fermentation of aromatic β-glucosides and lactose can be obtained by a second mutation (15, 16). Because of the phenotypic similarity of *Salmonella* mutants to *Citrobacter* wild-type strains, as well as other common characteristics, we considered *Salmonella* closer to *Citrobacter* than to *E. coli*.

The present investigation is an attempt to relate differences in the range of β-glucosides catabolized by different groups of *Enterobacteriaceae* to differences in active uptake, enzymatic hydrolysis, and inducibility. As no pertinent data are available on *Enterobacteriaceae* other than *E. coli* and *Aerobacter aerogenes* (3, 10, 11), a relatively simple screening test for phospho β-glucosidase and permease activity was applied for their detection in representatives of several genera of *Enterobacteriaceae*. In some instances the results of this screening test were supplemented by the determination of the active uptake of ^{14}C-thiophenyl β-glucoside and by enzymatic determinations with cell-free extracts.

Data obtained with *E. coli* and *A. aerogenes* indicate the existence of a complex catabolic system for the utilization of β-glucosides. In *A. aerogenes* (13), this system consists of three

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permeases with partly overlapping substrate specificities which accumulate phosphorylated β-glucosides: (i) the constitutive glucose permease which, in addition to glucose and α-methyl glucoside, accumulates also aliphatic β-glucosides and thioglucosides such as β-methyl glucoside and thioethyl β-glucoside, but not aromatic β-glucosides or cellobiose; (ii) the inducible β-glucoside permease I (aromatic permease) which accumulates aromatic β-glucosides such as salicin, thiophenyl β-glucoside (TPG), or *p*-nitrophenyl β-glucoside (PNP-glu), and also aliphatic β-glucosides (but not cellobiose) and is induced by aliphatic and aromatic β-glucosides; (iii) the inducible β-glucoside permease II (cellobiose permease), with high affinity for cellobiose, which accumulates also aliphatic and aromatic β-glucosides. β-Glucoside permease II is induced by cellobiose. The phosphorylation of β-glucosides during their active uptake occurs through the phosphoenol-pyruvate (PEP)-dependent phosphotransferase system (3, 13). In a first step, the phosphoryl group of PEP is transferred by enzyme I to a heat-stable protein. In a second step, substrate-specific membrane-bound enzymes II transfer the phosphoryl group to the substrates which are accumulated in a phosphorylated form. In the β-glucoside system there are three species of enzyme II with partly overlapping substrate specificities (Table 1), corresponding to the three permeases. After phosphorylation in the 6-position of their glucose moiety, aromatic β-glucosides are hydrolyzed into glucose-6-phosphate (G-6-P) and aglycone by two distinct enzymes, phospho-β-glucosidases A and B. Phospho-β-glucosidase A hydrolyzes PNP-glu, *o*-nitrophenyl β-glucoside, and other aromatic β-glucosides, with the exception of salicin. Phospho-β-glucosidase B is more thermolabile, has a substrate range similar to that of phospho-β-glucosidase A, and hydrolyzes phosphorylated salicin. Phospho-β-glucosidases A and B have a relatively low affinity for β-methyl glucoside and no detectable affinity for cellobiose. Preliminary investigations (S. Schaefler, *unpublished data*) indicate that cellobiose is hydrolyzed by a third enzyme, different from phospho-β-glucosidases A and B.

As the phosphorylation of β-glucosides by membrane-bound enzyme II is a prerequisite for their hydrolysis by phospho-β-glucosidases, the impairment of the integrity of the cell membrane by toluene treatment or sonic oscillation will inhibit the hydrolysis of β-glucosides. The determination of the hydrolysis of β-glucosides by cell-free extracts or toluenized cells requires, therefore, either the utilization of phosphorylated β-glucosides (3) or the coupling of the reaction with a phosphorylating system. In *A. aerogenes* (13; Schaefler and Schenkein, Proc. Nat. Acad. Sci. U.S.A., *in press*) but not in most *Enterobacteriaceae*, a soluble phosphotransferase was found (GL-phosphotransferase) which transfers inorganic phosphate from G-6-P and several other phosphate esters to glucose and α and β-glucosides. Addition of G-6-P to sonic extracts of *A. aerogenes* activates the hydrolysis of aromatic β-glucosides. In strains lacking GL-phosphotransferase, the phospho-β-glucosidase activity of toluenized cells or sonic extracts can be determined by the addition of G-6-P and GL-phosphotransferase from *A. aerogenes*.

Based on the assumption of an overall similarity of the substrate specificity of the β-glucoside permeases and phospho-β-glucosidases in *Enterobacteriaceae*, a screening test for the phospho-β-glucosidase activity of intact and toluenized cells was devised which also gives indirect indications for the presence of β-glucoside permeases I and II. By applying this screening test to several taxonomic groups of *Enterobacteriaceae*, it was found that some groups, such as *Citrobacter*, *Serratia*, and *Hafnia*, show a characteristic pattern of inducibility and enzymatic activity which could be of taxonomic significance. Investigations with β-glucoside-fermenting mutants obtained from β-glucoside-negative wild-type strains of *E. coli*, *Shigella*, and *Salmonella* indicated a high degree of similarity in mutants derived from wild-type strains of the same genus and characteristic differences among mutants of different genera. These differences reflect the presence of different cryptic genes in wild-type strains of each of these genera.

MATERIALS AND METHODS

Microorganisms. The present investigation was performed with four strains of *Alcalescens dispar*, 28 of *Citrobacter*, 7 of *Erwinia*, 14 of *E. coli*, 8 of *Hafnia*, 21 of *Klebsiella-Aerobacter* (*Enterobacter*), 25 of *Proteus*, 2 of *Providencia*, 18 of *Salmonella*, 7 of *Serratia*, and 24 of *Shigella*. The strains were obtained from B. Beame, W. Ewing, W. Martin, M. Doudoroff, D. Hildebrandt, F. Kauffmann, and from the collection of our Department.

