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Enzymatic Profiles of *Enterobacter sakazakii* and Related Species with Special Reference to the α-Glucosidase Reaction and Reproducibility of the Test System

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The enzymatic profiles of *Enterobacter sakazakii*, *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Enterobacter agglomerans* were determined with the API ZYM system (API System S.A., La Balme Les Grottes, France). Each assay was performed three times. A simple formula was derived and applied to assess the reproducibility of the API ZYM tests. In addition, a separate α-glucosidase test was performed. All *E. sakazakii* isolates produced α-glucosidase, in contrast to the other *Enterobacter* isolates. No phosphoamidase activity was detected in any of the *E. sakazakii* isolates, whereas it was present in 72% of *E. cloacae*, 89% of *E. agglomerans*, and 100% of *E. aerogenes* isolates. It was concluded that detection of α-glucosidase permits rapid and reliable differentiation between *E. sakazakii* and other *Enterobacter* species. The reproducibilities of α-glucosidase and phosphoamidase reactions were estimated to be 89 and 81%, respectively.

Enterobacter sakazakii (previously known as yellow-pigmented *Enterobacter cloacae*) was described as a new bacterial species in 1980 (3). This microorganism can cause neonatal meningitis (1, 3, 6, 7, 9–11) and has also been associated with bacteremia (5, 8). In addition, it can be found as a colonizer in sputum, feces, and wounds (3). A number of properties of *E. sakazakii* have already been described (2, 3). Only a few of these can be used to distinguish *E. sakazakii* from *E. cloacae*: the production of yellow-pigmented colonies on Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar at 25°C after 48 h and of extracellular DNase on toluidine blue agar at 36°C after 7 days and the failure to ferment either sorbitol or mucate at 36°C after 24 h. An additional distinguishing characteristic is the Tween 80-esterase reaction, which is positive in 97.3% of isolates after 3 to 8 days (2). As none of these tests is very rapid, we decided to evaluate an identification system based on microbial enzyme profiles (API ZYM system; API System S.A., La Balme Les Grottes, France). Only a short incubation time (4 h) is needed, as the enzymes are already present in the bacteria. In addition, a separate α-glucosidase test was performed.

MATERIALS AND METHODS

Bacterial strains. A total of 226 *Enterobacter* strains were used in the study. Of these, 129 were *E. sakazakii* strains, 113 of which were obtained from J. J. Farmer III, Enteric Bacteriology Section, Centers for Disease Control, Atlanta, Ga., and have been described elsewhere (3). The other 16 *E. sakazakii* strains, most of which have also been described before (10), were obtained from H. C. Zanen, Laboratory of Hygiene, University of Amsterdam, Amsterdam, The Netherlands, and Herman J. Sonderkamp, Stichting Pathologisch Laboratorium, Venlo, The Netherlands, or formed part of the collection of the Department of Medical Microbiology, Sint Radboud University Hospital, Nijmegen, The Netherlands. In addition, 60 *E. cloacae*, 19 *E. aerogenes*, and 18 *E. agglomerans* strains were tested. These strains were cultured from patients hospitalized at the Sint Radboud University Hospital and were identified with the API 20E system (Analytab Products, Plainview, N.Y.). In addition, one *E. gergoviae* strain and one *E. amnigenus* biogroup 1 strain were tested, both of which were identified at the Centers for Disease Control. All strains were stored on slants of heart infusion agar at room temperature in the dark. Bacteria were grown on Mueller-Hinton agar (BBL Microbiology Systems) for 24 h at 36°C. Each strain was then suspended in sterile distilled water. All suspensions were first adjusted to a McFarland no. 2 standard and after that concentrated to a turbidity between no. 5 and no. 6 standards.

Enzyme profiles. Enzyme assays were performed with the API ZYM system. Each plastic strip contains 20 cupules; 19 contain substrates and buffer, and 1 contains only buffer and acts as a negative control. The strip assays for alkaline phosphatase, butyrate esterase, caprylate esterase-lipase, myristate lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Each cupule of the strip was inoculated with 65 μl of the standardized bacterial suspension. After aerobic incubation in the dark at 36°C for 4 h, 1 drop each of reagent A (250 g of Tris, 110 ml of 37% HCl, 100 g of laurylsulfate, and distilled water to 1 liter) and reagent B (3.5 g of Fast Blue BB and 2-methoxyethanol to 1 liter) was added to each cupule. After 5 min, the strip was placed in daylight for a few minutes. The presence and degree of enzymatic activity were scored as color intensities 0, 1, 2, 3, 4, and 5, in accordance with the color comparison chart of the manufacturer. A limited number of strains (six strains of *E. sakazakii* [including a mucoid one] and four strains of each of the other *Enterobacter* species) were tested before and after ultrasonic treatment. The enzyme profile after ultrasonification was determined only once.

