JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1981, p. 483-490
0095-1137/81/030483-08$02.00/0

Vol. 13, No. 3

Rapid Identification of *Enterobacteriaceae* with Microbial Enzyme Activity Profiles

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A total of 539 clinical isolates belonging to 10 species of the *Enterobacteriaceae* family were identified by enzyme activity profiles within 30 min of test inoculation. Each isolate was grown at 37°C for 18 h on Mueller-Hinton agar and suspended to an optical density of 200 Klett units in 0.85% saline. Enzyme activity profiles were obtained by inoculating 18 fluorogenic substrates with the standardized bacterial suspension and monitoring initial rates of hydrolysis over the first 30 min of analysis. Individual enzyme activity profiles were entered into a coded data bank, and identifications were based on the Bayesian theory of probabilities. At a confidence level of 95%, five species were identified with a >90% efficiency, three species were identified between 83 and 88% efficiency, and two species demonstrated a 72 and 75% efficiency of identification. The enzyme activity profile method of bacterial identification is rapid, easily automated, and reproducible.

Identification of members of the *Enterobacteriaceae* family is commonly based on diagnostic schemes developed by Cowan and Steele (4) and Edwards and Ewing (6), using batteries of conventional tests. These tests require microbial growth and are therefore time consuming. In addition, they are inconvenient, requiring frequent handling, storage, and quality control of test media.

Recent improvements in microbial identification have involved test miniaturization, extended reagent shelf life, optimized test schemes, and computer-assisted data analysis. Microbial identification can now be achieved within 4 to 7 h of test inoculation, using improved kit technology (Micro-ID, General Diagnostics, Morris Plains, N.J.; API 20E Same Day procedure, Analytab Products, Plainview, N.Y.) and recent advances in automation (MS-2, Abbott Laboratories, Dallas, Tex; AMS, Vitek Systems Inc., St. Louis, Mo.).

Further reduction of microbial identification time to within 30 min of test inoculation was investigated by our laboratory. We focused on automation in order to minimize data acquisition and analysis times.

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A rapid, automated microbial identification system should contain at least three elements: (i) identifying parameters which are already present in the microbes to be identified, (ii) a detection system which is sufficiently sensitive that signal amplification through microbial growth is not required, and (iii) ease of automation. Microbial enzyme activity profiling contains these elements and could be the basis for a rapid and automated microbial analysis system.

Considerable literature is available on the use of microbial enzymes for the characterization of bacteria and fungi. Goldstein et al. (7) and Muftic (11) were among the first to synthesize chromogenic aminopeptidase substrates. Muftic stated that the resulting peptidase pattern could be an "important tool in the taxonomy of mycobacteria." Westley et al. (16) used commercially available aminopeptidase substrates to produce enzyme profiles of several *Bacillus* species and other bacteria within 4 h of test inoculation. Mulczyk and Szweczuk (12) identified L-pyrrolidonyl-β-naphthylamide as a new test for the rapid differentiation of enteric bacteria. McIntyre et al. (10) utilized aminopeptidase profiles to distinguish between virulent and avirulent phytopathogenic bacteria. Peterson and Hsu (13) and Godsey (M.S. thesis, University of Missouri, Kansas City, 1977) used aminopeptidase profiles to differentiate among clinically important gram-negative bacteria. Watson (15) recently reviewed this topic, pointing out the potential of aminopeptidase profiles for differ-

entiating among bacteria and fungi.

The use of chromogenic glycosidase substrates for the differentiation of *Enterobacteriaceae* was described by Kilian and Bülow (9). These authors demonstrated the ability of five glycosidases to rapidly distinguish major groups of enteric bacteria.

Waitkins (14) used a kit containing 20 lyophilized chromogenic substrates (ZYM strip, Analytab Products) for the differentiation of nonhemolytic streptococci. D'Amato et al. (5) then used a selected battery of these substrates to differentiate among *Neisseria gonorrhoeae*, *Neisseria meningitis*, and several closely related species within 4 h of test inoculation. None of the work described above, however, was incorporated into an automated system.

We sought test substrates which would provide the basis for a rapid, automated microbial identification system comprised of reagents, a sensitive detection system, and automatic data processing capacity. As limiting conditions, test substrates were required to react detectably with bacterial enzymes within 30 min, with limited inoculum ($\sim 10^7$ cells ml^{-1}), in a reliable, species-diagnostic manner.