Culture media. The strains were maintained on nutrient-agar slants. Fermentation tests and the isolation of fermenting mutants were made on the medium of Davis and Mingioli (1) supplemented with 0.075% yeast extract, 0.002% bromthymol blue, 2% agar, and 0.5% β-methyl glucoside, salicin, arbutin, or cellobiose, and also mixtures of β-methyl glucoside plus arbutin and cellobiose plus arbutin. This semisynthetic medium allows the detection of weak fermentations by growth response as well as by change in the indicator. The oxidation into quinone of hydroquinone liberated from arbutin allows the detection

of the fermentation of arbutin in the presence of other fermentable carbohydrates. Enzyme and permease determinations were made with cells grown in medium. LB [10 g of yeast extract, 20 g of Tryptone (Difco), 5 g of NaCl, and 1,000 ml of distilled water (*p*H 7.3), with NaOH] or in Davis and Mingioli medium with 0.5% sodium succinate and 0.2% yeast extract.

Isolation of fermenting mutants. Spontaneous β-glucoside-fermenting mutants were obtained either from papillae on solid semisynthetic medium or after an incubation of 3 to 9 days in liquid semisynthetic medium, followed by isolation on solid medium. The first method was used for the isolation of *E. coli* and *A. dispar* mutants and the second method for the isolation of *Salmonella* and *Shigella* mutants.

Screening test for phospho-β-glucosidases. Cells were grown overnight by shaking at 35 C (*Erwinia* strains were grown at 28 C) in 7 ml of LB medium without inducer and in the presence of 2×10^{-2} M β-methyl glucoside, 2×10^{-2} M cellobiose, and 2×10^{-4} M thiophenyl β-glucoside (TPG). After determination of the optical density at 560 nm (Coleman Junior spectrophotometer, 19-mm tubes), the cells were washed in 0.075 M phosphate buffer (*p*H 7.3) and suspended in 1.5 ml of buffer, and 1 ml of the suspension was then treated with 0.05 ml of toluene by shaking for 20 min at 35 C. The phospho-β-glucosidase activity was then tested in the following systems: (i) 0.2 ml of intact cells, 0.1 ml of water, and 0.1 ml of 2×10^{-2} M PNP-glu; (ii) 0.2 ml of toluenized cells, 0.1 ml of water, and 0.1 ml of PNP-glu; (iii) 0.2 ml of toluenized cells, 0.05 ml of water, 0.05 ml of 5×10^{-2} M G-6-P, and 0.1 ml of PNP-glu; (iv) 0.2 ml of toluenized cells, 0.05 ml of G-6-P, 0.05 ml of Gl-phosphotransferase from *A. aerogenes*, and 0.1 ml of PNP-glu; (v) identical to system (iv), but PNP-glu is replaced by salicin. In some instances the hydrolysis of salicin by intact cells was also determined. After incubation for 20 min at 35 C, the reaction was stopped by addition of 0.5 ml of 2 M Na_2CO_3. After completion to 5 ml with water, *p*-nitrophenol liberated from PNP-glu and saligenin liberated from salicin were tested as described previously (10). The liberation of 1 nmole per min of end product per mg (dry weight) of cells corresponds to 1 unit of activity. A cell suspension with an optical density of 0.60 at 560 nm (Coleman Junior spectrophotometer) is considered to contain the equivelent of 1 mg (dry weight) of cells. The interpretation of the screening test is given in Table 2.

Determination of phospho-β-glucosidase activity of sonic extracts. Cells were grown by shaking in 300 ml of LB broth in 1,000-ml Erlenmeyer flasks in the presence and absence of inducers. Washed cells, 50 mg (dry weight)/ml in 0.075 M phosphate buffer (*p*H 7.3), were sonically treated for 1 min at 4 amp in a Branson Sonifier. After centrifugation for 20 min at $9{,}000 \times g$, the supernatant fluid was used for phospho-β-glucosidase determinations. The activity was tested with PNP-glu and salicin as substrates in the following systems: (i) 0.05 or 0.1 ml of extract made up to 0.2 ml with 0.05 M phosphate buffer (*p*H 7.3), 0.1 ml of water, and 0.1 ml of substrate; (ii) 0.05 or 0.1 ml of extract made up to 0.2 ml with phosphate buffer, 0.05 ml of water, 0.05 ml of G-6-P, and 0.1 ml of substrate; (iii) 0.05 or 0.1 ml of extract made up to 0.2 ml with phosphate buffer, 0.05 ml of G-6-P, 0.05 ml of GL-phosphotransferase, and 0.1 ml of substrate. Incubation was for 20 min at 35 C. The substrate concentrations and the determination of the aglycone liberated were the same as for bacterial cells.

Preparation of phosphotransferase from A. aerogenes. Sonic extracts of *A. aerogenes* strain A_1 (13) cultivated in medium LB with 0.5% β-methyl glucoside were prepared as outlined for phospho-β-glucosidase determinations. Nucleic acids were precipitated with 0.4% protamine sulfate and were removed by centrifugation for 20 min at $9{,}000 \times g$. The supernatant fraction was heated for 20 min at 60 C and centrifuged; the supernatant fluid was used as the phosphotransferase preparation. The heated preparation had no phospho-β-glucosidase activity, whereas phosphotransferase activity was preserved. In the frozen state the phosphotransferase preparation can be preserved for at least 1 year. A single preparation was used during the present investigation. At *p*H 7.2 in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, the preparation hydrolyzes 3.6 μmoles of *p*-nitrophenyl phosphate per min per mg of protein and transfers 1.7 μmoles of phosphate per min per mg of protein from 2-deoxy glucose-6-phosphate to glucose, leading to the formation of G-6-P. Further data on the characteristics of the enzyme will be published elsewhere (Schaefler and Schenkein, Proc. Nat. Acad. Sci. U.S.A., *in press*).

Permease activity. Permease activity was determined as described previously (10, 13) by using bacterial suspensions of 0.5 mg (dry weight)/ml and a final concentration of 2×10^{-4} M TPG (11,000 counts per min per ml) as substrate. Chasing experiments were performed by using final concentrations of 10^{-2} M nonradioactive phenyl β-glucoside, cellobiose, glucose, and lactose.

