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Differentiation of *Corynebacterium* spp., *Listeria* spp., and Related Organisms by Using Fluorogenic Substrates

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A total of 228 strains of *Arcanobacterium haemolyticum*, *Corynebacterium* spp., *Erysipelothix rhusiopathiae*, and *Listeria* spp. were investigated for their abilities to hydrolyze 60 different fluorogenic 4-methylumbelliferyl-linked and β-naphthylamide-linked substrates within 6 and 24 h of incubation. The hydrolysis of a group of 16 fluorogenic substrates, and in particular, the glycosidase tests, in most cases showed high separation values at the genus level. When used in combination with other biochemical tests, these tests improved the differentiation of coryneform bacteria and phenotypically similar organisms.

Among the gram-positive organisms, *Corynebacterium diphtheriae* and related coryneform organisms have been reported to be involved in severe human infections (4, 15, 17, 19, 20), especially in immunosuppressed patients. Although they are not phylogenetically related, bacteria belonging to the genus *Listeria*, which includes the pathogenic species *Listeria monocytogenes* (8, 21) and *Listeria ivanovii* (a sheep pathogen), or *Erysipelothrix* (primarily a pathogen of swine and other animals) are physiologically very similar to coryneform bacteria and are frequently misidentified as *Corynebacterium* spp. Often, the identification of these organisms by conventional phenotypic methods is difficult and requires 24 or even 48 h of incubation.

The application of enzymatic profiling of microorganisms by using fluorogenic substrates takes advantage of the presence of enzymes, which can be detected very rapidly (often ≤4 h of incubation) (6). These qualitative enzyme tests are useful for differentiating between various bacteria (for reviews, see references 2 and 18), and some of these tests have been included in commercially available test kits for coryneforms and related bacteria (9, 10, 14, 23, 24). This study dealt with an evaluation of the separation potential of 60 different fluorogenic substrates for the clinically important *Corynebacterium* spp., *Listeria* spp., and physiologically similar species. All tests were evaluated for their reproducibilities, their separation potentials, and consequently, their usefulness in differentiation.

MATERIALS AND METHODS

Bacteria. A total of 228 strains representing 22 taxa of the genera *Arcanobacterium*, *Corynebacterium*, *Erysipelothix*, and *Listeria* were tested. All strains of the genus *Listeria* were obtained from the Special Listeria Culture Collection, Würzburg, Federal Republic of Germany, and have been listed elsewhere (12). Strains of the remaining genera were obtained from the National Collection of Type Cultures, London, England, and were identified by the methods used at National Collection of Type Cultures. Before testing, all strains were grown on sheep blood agar (Oxoid, Basingstoke, England) for 24 to 48 h at 37°C. To assess purity, cultures were checked throughout the study by colony morphology.

Tests. A total of 15 4-methylumbelliferyl (4-MU)-linked and 45 β-naphthylamide (β-NA)-linked substrates were tested. Tests were performed as described elsewhere (13), with the following exceptions. The fluorometer was blanked prior to measurement by autocalibration, and the fluorescence of a negative control containing no fluorogenic substrate was recorded. Results were read after 6 and 24 h of incubation, and after calculation of the differences ($S = T - C$) between the relative fluorescence intensities of the test (T) and the control (C) wells, a test was coded positive if the difference (S) exceeded the value of 10 for aminopeptidase substrates and 20 for glycosidase substrates (6 h of incubation). The threshold values were 10 (aminopeptidase substrates) and 30 (glycosidase substrates) after a 24-h incubation period. For the following tests, higher threshold levels were chosen, because spontaneous hydrolysis of the substrates was observed: 4-MU–phosphate ($S > 430$); 4-MU–β-D-glucopyranoside ($S > 230$); 4-MU–*N*-acetyl-β-D-galactosaminide, 4-MU–*N*-acetyl-β-D-glucosaminide, 4-MU–β-D-mannopyranoside ($S > 60$).

Selection of differentiating characteristics and reproducibility. Test selection for calculating the usefulness of the tests for separating the taxa was performed with a computer program by calculating the variance of separation potential (VSP) index and the consistency of separation potential (CSP) index of the CHARSEP program (25). The formulae of these indices are given by Sneath (25). The reproducibilities of the reactions were determined by testing 20 strains three times on separate days, and an overall probability of erroneous results was calculated as described by Sneath and Johnson (26).