This report describes a system for the automated acquisition of microbial enzyme activity profiles and generation of identification schemes. Substrates useful for *Enterobacteriaceae* identification are discussed. Using this system, clinical isolates of the family *Enterobacteriaceae* could be identified within 30 min of test inoculation.

MATERIALS AND METHODS

Chemicals. HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was obtained from Calbiochem (La Jolla, Calif.). Amino acyl derivatives of 7-amino-4-methylcoumarin (AMC; Coumarin 120; Eastman Kodak Co., Rochester, N.Y.) were synthesized as described below. All other chemicals and growth media were obtained commercially.

Synthesis of AMC derivatives. The AMC fluorogenic aminopeptidase substrates of alanine, arginine, glycine, hydroxyproline, and leucine were synthesized by coupling the benzyloxycarbonyl (CBZ)-protected amino acid with AMC by a mixed anhydride method (19, 20). To a solution of the CBZ-protected amino acid (20 mmol) in 20.0 ml of anhydrous tetrahydrofuran (distilled from calcium hydride) and 350 μl (252 mg; 2.5 mmol) of anhydrous triethylamine (distilled from calcium hydride) at 0°C, 300 μl (300 mg; 2.2 mmol) of isobutylchloroformate was added dropwise over 5 min with stirring. A white precipitate formed as stirring was continued for 20 min at 0°C. To this suspension, AMC (175 mg; 1 mmol) was added all at once. The resulting mixture was stirred at 0°C for 2 h and allowed to gradually warm to room temperature as stirring was continued overnight. The reaction mixture was then dissolved in ethylacetate and washed three times with ice-cold 0.1 N HCl (100 ml), followed by one wash with saturated sodium bicarbonate (100 ml). The ethyl acetate layer was dried with saturated sodium chloride (100 ml) and then with anhydrous sodium sulfate. The ethyl acetate was removed in vacuo to yield the desired 7-(*N*-CBZ-aminoacylamido)-4-methylcoumarin. The yields and melting points of these intermediates were as follows: *N*-CBZ-L-alanine, 77% (223–224°C); tri-*N*-CBZ-L-arginine, 69% (162–164°C); *N*-CBZ-glycine, 88% (230–232°C, decomposes); *N*-CBZ-hydroxyproline, 72% (non-crystallizable highly viscous oil); *N*-CBZ-L-leucine, 82% (150–152°C). Proton nuclear magnetic resonance and analytical data (C,H,N) of all compounds were fully compatible with the predicted structures.

The CBZ-protecting groups were removed by hydrogenolysis of 20 mmol of 7-(*N*-CBZ-aminoacylamido)-4-methylcoumarin in 100 ml of solvent containing 250 mg of 10% palladium on carbon. The 10% palladium on carbon catalyst was removed by filtration, and the solvent was removed in vacuo to yield the desired 7-aminoacylamido-4-methylcoumarin. Hydrogenolysis conditions and product yield are summarized in Table 1.

Fluorogenic substrate solutions. The substrates used in this study are listed in Table 2. All substrates except urea were derivatives of 4-methylumbelliferone (4-MeU), β-naphthylamine (β-NA), or AMC. Urea reactivity with microbial urease was assayed using the fluorogenic pH indicator 4-MeU (pK = 7; fluorescent in basic form).

Buffered solutions of fluorogenic substrates were prepared by first dissolving the crystalline solids in 1 ml of methanol or *N,N*-dimethylformamide, if necessary. The appropriate amount of 0.1 M HEPES buffer (pH 8.0) was then added to achieve a final substrate concentration of 0.1 mM, except for the phosphate ester, which was brought to 0.01 mM. The lower concentration of the phosphate ester was necessitated by the high fluorescent background of our preparations.

The urea solution contained 0.33 M urea, 0.15 M saline, and 1 M 4-MeU, preadjusted to pH 6.5.

All substrate solutions were stable at 4°C for from 5 days to 5 weeks, except for the phosphate ester. The latter was diluted daily from a frozen 1 mM stock solution.

Bacterial cultures. Bacterial isolates were obtained from Johns Hopkins Hospital (Baltimore, Md.), Good Samaritan Hospital (Suffern, N.Y.), and the New York City Department of Health. The species tested are listed in Table 2. Stock cultures were maintained on nutrient agar slants at 4°C and transferred as necessary. Reference cultures were stored in nutrient agar stabs, sealed with paraffin, and stored at room temperature. The identities of the *Enterobacteriaceae* isolates provided by the supplying laboratory were obtained by standard reference methods (Johns Hopkins Hospital and The New York City Department of Health) or kit technology (Good Samaritan Hospital, API 20E and Enterotube [Roche Diagnostics, Nutley, N.J.]).