RESULTS

Screening test for phospho-β-glucosidases. Based on the data obtained on β-glucoside permeases and phospho-β-glucosidases in *E. coli* (3, 10, 11) and *A. aerogenes* (13), a relatively simple screening test was devised for the detection of phospho-β-glucosidases in various *Enterobacteriaceae*. This screening test gives only a first indication of the presence and inducibility of phospho-β-glucosidases and β-glucoside permeases, and in representative strains its results were supplemented by determinations of active uptake of ^{14}C-TPG and by enzyme determinations with sonic extracts. The application of this screening test is based on the assumption of overall similarities in the catabolism of β-glucosides in *Enterobacteriaceae*. The data obtained so far in *Enterobacteriaceae* and some gram-positive bacteria (12) are consistent with this hypothesis. By the use of three different inducers and by testing the hydrolysis of both PNP-glu and salicin, evidence was obtained for the presence or absence of β-glucoside permeases I and II, phospho-β-glucosidases

A and B, and of a soluble phosphotransferase with a substrate specificity similar to the GL-phosphotransferase from *A. aerogenes*. The selection of the inducers β-methyl glucoside, cellobiose, and TPG is based on the substrate specificity and inducibility of the three permeases which accumulate β-glucosides (Table 1). The hydrolysis by intact cells of aromatic β-glucosides such as PNP-glu is indicative of the presence of either β-glucoside permease I or of permease II. Induction by β-methyl glucoside and especially TPG indicate the presence of permease I and induction by cellobiose of permease II (13). β-Methyl glucoside is accumulated by the two β-glucoside permeases and also by the constitutive glucose permease. β-Methyl glucoside can therefore serve as inducer of cryptic phospho-β-glucosidases in strains unable to catabolize aromatic β-glucosides due to the lack of β-glucoside permeases I and II.

The phosphorylation during their active uptake is required for the further catabolism of β-glucosides. Therefore, loss of activity after toluene treatment or sonic treatment and detection of activity after addition of G-6-P or G-6-P + GL-phosphotransferase from *A. aerogenes* is indicative of the presence of phospho-β-glucosidases. The addition of G-6-P and GL-phosphotransferase allows also the study of cryptic phospho-β-glucosidases in strains unable to catabolize aromatic β-glucosides due to the lack of β-glucoside permeases I and II (13). The hydrolysis of phosphorylated PNP-glu but not of phosphorylated salicin is indicative of the presence of phospho-β-glucosidase A and the absence of phospho-β-glucosidase B. If both phosphorylated PNP-glu and salicin are hydrolyzed by toluenized cells, the induction of the hydrolysis of PNP-glu and salicin by two different inducers can be considered indicative for the presence of two distinct phospho-β-glucosidases. Based on the above characteristics of the β-glucoside system, a schematic interpretation of the screening test outlined in the Methods section is given in Table 2. By applying this screening test to representatives of several groups of *Enterobacteriaceae* we found that strains of *Hafnia*, *Citrobacter*, and *Serratia* show a characteristic pattern in their phospho-β-glucosidase activity, whereas others such as *A. aerogenes* and *Erwinia* show a wider range of variation.

Hafnia. The eight *Hafnia* strains analyzed can be divided into two groups: six strains are β-glucoside-negative, showing only a weak fermentation of β-methyl glucoside, and two strains are β-glucoside-positive, fermenting salicin and arbutin but not cellobiose. The first group is represented in Table 3 by strain 1006-68 and the second group by strain 1950-68. Intact cells of the β-glucoside-negative strain showed no detectable hydrolysis of PNP-glu. In the β-glucoside-positive strain, the hydrolysis of PNP-glu by intact cells was induced by β-methyl glucoside and TPG but not by cellobiose, indicating the presence of an inducible β-glucoside permease I. Toluene treatment inactivated the hydrolysis of PNP-glu by the β-glucoside-positive strain, and the addition of G-6-P restored the activity. In the β-glu-

TABLE 1. *Active uptake of β-glucosides by E. coli and A. aerogenes*[a]

Permease	Substrate				Inducer
	α-Methyl glucoside, glucose	Aliphatic β-glucosides and thioglucosides	Aromatic β-glucosides and thioglucosides	Cellobiose	
Glucose permease[b]	++	+	−	−	Constitutive
β-Glucoside permease I[c]	−[e]	+	++	−	Aliphatic and aromatic β-glucosides
β-Glucoside permease II[d]	+	+	+	++	Cellobiose

[a] The uptake of α-methyl glucoside and β-glucosides was determined by the accumulation of ^{14}C-α-methyl glucoside, ^{14}C-TPG, and ^{14}C-thioethyl β-glucoside. The substrate specificity was determined by chasing of the radioactive compound by nonradioactive α-methyl glucoside, glucose, phenyl-β-glucoside, and cellobiose. Symbols indicate relative affinities for tested substrates.

[b] Wild-type cells of *E. coli* K-12, β-glucoside-fermenting mutants of *E. coli* K-12, and wild-type cells of *A. aerogenes* A_1.

[c] β-Glucoside-fermenting mutants of *E. coli* K-12 and wild type cells of *A. aerogenes*.

[d] Wild-type cells of *A. aerogenes*.

[e] α-Methyl glucoside and glucose increase the accumulation of ^{14}C-TPG by β-glucoside permease I.

TABLE 2. *Presumptive interpretation of the hydrolysis of PNP-glu and salicin by intact and toluenized cells*

Characteristic	Interpretation
(A) Hydrolysis of PNP-glu by intact cells	Indicates presence of β-glucoside permease I or II and at least one phospho-β-glucosidase.
(B) Hydrolysis of PNP-glu by toluenized cells	Activity lower than in systems (C) and (D); indicates that phosphorylation of aromatic β-glucosides is required for their enzymatic hydrolysis
(C) Hydrolysis of PNP-glu by toluenized cells + G-6-P	Activity higher than in system (B); indicates presence of at least one phospho-β-glucosidase and of a phosphotransferase which can transfer P_i from G-6-P to PNP-glu.
(D) Hydrolysis of PNP-glu by toluenized cells + G-6-P and phosphotransferase	Activity higher than in system (C); indicates presence of at least one phospho-β-glucosidase. Phosphotransferase activity of toluenized cells is low or absent. If system (D) is active and system (E) is inactive, only phospho-β-glucosidase A is present.
(E) Hydrolysis of salicin by toluenized cells + G-6-P and phosphotransferase	Indicates presence of phospho-β-glucosidase B or phospho-β-glucosidase A and B. Additional information on presence of one or two phospho-β-glucosidases can be obtained by comparing activities of systems (D) and (E) after growth in presence of inducers and the thermolability of hydrolysis of PNP-glu and salicin.