RESULTS

Experimental test error was calculated from the data for the 20 duplicate strains. The average probability of an erroneous result (P) was 13.6% after 6 h of incubation and 6.9% after 24 h of incubation and was calculated from the pooled variance of all characters (formula 4 of Sneath and Johnson [26]). In general, the 14 glycosidase tests were more reliable and showed P values of 5.6% (6 h) and 4.1% (24 h). The majority of aminopeptidase tests were less reproducible. In addition, a comparison of test results after 24 h of incubation with those obtained after 6 h of incubation showed unstable results after 6 h of incubation, especially within the aminopeptidase reactions. The results of the

TABLE 1. Enzyme profiles of *Arcanobacterium haemolyticum*, *Corynebacterium* spp., *Erysipelothrix rhusiopathiae*, and *Listeria* spp. after 6 and 24 h of incubation

Species (no. of strains)	% Positive for hydrolysis of the following[a]:															
	D-ALA	BEN-L-ARG	L-ASP	GLY	L-PROL	ARA[b]	CEL[b]	βGAL	αGLU[b]	βGLU	βGLUO	N-Ac-βGLU	βFUC[b]	αMAN	βMAN[b]	βXYL
Arcanobacterium haemolyticum (5)	0 (40)[c]	100	0	100	100	100	0	100	100	0	0	100	100	100	0	0
Corynebacterium diphtheriae, untyped (6)	17	100	17	50 (67)	83 (100)	0	0	0	100	0	0	0	0	0	0	0
Corynebacterium diphtheriae gravis (9)	0	100	0	44 (100)	67 (78)	0	0	0	100	0	0	11	0	0	0	0
Corynebacterium diphtheriae intermedius (6)	0	100	0	67 (100)	83	0	0	0	100	0	0	0	0	0	0	0
Corynebacterium diphtheriae mitis (4)	0	100	0	50	100	0	0	0	100	0	0	0	0	0	0	0
Corynebacterium diphtheriae belfanti (7)	0	100	0	86 (100)	71 (86)	0	0	0	86	0	0	0	14	0	0	0
"*Corynebacterium ulcerans*" (5)	0	100	0	100	100	0	0	0	100	0	0	60	0	0	0	0
Corynebacterium jeikeium/*Corynebacterium "genitalium"*[d] (15)	0	67 (80)	0	60 (87)	7 (13)	0	0	0	13	0	0	0	40	0	0	0
Corynebacterium kutscheri (8)	0	100	0 (12)	100	75	0	0	0	100	100	0	0	0	0	0	0
Corynebacterium matruchotii (6)	0	100	0	83	83 (100)	0	0	0	0	0	17	17	0	0	0	0
Corynebacterium minutissimum (4)	0	100	0	75 (100)	75	0	0	0	0	0	0	0	0	0	0	0
Corynebacterium pseudodiphtheriticum (7)	0	100	0	29 (71)	86 (100)	0	0	0	0	0	0	0	0	0	0	0
Corynebacterium pseudotuberculosis (7)	0	100	14	86 (100)	43 (100)	0	0	0	86	0	0	0	0	0	0	0
Corynebacterium renale (7)	0	100	0	0	14 (100)	0	0	0	0	0	100	86	0	0	0	0
Corynebacterium striatum (2)	0	100	0	100	100	0	0	0	0	0	0	100	0	0	0	0
Corynebacterium xerosis (8)	12	100	0	38 (62)	62 (100)	0	0	0	0	0	0	12	0	0	0	0
"*Corynebacterium aquaticum*" (4)	0 (50)	100	0	100	0 (100)	50	100	100	100	100	75	100	100	50	100	100
Erysipelothrix rhusiopathiae (12)	0	0	92	0	0 (75)	0	0	100	0	50	0	100	100	0	8	0
Listeria innocua (32)	9 (97)	0	0	6 (50)	0	0	100	0	100	100	0	100	3	47	100	3
Listeria ivanovii (22)	59 (100)	0	0	5 (68)	0	0	100	0	100	100	0	100	59	0	100	32
Listeria monocytogenes (14)	0 (7)	0	0	0 (7)	0	0	100	0	100	100	0	100	21	14	100	0
Listeria murrayi (4)	0 (100)	0	0	0 (75)	0	0	100	0	50	100	0	100	0	0	100	0
Listeria seeligeri (22)	27 (100)	0	5 (9)	5 (95)	0	9	100	0	100	100	0	100	100	0	100	100
Listeria welshimeri (12)	33 (100)	0	0 (25)	0 (92)	0	0	100	0	100	100	0	100	92	58	100	92