α-Glucosidase assay. A suspension adjusted to a turbidity between McFarland no. 5 and no. 6 standards was prepared from each strain in saline. A 0.25-ml amount of the suspen-

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sion was added to each of two tubes. One tube contained a 300-μg tablet of *p*-nitrophenyl-α-D-glucoside in 0.05 M phosphate buffer (pH 8.0) (Rosco Diagnostica, Taastrup, Denmark). The other tube served as a control of color. This tube was included because *E. sakazakii* is able to produce a yellow pigment. After the closed tubes were agitated vigorously for a few seconds, they were incubated at 36°C for 4 h. A yellow color in the supernatant, caused by released nitrophenol, indicated the presence of α-glucosidase.

Reproducibility of the enzyme test system. Each assay was performed three times on separate days and read (color intensities 0, 1, 2, 3, 4, and 5) by the same observer, who was unaware of the previous results. To assess the reproducibility of each of the 19 enzyme reactions, we made three assumptions: if the same result was obtained three times, then this result was correct; if the same reaction was observed two times, then either this observation or the other observation was correct; and if three different results were obtained, one was correct. Under these assumptions the probability (P) that the result of one test chosen at random is correct is as follows:

$$P = w_a + P_b w_b + 1/3 w_c \quad (1)$$

where w_a, w_b, and w_c are the relative frequencies of test triples with, respectively, three consistent, two consistent, and three different results and P_b is the part of the results which is correct in test triples with two consistent results.

TABLE 1. Enzymatic profiles of *Enterobacter* spp. as determined by the API ZYM system

Enzyme	Reaction	% of strains producing indicated enzyme			
		E. sakazakii (n = 129)	*E. cloacae* (n = 60)	*E. aerogenes* (n = 19)	*E. agglomerans* (n = 18)
Alkaline phosphatase	+	100	90	11	72
	++	0	7	89	28
Butyrate esterase	+	94	63	89	72
	++	6	0	0	11
Caprylate esterase-lipase	+	9	100	95	100
	++	91	0	0	0
Leucine arylamidase	+	90	73	26	44
	++	10	27	74	56
Acid phosphatase	+	72	7	0	0
	++	28	93	100	100
Phosphoamidase	+	0	70	95	61
	++	0	2	5	28
α-Galactosidase	+	25	20	5	61
	++	5	0	0	0
β-Galactosidase	+	37	7	84	11
	++	63	92	16	83
α-Glucosidase	+	100	0	0	0
	++	0	0	0	0
β-Glucosidase	+	83	15	58	50
	++	1	0	0	0
N-Acetyl-β-glucosaminidase	+	79	40	68	33
	++	0	0	0	0

TABLE 2. Reproducibility of API ZYM reactions for the strains tested[a]

Enzyme	w_a	w_b	w_c	P
Alkaline phosphatase	72	26	2	89
Butyrate esterase	28	60	12	65
Caprylate esterase-lipase	49	50		79
Myristate lipase	99	1		100
Leucine arylamidase	10	60	30	50
Valine arylamidase	79	21		92
Cystine arylamidase	100			100
Trypsin	98	2		99
Chymotrypsin	100			100
Acid phosphatase	28	63	9	66
Phosphoamidase	52	41	7	81
α-Galactosidase	67	29	4	88
β-Galactosidase	42	46	13	72
β-Glucuronidase	100			100
α-Glucosidase	71	27	2	89
β-Glucosidase	47	44	9	75
N-Acetyl-β-glucosaminidase	46	49	5	78
α-Mannosidase	100			100
α-Fucosidase	100			100

[a] w_a, w_b, w_c are the relative frequencies (percentages) in test triples of, respectively, three consistent, two consistent, and three different results; P is the approximate probability that the result of one test chosen at random is correct.