coside-negative strain, the addition of G-6-P to toluenized cells resulted in the hydrolysis of PNP-glu by a cryptic phospho-β-glucosidase which was not detected in intact cells. The activity of toluenized cells increased further on addition of G-6-P + GL-phosphotransferase. Toluenized cells hydrolyzed phosphorylated PNP-glu as well as phosphorylated salicin. The activity of toluenized cells after addition of G-6-P without GL-phosphotransferase from *A. aerogenes* indicates the presence in *Hafnia* of a soluble phosphotransferase which transfers the phosphoryl group from G-6-P to aromatic β-glucosides. In all strains tested, β-methyl glucoside induces the hydrolysis of phosphorylated PNP-glu and salicin, whereas TPG is an inducer only in the β-glucoside-positive strains. This can be explained by the uptake of TPG by the β-glucoside permease I which is present only in β-glucoside-positive strains. β-Methyl glucoside which can be accumulated by the glucose permease acts as inducer in positive and negative strains. Determination of the active uptake of ^{14}C-TPG by the β-glucoside-positive strain 1950–68 indicated a maximal accumulation of 3.6 μmoles of ^{14}C-TPG/g (dry weight) by uninduced cells, which increased to 9.2 μmoles/g after growth in the presence of TPG. The labeled compound was chased by phenyl β-glucoside but not by cellobiose, indicating the presence of β-glucoside permease I. The β-glucoside-negative strain 1006-68 accumulated 1.8 μmoles of ^{14}C-TPG/g and the accumulation did not increase after growth in the presence of TPG or β-methyl glucoside. The activity of the second β-glucoside-positive strain is similar to that of strain 1950–68, and the activity of five β-glucoside-negative strains is similar to that of strain 1006-68. It appears, therefore, that common elements of all *Hafnia* strains analyzed are a semiconstitutive phospho-β-glucosidase which is further induced by β-methyl glucoside and a soluble phosphotransferase. The β-glucoside-positive strains have in addition an inducible β-glucoside permease I.

Citrobacter. The 28 *Citrobacter* strains can be divided into two groups: 21 strains which are inducible for the hydrolysis of PNP-glu and salicin by intact cells and 7 which are semiconstitutive but show the same induction pattern as the inducible strains. In Table 3, the inducible strains are represented by strain C24 and the semiconstitutive strains by strain C28. Common elements of all tested *Citrobacter* strains are as follows. The hydrolysis of PNP-glu and salicin by intact cells is induced by cellobiose but not by β-methyl glucoside and TPG. The activity of toluenized cells can be detected only after the addition of G-6-P and glucosylphosphotransferase. The hydrolysis of phosphorylated PNP-glu is semiconstitutive and increases after growth in the presence of β-methyl glucoside, whereas the hydrolysis of phosphorylated salicin is induced by cellobiose. TPG, arbutin, and salicin have no inducer activity. The difference in the inducer specificity of the hydrolysis of phosphorylated PNP-glu and salicin could be due to the presence of two different phospho-β-glucosidases. This is also indicated by the higher temperature sensitivity of the hydrolysis of salicin by sonic extracts than that of PNP-glu (11).

Prior investigations indicated the presence in *Citrobacter* of β-glucoside permease II with high affinity for both phenyl β-glucoside and cellobiose (13). Similar results were obtained in the present

investigation with the strains C24 and C28. Uninduced cells of strain C24 showed a maximal accumulation of 7.6 μmoles of ^{14}C-TPG per g and uninduced cells of strain C28 accumulated 13.8 μmoles of ^{14}C-TPG per g. TPG was chased by phenyl β-glucoside and cellobiose. Growth in the presence of cellobiose did not produce a significant increase in the accumulation of TPG (8.7 and 15.3 μmoles/g) but appeared to increase the affinity for cellobiose. With cells of strain C24, the chasing of TPG by cellobiose increased from 46 to 63%, and with cells of strain C28 it increased from 53 to 76%.

As the differentiation of *Citrobacter* from *Salmonella* is important from a diagnostic point of view, an attempt was made to develop fermentation tests based on the characteristics of the β-glucoside system in these two genera. With some exceptions (4), *Salmonella* does not ferment cellobiose, salicin, and arbutin, and ferments β-methyl glucoside only weakly. Literature on *Citrobacter* (3, 4, 14, 16) indicates that most strains show a prompt or delayed fermentation of cellobiose and some strains ferment salicin and arbutin. Among the 28 strains analyzed, 22 ferment cellobiose in 24 to 48 hr, 8 showed a weak fermentation of arbutin, and 6 showed a weak fermentation of salicin. All tested strains ferment β-methyl glucoside, however. A prompt fermentation of β-methyl glucoside was obtained on semisynthetic medium with β-methyl glucoside as carbon source as well as on MacConkey and phenol red media. This is consistent with the induction of P-β-glucosidase A by β-methyl glucoside. Although the fermentation of arbutin is weak and varies with the strain tested, arbutin was fermented by all strains when added together with β-methyl glucoside and especially with cellobiose. The fermentation of arbutin can be easily detected on solid medium by the oxidation of hydroquinone liberated in the presence of β-methyl glucoside or cellobiose. The catabolism of arbutin in the above mixtures can be explained by the induction of phospho-β-glucosidase A by β-methyl glucoside and of phospho-β-glucosidase B by cellobiose, which in turn hydrolyze phosphorylated arbutin. Arbutin per se shows no detectable inducer activity and therefore its hydrolysis in the absence of the above inducers is weak or not detectable.