[a] D-ALA, D-alanine–β-NA; BEN-L-ARG, *N*-α-benzoyl-L-arginine–β-NA; L-ASP, L-aspartic acid–β-NA; GLY, glycine–β-NA; L-PROL, L-proline–β-NA; ARA, 4-MU–α-L-arabinopyranoside; CEL, 4-MU–β-D-cellopyranoside, βGAL, 4-MU–β-D-galactopyranoside; αGLU, 4-MU–α-D-glucopyranoside; βGLU, 4-MU–β-D-glucopyranoside; βGLUO, 4-MU–β-D-glucuronide; N-Ac-βGLU, 4-MU–*N*-acetyl-β-D-glucosaminide; βFUC, 4-MU–β-D-fucopyranoside; αMAN, 4-MU–α-D-mannopyranoside; βMAN, 4-MU–β-D-mannopyranoside; βXYL, 4-MU–β-D-xylopyranoside.

[b] Substrates were obtained from (Sigma, Deisenhofen, Federal Republic of Germany); all other substrates were obtained from (Serva, Heidelberg, Federal Republic of Germany).

[c] Numbers in parentheses are results after an incubation period of 24 h; otherwise, results were identical after 6 and 24 h of incubation.

[d] Strains of *Corynebacterium jeikeium* (11) and *Corynebacterium "genitalium"* were shown to be very similar (7, 15).

TABLE 2. Comparison of results of 12 fluorogenic substrate tests for 20 species with growth-dependent chromogenic substrate tests

Substrate[a]	Discrepant species[b]	Agreement[c]
D-Alanine-β-NA	*Corynebacterium minutissimum*, *"Corynebacterium aquaticum"*	18/20 (90)
Glycine-β-NA	*Arcanobacterium haemolyticum*, *Corynebacterium diphtheriae*, *Corynebacterim jeikeium/Corynebacterium "genitalium,"* *Corynebacterium kutscheri*, *"Corynebacterium aquaticum"*	15/20 (75)
L-Proline-β-NA	*Corynebacterium jeikeium/Corynebacterium "genitalium,"* *Corynebacterium kutscheri*, *C. xerosis*	17/20 (85)
4-MU-α-D-glucopyranoside	*Corynebacterium diphtheriae*, *Corynebacterium xerosis*	18/20 (90)
4-MU-*N*-acetyl-β-D-glucosaminide	*Corynebacterium "ulcerans"*, *Corynebacterium renale*, *Corynebacterium striatum*	17/20 (85)
4-MU-α-D-mannopyranoside	*"Corynebacterium aquaticum"*, *Listeria innocua*, *Listeria monocytogenes*	17/20 (85)

[a] The substrates used by Kämpfer et al. (12, 13a) were the corresponding *p*-nitrophenyl- or *p*-nitroanilide-linked substrates (results were read after 7 days of incubation). Results obtained for the hydrolysis of 4-MU-α-L-arabinopyranoside, 4-MU-β-D-cellopyranoside, 4-MU-β-D-galactopyranoside, 4-MU-β-D-glucopyranoside, 4-MU-β-D-glucuronide, and 4-MU-β-D-xylopyranoside were identical for all species that were compared.

[b] Discrepancies were noted if the percentages given in Table 1 showed ±10% deviation to the corresponding results. If only 10 strains were tested per species, ±15% deviation was tolerated.

[c] Values indicate number in agreement/total number compared; values in parentheses indicate percent. Average agreement was 92.5%.

glycosidase reactions remained stable after 24 h of incubation.

The following 13 substrates showed high differences after 6 and 24 h of incubation and had low VSP and CSP indices (VSP < 0.2; CSP < 0.3) for hydrolysis of DL-alanine-β-NA, L-alanine-β-NA, L-alanine-4-methoxy-β-NA, L-aspartyl-L-alanine-β-NA, L-glutamic-acid-α-β-NA, glycyl-L-arginine-β-NA, glycyl-L-prolyl-L-leucine-β-NA, L-glutamine-β-NA, L-histidine-β-NA, L-histidyl-L-leucine-β-NA, D-leucine-β-NA, L-serine-4-methoxy-β-NA, and L-seryl-L-tyrosine-β-NA.