Again, with P being the probability of obtaining a correct result in one test, P_b can be estimated by using the conditional binomial distribution. The unconditional probabilities of exactly one and two correct results are calculated as $P_1 = 3P(1 - P)^2$ and $P_2 = 3P^2(1 - P)$, respectively. Therefore, the conditional probability of two correct results in a 2/1 test triple can be estimated by $P_2/(P_1 + P_2)$, which equals P. It follows immediately that the number of correct results in test triples with two consistent results is calculated as $2Pn_b + (1 - P)n_b$, where n_b is the number of these test triples. Hence, the part (P_b) of the results which is correct in test triples with two consistent results is estimated as follows:

$$P_b = [2Pn_b + (1 - P)n_b]/3n_b = 1/3(1 + P) \quad (2)$$

Substitution of P_b in formula 1 by formula 2 and solving for P results in the following expression for P:

$$P = [w_a + 1/3(w_b + w_c)]/(1 - 1/3w_b) \quad (3)$$

When w_a, w_b, and w_c are determined, P can be calculated. This value can be interpreted as the approximate probability of obtaining a correct test result under the assumptions stated above.

RESULTS

Enzyme profiles. Myristate lipase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, β-glucuronidase, α-mannosidase, and α-fucosidase activities were practically absent in all strains. The results obtained for the remaining 11 tests are shown in Table 1. The presence and degree of enzymatic activity are summarized as a moderate positive reaction (+; color intensity 1 or 2) or a strong positive reaction (++; color intensity 3 to 5), in accordance with the chart. To assess the reproducibility of each of the 19 enzyme reactions with color intensities 0, 1, 2, 3, 4, and 5, we used formula 3. The approximate probability that the result of one test, chosen at random, is correct, is shown in Table 2.

Differences in intensity were observed between the various *Enterobacter* species. Although most strains possessed alkaline phosphatase, only *E. aerogenes* strains showed

strong positive reactions. In contrast was the observation that although a majority of *E. sakazakii*, *E. cloacae*, and *E. agglomerans* strains produced a strong positive β-galactosidase reaction, most of the *E. aerogenes* reactions were only moderately positive. *E. sakazakii* was usually associated with a strong caprylate esterase-lipase reaction (the other species were only moderately positive); in contrast, only a moderate acid phosphatase activity was expressed by a majority of the *E. sakazakii* strains, whereas the other species were strongly positive. The absence or presence of an enzyme is perhaps of more importance than the intensity of the reaction; all *E. sakazakii* strains produced α-glucosidase, but in no other *Enterobacter* strain could this enzyme be found. No phosphoamidase activity was detected in any of the *E. sakazakii* strains, whereas this enzyme was present in 72% of *E. cloacae*, 89% of *E. agglomerans*, and 100% of *E. aerogenes* strains. The reproducibilities of the α-glucosidase and phosphoamidase reactions were estimated to be 89 and 81%, respectively (Table 2).

Ultrasonification. A different enzyme profile was observed after ultrasonic treatment: leucine arylamidase and β-galactosidase reactions, which were positive in nearly all *Enterobacter* strains, became negative after ultrasonification. There was also a total loss of β-glucosidase activity in the β-glucosidase-positive strains (two *E. sakazakii*, two *E. aerogenes*, and two *E. agglomerans*). α-Glucosidase, acid phosphatase, and *N*-acetyl-β-glucosaminidase reactions became negative in some of the *E. sakazakii* strains. Phosphoamidase activity remained detectable in the *E. aerogenes* strains but was lost in most *E. cloacae* and *E. agglomerans* strains.

α-Glucosidase assay. α-Glucosidase activity was demonstrated in all *E. sakazakii* strains but in no other *Enterobacter* strain. The inclusion of a control tube without substrate in this test is not necessary, because no pigment was detected in any of the suspensions. The *E. amnigenus* strain was phosphoamidase positive (+) but α-glucosidase negative, whereas neither enzyme was demonstrated in the *E. gergoviae* strain.