TABLE 3. *Phospho-β-glucosidase activity of Hafnia, Citrobacter, and Serratia strains*[a]

Strain	Inducer[b]	Intact cells: PNP glu[c]	Toluenized cells[d]			
			PNP glu	PNP glu + G-6-P	PNP-glu + G-6-P and phosphotransferase	Salicin + G-6-P and phosphotransferase
Hafnia 1006-68	None			3	3	2
	β-Methyl			28	34	27
	Cellob			3	2	3
	TPG			3	2	2
Hafnia 1050-68	None	2	2	4	3	3
	β-Methyl	39	3	31	61	63
	Cellob	3		3	3	2
	TPG	41	3	34	65	61
Citrobacter C-24	None				3	
	β-Methyl				12	
	Cellob	5			6	14
	TPG				4	
Citrobacter C-28	None	3			6	4
	β-Methyl	2			14	5
	Cellob	8		2	7	17
	TPG	2			5	4
Serratia marcescens 1201-65	None				6	2
	β-Methyl				49	3
	Cellob				7	2
	TPG	26		3	19	26

[a] Overnight cultures grown in LB medium and washed with 0.05 M phosphate buffer were tested for phospho-β-glucosidase activity. Activity is expressed in nanomoles per minute per milligram (dry weight) of cells. Incubation was for 20 min at 35 C.

[b] Abbreviations: β-Methyl, β-methyl glucoside; Cellob, cellobiose.

[c] Suspension of 0.2 ml of intact cells.

[d] Suspensions of 0.2 ml of toluene-treated cells.

Serratia. The hydrolysis of PNP-glu by intact cells of strain 1201-65 (Table 3) is induced by TPG but not by β-methyl glucoside or cellobiose. Toluenized cells showed no increase in activity after the addition of G-6-P; their activity was restored by addition of G-6-P + Gl-phosphotransferase. As in *Citrobacter*, β-methyl glucoside induces cryptic phospho-β-glucosidase A but not phospho-β-glucosidase B which is induced by TPG. The inducer activity of TPG indicates the presence of a β-glucoside permease I. This was confirmed by the determination of the uptake of ^{14}C-TPG. The permease induced by TPG has a high affinity for phenyl β-glucoside and a low affinity for cellobiose. The enzymatic activity of six additional *Serratia* strains was found similar to that in strain 1201-65. In two of these strains, in addition to TPG, β-methyl glucoside is also a weak inducer of the hydrolysis of PNP-glu by intact cells, and in one strain cellobiose is a weak inducer of cryptic phospho-β-glucosidase B. All *Serratia* strains tested ferment salicin and arbutin and showed a weak fermentation of cellobiose.

Klebsiella-Aerobacter (Enterobacter). Common features of the strains of *A. aerogenes* (*Enterobacter* B), *A. cloacae* (*Enterobacter* C), *A. liquefaciens* (*Enterobacter* A), and *K. rhinoscleromatis* are the semiconstitutive enzymatic activity of intact cells and the presence of a semiconstitutive phospho-β-glucosidase B (Table 4). The fermentation of salicin by wild-type cells of all strains tested is consistent with the presence of phospho-β-glucosidase B. The strains differ, however, in their inducibility and the activity of the soluble phosphotransferase. Intact and toluenized cells of *Klebsiella-Aerobacter* are characterized by a relatively high phospho-β-glucosidase activity. Depending on the strain, fully induced intact cells have an activity of 34-123 units/mg with PNP-glu as substrate. Induced toluenized cells have an activity of 53-108 units with phosphorylated PNP-glu and 37-84 units with phosphorylated salicin. Among the 15 strains of *A. aerogenes* analyzed, 10 strains are induced by β-methyl glucoside, cellobiose, and TPG; in this respect they are similar to the previously investigated *A. aerogenes* strain A_1 (13). Strains R5, 17, and two additional strains are induced by β-methyl glucoside and cellobiose but not by TPG, and strain 2002 is induced by TPG and cellobiose but not by β-methyl glucoside. All tested *A. aerogenes* strains possess a soluble phosphotransferase, and the activity of toluenized cells parallels that of intact cells. Permease determinations on four strains induced by all three inducers showed, as found in *A. aerogenes* A_1, the induction of permease I activity by β-methyl glucoside and TPG and of permease II activity by cellobiose. In strains R5 and 17 both β-methyl glucoside and cellobiose induce a permease with affinity for phenyl β-glucoside and cellobiose. Whether the permease induced by β-methyl glucoside is identical with β-glucoside permease II induced by cellobiose requires further investigation. In strain 2002, TPG, but not β-methyl glucoside, induces permease I and cellobiose induces premease II. The activity of the strains of *A. cloacae*, *A. liquefaciens*, and *K. rhinoscleromatis* grown in the presence and absence of inducers is shown in Table 4. The strains belonging to the same species showed a similar induction pattern. Phosphotransferase activity was found only in *K. rhinoscleromatis*.

Erwinia. Prior investigations by Schroth and Hildebrandt (17) showed no detectable β-glucosidase activity in sonic extracts of *Erwinia amylovora*, although their strains catabolized arbutin. In the present investigation we found phospho-β-glucosidase activity in all strains tested, including three strains obtained through the courtesy of D. Hildebrandt. The strains showed differences in activity and inducibility (Table 4). The apparent lack of activity of sonic extracts found by Schroth and Hildebrandt could be explained by their use of nonphosphorylated β-glucosides.

Proteus-Providencia. No detectable phospho-β-glucosidase activity was found with the *Providencia*, *Proteus morganii*, and *P. mirabilis* strains (Table 4). When the reaction time was prolonged from 20 min to 1 hr or longer, weak activity was detected with most strains. β-Glucoside-positive and β-glucoside-negative strains were found among the *P. vulgaris* and *P. rettgeri* strains. The positive strains are induced by β-methyl glucoside and TPG and ferment salicin and arbutin, but not cellobiose. Premease determinations with the *P. vulgaris* strain Pr-1 and the *P. rettgeri* strain 104 indicated the presence of β-glucoside permease I, induced by β-methyl glucoside and TPG.