None of the 228 strains hydrolyzed β-alanine-β-NA, benzoyl-L-alanine-β-NA, *S*-benzoyl-L-cysteine-β-NA, L-cysteine-bis-β-NA, L-glutamic acid-α-4-methoxy-β-NA, L-glutamic acid-γ-4-methoxy-β-NA, glutaminyl-glycyl-glycyl-phenylalanine-β-NA, *N*-chloroacetyl-γ-L-glutamic acid-4-methoxy-β-NA, glycyl-L-proline-β-NA, L-threonine-β-NA, L-valine-β-NA, L-valine-4-methoxy-β-NA, 4-MU-α-D-galactopyranoside, or 4-MU-β-L-fucopyranoside.

The following 17 tests revealed a higher stability and a good reproducibility for hydrolysis of L-arginine-β-NA, *N*-α-benzoyl-DL-arginine-4-methoxy-β-NA, *N*-α-benzoyl-L-arginine-4-methoxy-β-NA, *N*-α-benzoyl-L-arginine-β-NA, *N*-α-*Z*-L-arginine-β-NA, L-aspartic acid-L-arginine-β-NA, L-isoleucine-β-NA, L-leucine-4-methoxy-β-NA, L-lysine-β-NA, L-lysyl-L-alanine-4-methoxy-β-NA, DL-methione-β-NA, glycyl-L-phenylalanine-β-NA, L-proline-L-arginine-4-methoxy-β-NA, *trans*-4-hydroxy-L-proline-β-NA, L-hydroxyproline-β-NA, 4-MU-phosphate, and 4-MU-*N*-acetyl-β-D-galactosaminide. However, the separation indices of these tests were low (VSP < 0.3; CSP < 0.4).

Sneath (25) recommended that CSP values of less than 50% and VSP values of less than about 25% are of little value in an identification matrix. Sixteen fluorogenic tests had VSP values greater than 30% and CSP values greater than 50%. These remaining 16 substrates had high reproducibilities and results are given in Table 1 as the percentage of positive tests for each taxon.

Several fluorogenic substrates were able to separate organisms at the genus level. All strains of the genera *Corynebacterium* (with the exception of *"Corynebacterium aquaticum"*, which is not a true member of the genus [15]) and *Arcanobacterium* tested hydrolyzed *N*-α-benzoyl-L-arginine-β-NA, whereas none of the *Listeria* or *Erysipelothrix* strains did so (Table 1). In contrast, *Listeria* spp. were positive for the hydrolysis of 4-MU-β-D-cellopyranoside and 4-MU-β-D-mannopyranoside. These substrates were not hydrolyzed by members of the other genera tested. The hydrolysis of 4-MU-α-L-arabinopyranoside was positive for *Arcanobacterium haemolyticum*, and only two strains of *"C. aquaticum"* showed a positive reaction in this test (Table 1).

Within the genus *Corynebacterium*, there were some tests which showed differentiation potential (hydrolysis of 4-MU-α-D-glucopyranoside, 4-MU-β-D-glucopyranoside, 4-MU-β-D-glucuronide, 4-MU-*N*-acetyl-β-D-glucosaminide); however, all species could not be differentiated by using the 16 different chromogenic substrates listed in Table 1. Strains of

TABLE 3. Comparison of selected glycosidase profiles with those obtained by commercially available test kits reported in literature

Test kit	Reference	Agreement[a]	Discrepancy[b]
API ZYM	16[c]	4/6 (66)	αGLU, αMAN (*Arcanobacterium haemolyticum*)
API ZYM	23[d]	33/36 (92)	αMAN (*Listeria innocua*, *Listeria monocytogenes*, *Listeria welshimeri*)
API Coryne	9[e]	59/64 (92)	βGAL (*Erysipelothrix rhusiopathiae*), αGLU (*Corynebacterium pseudotuberculosis*, *Listeria innocua*), N-Ac-βGLU (*Corynebacterium renale*, *Corynebacterium striatum*)

[a] Values indicate number in agreement/total number compared; values in parentheses indicate percent.