Preparation of inoculum. Growth from a Muel-

TABLE 1. *Substituted AMC substrates*[a]

Substrate[a]	Hydrogenolysis conditions[b]	Yield (%)	MP (°C)
Alanyl-AMC	Methanol; 1 h	93	192 (dec.)
Arginyl-AMC	Methanol-tetrahydrofuran-trifluoroacetic acid, 74:21:1; 2 h	93	—[c]
Glycyl-AMC	Methanol-tetrahydrofuran, 2:1, 1 h	94	222–224 (dec.)
Hydroxyprolyl-AMC	Methanol; 1 h	91	191–193 (dec.)
Leucyl-AMC	Methanol; 1 h	95	155–157 (dec.)

[a] Amino acids coupled via amido linkage to 7-amino group of AMC.

[b] Conditions required to remove the CBZ group from 7-(*N*-CBZ-aminoacylamido)-4-methylcoumarin intermediates.

[c] Trifluoroacetate salt; very hygroscopic.

ler-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.) plate was suspended in 0.85% NaCl to achieve a standard turbidity of 200 units on a Klett-Summersen colorimeter and used immediately or stored at 4°C until used. This procedure yielded suspensions of 5×10^8 to 5×10^9 viable colony-forming units per ml, depending on the species.

Reaction mixture. A 1.9-ml sample of each substrate was dispensed into individual acid-washed borosilicate glass test tubes (10 by 75 mm; Kimble, Toledo, Ohio). Substrate solutions were prewarmed at 37°C before each assay and maintained at this temperature throughout the assay. The reaction was initiated with 100 μl of the inoculum. Hydrolysis of glycosidic, peptide, and ester linkages was detected by monitoring liberation of the aryl group.

Detection of enzyme activity. The data acquisition system included two Aminco Fluorocolorimeters (American Instrument Co., Silver Spring, Md.), each equipped with a 20-sample turret, automatic sample changer, and temperature control and interfaced with a programmable calculator (Hewlett-Packard 9820A, 3050A; Hewlett Packard Calculator Products Div., Loveland, Colo.). This system was later supplanted by a PDP 11/03 computer (Digital Equipment Corp., Maynard, Mass.) which stored data on a floppy disk for eventual transmission to a time-sharing system.

The fluorescence of each tube was monitored once (five readings were taken over a 2-s period and averaged) every 5 min over a 30-min assay period. Reaction rates were then calculated (least squares) from the five points between 6 and 26 min. This method provides "initial rate" data under the assay conditions used.

Units. Substrate reaction rates were expressed in nanomoles of product per milliliter per minute. The conversion of "relative fluorescence intensity" to nanomoles of product per milliliter was accomplished by comparing the data collected with each fluor (millivolts per milliliter) with its appropriate standard curve (millivolts per nanomole). A fluorescence standard (0.2×10^{-7} M quinine sulfate in 0.1 N sulfuric acid) and blank (0.1 N sulfuric acid) were included in each experiment to avoid the necessity of performing a standard curve each day. The ratio of quinine sulfate (QS) fluorescence on the experimental day ($QS_{exp} - blank_{exp}$) to quinine sulfate fluorescence on the reference day (when the fluor standard curves were determined; $QS_{ref} - blank_{ref}$) was then used to correct the fluor curve slope (α_{ref}) to experimental conditions (α_{exp}):

$$\alpha_{exp} = \alpha_{ref}\frac{(QS_{exp} - blank_{exp})}{(QS_{ref} - blank_{ref})}$$

$$\text{Rate (nanomoles per milliliter per minute)} = \frac{\text{rate (millivolts per milliliter per minute)}}{\alpha_{exp}\text{(millivolts per nanomole)}}$$

In practice, $\alpha_{ref}/(QS_{ref} - blank_{ref})$ was stored in the calculator memory for each fluor.

Thus, a standard protocol involving more than one fluorophore with a single optical system could be handled by the data acquisition system. The use of a single optical system for multiple fluorophores greatly simplified data acquisition instrumentation and protocol.