Wild-type strains and mutants of E. coli, A. dispar, Shigella, and Salmonella. Wild-type strains of *E. coli*, *A. dispar*, *Shigella*, and *Salmonella* do not catabolize aromatic β-glucosides and cellobiose and lack β-glucoside permeases I and II. With the exception of most *Shigella boydii* and *Shigella flexneri* strains, all wild-type strains tested possess a low constitutive phospho-β-glucosidase A activity and lack phospho-β-glucosidase B. In *S. boydii* and *S. flexneri*, phospho-β-glucosidase A activity can be detected after incubation for 1 hr or longer. The cryptic phospho-β-glucosidase A activity of *E. coli*, *Shigella sonnei*, *A. dispar*, and *Salmonella* is 2 to 6 units/mg and is reflected in most strains in a

TABLE 4. *Constitutive and inducible phospho-β-glucosidase activity of wild-type strains*[a]

Organism	Intact cells PNP glu	Toluenized cells	
		Phosphorylated PNP glu	Phosphorylated salicin
Hafnia (8)[b]	Ind, β-me, TPG (2)	Scon, β-me, TPG (2)	Scon, β-me, TPG (2)
	— (6)	Scon, β-me (6)	Scon, β-me (6)
Citrobacter (28)	Ind, Cell (22)	Scon, β-me (28)	Ind, Cell (22)
	Scon, Cell (6)		Scon, Cell (6)
Serratia (7)	Ind, TPG[c]	Scon, β-me (7)	Scon, TPG (7)
Aerobacter aerogenes (15)	Scon, β-me, Cell TPG (10)	Scon, β-me Cell TPG (10)	Scon, β-me, Cell TPG (10)
	Scon, β-me, Cell (4)	Scon, β-me Cell (4)	Scon, β-me, Cell (4)
	Scon, Cell, TPG (1)	Scon, Cell, TPG (1)	Scon, Cell, TPG (1)
A. cloacae (2)	Scon, β-me, Cell (2)	Scon, β-me, Cell (2)	Scon, β-me, Cell (2)
A. liquefaciens (2)	Scon, Cell, TPG (2)	Scon, β-me, Cell TPG (2)	Scon, β-me, Cell TPG (2)
Klebsiella rhinoscleromatis (2)	Scon, β-me, TPG (2)	Scon, β-me, TPG (2)	Scon, β-me, TPG (2)
Proteus vulgaris (3)	Ind. β-me, TPG (1)	Scon, β-me, TPG (1)	Scon, β-me, TPG (1)
	— (2)	— (2)	— (2)
P. rettgeri (3)	Ind. β-me, TPG (2)	Scon, β-me, TPG (2)	Ind, β-me, TPG (2)
	— (1)	— (1)	— (1)
P. morganii (9)	— (9)	— (9)	— (9)
P. mirabilis (10)	— (10)	— (10)	— (10)
Erwinia (7)	Scon, β-me, TPG (4)	Scon, β-me, TPG (4)	Scon, β-me, TPG (4)
	Scon, β-me, Cell (2)	Scon, β-me, Cell (2)	Scon, β-me, Cell (2)
	— (1)	Const (1)	— (1)
Escherichia coli	— (14)	Const (14)	— (14)
Alcalescens dispar (4)	— (4)	Const (4)	— (4)
Shillega ambigua (2)	— (2)	Const (2)	— (2)
S. sonnei (5)	— (5)	Const (5)	— (5)
S. flexneri (5)	— (5)	Const (2)	— (5)
		— (3)	
S. boydii (9)	— (9)	Const (3)	— (9)
		— (6)	
Salmonella (18)	— (18)	Const (18)	— (18)

[a] Abbreviations used: Ind, inducible; Const, constitutive; Scon, semiconstitutive (maximal level of activity after induction is less than four times the basal activity); β-me, induced by β-methyl glucoside; Cell, induced by cellobiose; TPG, induced by TPG;—, no detectable activity. Values in parentheses indicates numbers of strains.

[b] Number of strains tested.

[c] Two strains showed a weak induction by β-methyl glucoside.

weak fermentation of β-methyl glucoside, which can be accumulated by the constitutive glucose permease.

Prior investigations of one of us (14, 16) showed that *E. coli* and *Salmonella*, although phenotypically identical in their inability to catabolize β-glucosides, differ in the substrate range of their spontaneous β-glucoside-fermenting mutants. Genetic and biochemical data obtained with *E. coli* K-12 (3, 10, 11, 13) indicate that the *β-gl*$^-$ $\rightarrow$ *β-gl*$^+$ mutation, resulting in the fermentation of salicin and arbutin, is accompanied by the appearance of inducible β-glucoside permease I and phospho-β-glucosidase B activities. The permease and phospho-β-glucosidase are induced by aliphatic and aromatic β-glucosides and thioglucosides. The structural gene for phospho-β-glucosidase B is present in wild-type cells and in mutants, but is expressed only in mutants. Secondary arbutin$^+$ salicin$^-$ mutants (*β-gl*$^+$ *sal*$^-$) which lack phospho-β-glucosidase B and retain the permease activity can be obtained by mutagenesis (11). Investigations in progress indicate the existence of two additional classes of mutants. Mutants with a three- to ninefold increase of the constitutive phospho-β-glucosidase A activity, without simultaneous fermentation of salicin and arbutin (*β-me*$^+$), were obtained from β-gl$^-$ wild-type strains by selection for increased growth rate on β-methyl glucoside. Mutants which lack both phospho-β-glucosidases were obtained by mutagenesis from β-gl$^+$ sal$^-$ mutants. These

mutants also show a very low β-glucoside permease activity.

In the present study, β-gl^+ mutants obtained from 10 different *E. coli* wild-type strains showed an inducible hydrolysis of PNP-glu and salicin by intact cells. One strain showed a very low rate of hydrolysis of salicin. As in *E. coli* K-12, β-methyl glucoside and TPG induce the hydrolysis of PNP-glu and salicin by intact cells and the biosynthesis of phospho-β-glucosidase B. Secondary β-gl^+ sal^- mutants were obtained from β-gl^+ mutants of eight strains. Active uptake determinations made with two β-gl^+ and two β-gl^+ sal^- mutants showed that both β-methyl glucoside and TPG induce β-glucoside permease I. It appears, therefore, that *E. coli* mutants of different origin are of the phenotype previously found for *E. coli* K-12.

Among the four *A. dispar* strains tested, two gave β-gl^+ mutants inducible by β-methyl glucoside and TPG and possessed an inducible phospho-β-glucosidase B and β-glucoside permease I. One strain (02-7040-59) gave only β-methyl glucoside-fermenting mutants which do not ferment salicin and arbutin. As in β-me^+ mutants of *E. coli*, intact cells of these mutants do not hydrolyze PNP-glu and the cryptic phospho-β-glucosidase A activity is 14.7 units/mg as compared with 2.8 units in the wild-type strain.