DISCUSSION

It is possible to use a heavy inoculum of microorganisms instead of growing organisms to detect specific enzymes. The activity of enzymes is demonstrated much faster with a heavy inoculum (in 4 h) than with methods based on growing bacteria, which require an incubation period of 24 h or more. Methods based on direct detection of enzymes have a value as separate tests, because primarily only constitutive enzymes are measured and interference by other reactions is minimized. This can be illustrated with the α-glucosidase enzyme, which can hydrolyze a number of α-D-glucopyranosides, including sucrose and maltose. Although both *E. sakazakii* and *E. cloacae* tested in the usual way are able to ferment these disaccharides, the effect of α-glucosidase was only demonstrated in *E. sakazakii*. Mention has been made of a total loss of leucine arylamidase and occasionally diminished activity of other enzymes after ultrasonic treatment of a limited number of gram-negative strains, including one *E. cloacae* strain (4). The same observation was made in this study, but there was a total loss of β-glucosidase activity in the β-glucosidase-positive strains, too.

Relatively little attention has been paid to the reproducibility of the API ZYM tests; figures for the statistical validity of the test results are not available. If it can be assumed that at least one test obtained in a test triple is correct, formula 3 can be used to describe the reproducibility of the system. If the probability of obtaining an incorrect test result in one test (color intensity) is too large, formula 3 needs some modification. This may occur in situations in which P is smaller than, for example, 70% (Table 2). Of course, one could always merge the adjacent color intensities to meet the required condition more satisfactorily. However, merging codes 0 and 1 in the α-glucosidase reaction would mask the discriminative value of this test with respect to the detection of *E. sakazakii*. A more general and sophisticated statistical examination of the reproducibility of the API ZYM tests will be presented elsewhere; the reproducibility study presented here seems adequate for the present purpose. Clearly, the reproducibility of the leucine arylamidase test is very poor (Table 2). This enzyme, however, was not important in differentiating between *E. sakazakii* and other *Enterobacter* species because it was demonstrated in all *Enterobacter* strains.

Two major differences between *E. sakazakii* and the other *Enterobacter* species are the presence of α-glucosidase and the absence of phosphoamidase in *E. sakazakii*. The α-glucosidase reaction is the most important. In fact, it is possible to use the α-glucosidase reaction as a single, simple, and rapid test to distinguish *E. sakazakii* from other *Enterobacter* species.

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LITERATURE CITED

1. **Adamson, D. H., and J. R. Rogers.** 1981. *Enterobacter sakazakii* meningitis with sepsis. Clin. Microbiol. Newsl. **3**:19–20.
2. **Aldová, E., O. Hausner, and R. Postupa.** 1983. Tween-esterase activity in *Enterobacter sakazakii*. Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. A **256**:103–108.
3. **Farmer, J. J., III, M. A. Asbury, F. W. Hickman, D. J. Brenner, and the *Enterobacteriaceae* Study Group.** 1980. *Enterobacter sakazakii*: a new species of "*Enterobacteriaceae*" isolated from clinical specimens. Int. J. Syst. Bacteriol. **30**:569–584.
4. **Humble, M. W., A. King, and I. Phillips.** 1977. API ZYM: a single rapid system for the detection of bacterial enzymes. J. Clin. Pathol. **30**:275–277.
5. **Jiménez, E. B., and C. Giménez.** 1982. Septic shock due to *Enterobacter sakazakii*. Clin. Microbiol. Newsl. **4**:30.
6. **Jøker, R. P., T. Nørholm, and K. E. Siboni.** 1965. A case of neonatal meningitis caused by a yellow *Enterobacter*. Dan. Med. Bull. **12**:128–130.
7. **Kleiman, M. B., S. D. Allen, P. Neal, and J. Reynolds.** 1981. Meningoencephalitis and compartmentalization of the cerebral ventricles caused by *Enterobacter sakazakii*. J. Clin. Microbiol. **14**:352–354.
8. **Monroe, P. W., and W. L. Tift.** 1979. Bacteremia associated with *Enterobacter sakazakii* (yellow-pigmented *Enterobacter cloacae*). J. Clin. Microbiol. **10**:850–851.
9. **Muytjens, H. L., and L. A. A. Kollée.** 1982. Neonatal meningitis due to *Enterobacter sakazakii*. Tijdschr. Kindergeneeskd. **50**:110–112.
10. **Muytjens, H. L., H. C. Zanen, H. J. Sonderkamp, L. A. Kollée, I. K. Wachsmuth, and J. J. Farmer III.** 1983. Analysis of eight cases of neonatal meningitis and sepsis due to *Enterobacter sakazakii*. J. Clin. Microbiol. **18**:115–120.
11. **Urmenyi, A. M. C., and A. W. Franklin.** 1961. Neonatal death from pigmented coliform infection. Lancet **i**:313–315.