[b] Discrepancies were noted if the percentages given in Table 1 showed ±10% deviation to the corresponding results. If only 10 strains were tested per species, ±15% deviation was tolerated. For abbreviations of tests, see footnote *a* of Table 1.

[c] Results of hydrolysis of βGAL, αGLU, βGLU, βGLUO, N-Ac-βGLU, and αMAN for *Arcanobacterium haemolyticum* were compared.

[d] Results of hydrolysis of βGAL, αGLU, βGLU, βGLUO, N-Ac-βGLU, and αMAN for the six tested *Listeria* spp. (Table 1) were compared.

[e] Results of hydrolysis of βGAL, αGLU, βGLUO, and N-Ac-βGLU for all tested *Corynebacterium* spp. (with the exception of *Corynebacterium matruchotii*) listed in Table 1, *Arcanobacterium haemolyticum*, *Erysipelothrix rhusiopathiae*, *Listeria monocytogenes/Listeria innocua*, and *Listeria murrayi* were compared with the percentages of the identification table provided by the manufacturer (API, Bio Mérieux S. A., Marcy-l'Etoile, France).

Listeria spp. exhibited very similar reaction patterns. The hydrolysis of D-alanine–β-NA was positive for all *Listeria* species, with the exception of one *Listeria innocua* strain and all but one *L. monocytogenes* strains (after 24 h of incubation).

DISCUSSION

The major advantage of fluorogenic tests for diagnostic purposes is the short incubation time in which reaction profiles can be obtained. However, results of the present study indicate that the activities of preformed enzymes can be very low after a short incubation time, such that positive reactions can be observed only after a prolonged incubation period. This was demonstrated for the aminopeptidases (Table 1), which were shown to produce more positive results after a longer incubation period. Especially for the D-alanine-aminopeptidase, which has been shown to differentiate between *L. monocytogenes* and *L. innocua* in growth-dependent tests by using D-alanine-*p*-nitroanilide as the substrate (12), it could be demonstrated that a longer incubation time is necessary for the differentiation of the two species (Table 1).

A comparison of the results of this study with those obtained from growth-dependent enzyme tests for *Listeria* spp. (12) and strains belonging to the genera *Arcanobacterium*, *Corynebacterium*, and *Erysipelothrix* (13a) showed no or minor discrepancies (Table 2). Furthermore, the results obtained with some glycosidase substrates were essentially in line with those of other studies partly obtained with the API ZYM system for *Arcanobacterium* spp. (5, 16) and *Listeria* spp. (23) and with the API Coryne system (9) (Table 3).

The differences obtained for some substrates (Tables 2 and 3) can be explained by various parameters, like incubation temperature, inoculum size, and duration of incubation, which may influence the test results. Moreover, the lower level of agreement found for the substrates 4-MU–*N*-acetyl-β-D-glucosaminide and 4-MU–*N*-acetyl-β-D-galactosaminide was probably caused by the unstable natures of those substrates.

For evaluation of the separation potential, it is essential to consider the probabilities of the positive or negative results for each test, which can be obtained by testing as many strains of each taxon as possible (13). Furthermore, the separation values and the reproducibilities of the different tests should be calculated (6).

The identification of gram-positive bacteria in a routine laboratory is still difficult in some cases. The use of cellular fatty acid profiles for the differentiation of clinically relevant gram-positive rods can group an unknown at the genus level (1, 27); however, as pointed out by Von Graevenitz et al. (27), biochemical testing is still required for accurate identification. Some commercially available test systems that use biochemical tests for the identification of corynebacteria (10, 14) provide sufficient identification results within 1 to 4 h of incubation. However, these systems have been evaluated with a limited number of species. The API Coryne strip (9), which was recently evaluated with a representative set of different species of coryneform bacteria, requires incubation times of 24 to 48 h. That amount of time is probably required for a reliable interpretation of the carbohydrate fermentation tests; however, the enzyme tests could be read after a shorter period of incubation (see Table 3).

In the present analysis, it was shown that few glycosidase substrates can group an unknown coryneform or similar organism at the genus level within 6 h of incubation. The use of fluorogenic substrate tests is not sufficient for the identification of all taxa of gram-positive bacteria. They are, however, useful for supplementing other tests recommended for coryneforms (3, 5, 9, 15) or listeriae (4, 12, 23) to achieve more precise identifications.

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