β-Glucoside-fermenting mutants were obtained from three of the five *S. sonnei* strains tested. All mutants isolated so far ferment arbutin but not salicin, resembling in this respect the β-gl^+ sal^- secondary mutants of *E. coli* (Table 5). The hydrolysis of PNP-glu by intact cells of two mutants is semiconstitutive and is further induced by β-methyl glucoside and TPG. The mutant of the third strain is low constitutive (7 units of PNP-glu/mg, dry weight). From β-gl^+ sal^- mutants of all three strains, β-gl^+ mutants were obtained by a second spontaneous mutation. The β-gl^+ mutants possess a semiconstitutive phospho-β-glucosidase B. The determination of the active uptake of ^{14}C-TPG by a semiconstitutive and a constitutive β-gl^+ sal^- mutant indicated the presence of β-glucoside permease I.

Prior investigations showed that *Salmonella* strains of different antigenic groups give mutants which ferment cellobiose (clb^+) but not arbutin and salicin (14, 15, 16). From cellobiose-fermenting mutants, arbutin-fermenting mutants (arb^+) can be obtained by a second mutation. In the present investigation, clb^+ mutants were obtained from 15 out of 18 *Salmonella* wild-type strains. With the exception of a weak constitutive activity of the mutants isolated from *S. anatum* and *S. senftenberg*, intact cells of clb^+ mutants showed no detectable hydrolysis of PNP-glu and toluenized cells showed no detectable phospho-β-glucosidase B activity. The cryptic hydrolysis of PNP-glu of 3 to 5 units/mg found in toluenized cells of the wild-type strains increased to 8 to 14 units/mg in clb^+ mutants. After 5 to 9 days of incubation of clb^+ mutants in liquid semisynthetic medium with arbutin, arb^+ mutants were obtained from nine clb^+ mutants of different serotypes. The intact cells of all arb^+ mutants showed a

TABLE 5. *Catabolism of β-glucosides by wild-type strains and fermenting mutants of E. coli, S. sonnei, and Salmonella*[a]

Organism	Mutation	Derived from	Permease I	Permease II	Phospho-β-glucosidase A	Phospho-β-glucosidase B
Escherichia coli	WT				Const	
	β-gl+	WT	Ind		Const	Ind
	β-gl+sal−	*β-gl+*	Ind		Const	
	β-me+	WT			Const+[b]	
Shigella sonnei	WT				Const	
	β-gl+sal−	WT	Scon or Const		Const	
	β-gl+	*β-gl+sal−*	Scon or Const		Const	Scon
Salmonella	WT				Const	
	clb+	WT		Const	Const+[c]	
	arb+	*clb+*		Const+[d]	Const+[c]	Const or Scon

[a] Abbreviations used: WT, wild type; β-gl^+, arbutin+, salicin+ mutant; β-gl^+sal^-, arbutin+, salicin− mutant; β-me^+, β-methyl glucoside+ mutant; clb^+, cellobiose+ mutant; arb^+ cellobiose+, arbutin+ mutant; Ind, inducible; Scon, semiconstitutive; Const, constitutive.

[b] Increase of three to nine times the constitutive phospho-β-glucosidase A activity of wild-type strains.

[c] Increase of two to three times the constitutive phospho-β-glucosidase A activity of wild-type strains.

[d] Increase of three to four times the active uptake of TPG of clb^+ mutants.

constitutive hydrolysis of PNP-glu with an activity of 13 to 29 units/mg, depending on the strain. A weak constitutive phospho-β-glucosidase B activity was found in arbutin-fermenting mutants of *S. anatum*, *S. montevideo*, and *S. senftenberg*. The arb^+ mutants showed a weak fermentation of lactose and may be analogous to the previously described lactose-fermenting mutants of *Salmonella* (15). Active uptake experiments with wild-type strains, clb^+ mutants, and arb^+ mutants of four *Salmonella* strains showed lack of permease activity in the wild-type strains, a low constitutive β-glucoside permease II activity in clb^+ mutants and an increase of this constitutive activity in arb^+ mutants. For *S. senftenberg* the maximal accumulation of ^{14}C-TPG was 2.8 μmoles/g, with low affinity for cellobiose. It increased to 6.4 μmoles/g in a clb^+ mutant of this strain and to 23.7 μmoles/g in an arb^+ mutant derived from the clb^+ mutant. The constitutive hydrolysis of PNP-glu by arb^+ mutants of *Salmonella* can be explained by their high β-glucoside permease II activity. A comparison of the *E. coli*, *S. sonnei*, and *Salmonella* mutants is made in Table 5. The main difference between *Salmonella* mutants and *E. coli* and *Shigella* mutants is the presence of different permeases: β-glucoside permease II in *Salmonella* and β-glucoside permease I in *E. coli* and *Shigella*. This accounts for the difference in substrate range of *E. coli* and *Salmonella* mutants.

DISCUSSION

This investigation shows that in strains of *Enterobacteriaceae*, β-glucosides are hydrolyzed by phospho-β-glucosidases, after their active transport into the cells in a phosphorylated form by β-glucoside permeases. Among other gram-negative bacteria, phospho-β-glucosidases are also found in *Aeromonas* and *Arthrobacter* (S. Schaefler, *unpublished data*). The complete system consisting of three permeases and two phospho-β-glucosidases is found in *Klebsiella-Aerobacter* (13), whereas most other taxonomic groups possess only parts, either phenotypically expressed or in a cryptic form. Among strains belonging to the same taxonomic group there is often a sufficiently high degree of similarity in the catabolism of β-glucosides to be useful in their taxonomic characterization. Among such groups are *Citrobacter*, *Hafnia*, *Serratia*, and probably different species of *Klebsiella-Aerobacter* (*Enterobacter*). In some instances differences in the catabolism of β-glucosides could be of diagnostic value, as in the differentiation of *Citrobacter* from *Salmonella*.

The criteria for the differentiation of *Enterobacteriaceae* by their catabolism of β-glucosides are the presence of β-glucoside permeases, inducibility, and the presence of phospho-β-glucosidases A and B. Among these criteria, β-glucoside permeases I and II are probably the most important from the taxonomic point of view. The screening test used in the present investigation gives a first indication for their presence and inducibility. As phosphorylation during uptake is required for the catabolism of aromatic β-glucosides, the hydrolysis of β-glucosides by intact cells can be linked to the presence of β-glucoside permease I, β-glucoside permease II, or both permeases. Induction by β-methyl glucoside and especially by TPG of the hydrolysis of PNP-glu by intact cells, the fermentation of arbutin or arbutin and salicin, and the lack of fermentation of cellobiose can be related to the presence of only β-glucoside permease I. The accumulation of β-glucosides by this permease occurs in wild-type strains of *Serratia*, β-glucoside-fermenting strains of *Hafnia* and *Proteus*, and β-glucoside-fermenting mutants of *E. coli* and *S. sonnei*. The fermentation of cellobiose and induction by cellobiose but not by TPG are indicative of the presence of permease II. Permease II is found in wild-type strains of *Citrobacter* and β-glucoside-fermenting mutants of *Salmonella*. Most strains possessing only β-glucoside permease II show a weaker fermentation of aromatic β-glucosides than strains possessing β-glucoside permease I. This could be explained by the lower affinity of β-glucoside permease II for aromatic β-glucosides (13). In strains lacking β-glucoside permease I and II or in uninduced cells of inducible strains (10), β-methyl glucoside as well as thiomethyl β-glucoside and thioethyl β-glucoside are accumulated by the constitutive glucose permease. β-Methyl glucoside can therefore serve as inducer for cryptic phospho-β-glucosidase A in *Citrobacter* and *Serratia* and phospho-β-glucosidase B in *Hafnia*.

Strains of *E. coli*, *S. sonnei*, and *Salmonella*, although apparently phenotypically similar because of the presence of a cryptic phospho-β-glucosidase A and lack of β-glucoside permeases I and II, can be differentiated by the phenotype of their β-glucoside-fermenting mutants. This is based on the high degree of similarity of the mutants derived from strains of each of these taxonomic groups. All β-gl^+ mutants of *E. coli* tested possess an inducible β-glucoside permease I and an inducible phospho-β-glucosidase B. The clb^+ mutants of *Salmonella* possess a low constitutive β-glucoside permease II and show an increase in the phospho-β-glucosidase A activity. Secondary mutants derived from these mutants show also a high degree of similarity as found for the arb^+ mutants of *Salmonella* and β-gl^+ mu-

tants of *S. sonnei*. These observations could be explained by the presence of point mutations in genes such as the *β-gl*-B gene in the wild-type *E. coli* (11) which do not preclude the restoration of their function by back mutations or suppressor mutations, and the presence of intact cryptic genes, such as the structural gene for phospho-β-glucosidase B in *E. coli*, which are expressed only in mutants. The preservation of intact or mutationally modified cryptic genes can be found also in other catabolic systems of *Enterobacteriaceae*. The presence of cryptic genes can be detected either by genetic recombination, as for the *z* and *i* genes of the lactose system in *Shigella* (6, 7) or by the isolation of fermenting mutants, as for example the isolation by two successive mutations of rhamnose-fermenting mutants from FIRN strains of *S. typhimurium* (9). The presence of cryptic genes in *Salmonella* is also indicated by the isolation of mutants which ferment xylose, D-arabinose, sorbose, and raffinose (4, 16). In some instances, however, the mutation results in the activation of an existing alternative pathway, as described for mannitol-fermenting mutants of *A. aerogenes* (18).

In some instances, cryptic genes modified by one or several point mutations may represent relics of functional genes of ancestral strains and could therefore be used for the investigation of possible evolutionary relationships among *Enterobacteriaceae*. If one considers the loss of individual elements of the β-glucoside system as the result of a regressive physiological evolution (8, 19), then species of *Klebsiella-Aerobacter* which possess both β-glucoside permeases and both phospho-β-glucosidases are in this respect relatively close to the hypothetical primitive *Enterobacteriaceae*. The loss of β-glucoside permease II and retention of permease I resulted in wild-type strains which ferment salicin and arbutin but not cellobiose. *E. coli* and *A. dispar* strains are at least one further mutational step apart. They lack β-glucoside permease I activity, which in most strains appears as the result of spontaneous mutations, and possess the cryptic intact, but nonfunctional, gene for phospho-β-glucosidase B. The similarity in this respect of *E. coli* with *A. dispar* is consistent with their present classification (2, 4). An additional mutation probably occurred in the structural gene of phospho-β-glucosidase B of *S. sonnei*, as the appearance of the β-gl^+ phenotype requires two successive mutations instead of a single mutation as in *E. coli*. *S. flexneri* and *S. boydii* strains seem to have undergone further mutations affecting the biosynthesis of the cryptic phospho-β-glucosidase A. Deoxyribonucleic acid-annealing data, genetic exchange (7), and physiological and antigenic characteristics indicate the relatedness of *Shigella* to *E. coli*. The delayed fermentation of lactose and sucrose by *S. sonnei* indicates its close relationship to nonpathogenic *Enterobacteriaceae*.

The loss of β-glucoside permease I activity and retention of permease II would result in a phenotype similar to the present *Citrobacter*, characterized by the fermentation of cellobiose and a weak fermentation of aromatic β-glucosides. *Salmonella* are at least two mutations apart, the expression of the full β-glucoside permease II activity in arb^+ strains requiring two successive mutations. Another mutation probably occurred in the structural gene of phospho-β-glucosidase B. Although differences such as lysine decarboxylase activity and resistance to KCN make the filiation of *Salmonella* from ancestors similar to the present *Citrobacter* improbable, the relative frequency of common antigens, common physiological characteristics, and the phentotype of β-glucoside and raffinose-fermenting mutants of *Salmonella* led us to consider *Salmonella* closer to *Citrobacter* than to *E. coli* (16). This is consistent with the present classification of *Citrobacter* (4). It appears therefore that, when combined with other physiological and genetic criteria, the study of the phenotype of β-glucoside-fermenting mutants could give indications on possible evolutionary relationships in *Enterobacteriaceae*.

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