The optical conditions used to obtain these values included a tungsten light source filtered through a Corning 7-60 filter (transmits 300 to 400 nm) for excitation and a Wratten 2A filter (transmits >400 nm) for emission.

Data manipulation. Standardized reaction rates and identifying information were stored in a data bank (MASTERFILE) in the time-share system. Several data analysis packages were developed to generate identification schemes from the data in MASTERFILE. These included algorithms for matrix cluster analysis (8) and Bayesian probability analysis (1, 3) with simultaneous pattern recognition.

The Bayesian algorithm assumes that the likelihood of occurrence of a particular taxon is equal to that of other taxa. The probability values for each species were generated from substrate hydrolysis profiles, R, where the log of the hydrolysis rate for each substrate represents an independent test result (r). An identification score was computed by the procedure of Wilcox and Lapage (17). Specifically, if $P(t_i/R)$ is the probability that an organism giving a substrate hydrolysis profile (R) is a member of taxon (t_i), then $P(t_i/R) = [P(R/t_i)]/[\sum_i^i P(R/t_i)]$, where $P(R/t_i) = P(r_1/t_i)\,P(r_2/t_i) \ldots P(r_n/t_i)$. $P(R/t_i)$ is the probability that a member of taxon (t_i) will give results (R) with n characters and $r_1 \ldots r_n$ individual character results. The taxon with the highest probability of giving the substrate hydrolysis profile (R) is the identification, with the value of the score indicating its reliability.

RESULTS

Initial substrate screen. Thirty-six fluorogenic substrates were tested for their ability to

TABLE 2. *Log mean hydrolysis rates*[a]

Species	No. of isolates	Log mean hydrolysis rate on substrate:[b]																
		Ara	Xyl	α-Gal	Phos	NAGlu	β-Glu	β-Gal	NAGal	Glucu	SO_4	Pyrr	Arg	Pro	Tyr	Ala	Leu	Ser	Urea
E. coli	69	−2.1	−4.8	−2.7	−2.3	−4.6	−4.5	−1.5	−4.1	**−3.2**	−4.4	−3.6	−3.1	−3.1	−2.1	−0.4	−0.7	−2.0	−3.6
S. sonnei	30	−3.3	−4.6	−3.5	−2.0	−4.2	−3.1	−2.4	−4.1	**−3.3**	−5.5	−3.7	−3.1	−3.0	−1.8	−0.3	−0.6	−1.8	−3.6
C. freundii	26	−2.6	−4.8	−2.9	−2.2	−4.2	−3.1	−2.0	−3.9	−4.6	−5.6	−2.1	−2.8	−2.9	−1.9	−0.1	−0.6	−1.7	−3.2
K. pneumoniae	73	−2.9	−4.1	−2.7	−2.2	−3.9	−2.3	−2.1	−4.0	−4.8	−3.9	−2.1	−2.9	−2.9	−2.1	−0.3	−1.0	−1.9	−3.5
E. aerogenes	39	−3.7	−4.4	−3.9	−2.2	−3.6	−2.4	−2.9	−3.9	−4.9	−3.7	−2.0	−2.9	−3.0	−2.1	−0.2	−1.0	−1.9	−3.6
E. cloacae	54	−2.5	**−2.9**	−2.8	−3.1	−3.9	−3.0	−1.9	−3.8	−4.8	−5.4	−3.1	−2.9	−3.2	−2.4	−0.4	−1.0	−2.1	−3.7
S. marcescens	61	−3.8	−4.7	−4.3	−2.9	**−2.4**	−3.3	−2.8	**−3.3**	−4.6	−4.9	−2.2	−2.5	**−1.4**	−2.4	−0.4	−1.2	−2.1	−3.7
S. typhimurium	46	−4.6	−4.7	−3.4	−2.3	−4.8	−4.6	−4.6	−4.0	−4.6	−5.4	−3.7	−2.5	−3.4	−2.6	−0.7	−1.2	−2.4	−3.2
P. mirabilis	49	−4.6	−4.7	−4.2	−2.8	−4.5	−4.7	−4.6	−4.2	−4.8	−4.1	−3.8	−2.4	−3.1	−2.1	−0.4	−0.8	−2.2	−3.2
M. morganii	23	−4.4	−4.3	−4.3	−1.8	−4.0	−4.6	−4.7	−4.0	−4.7	−5.1	−4.0	−3.0	−3.4	−2.4	−0.5	−1.0	−2.3	**−2.1**

[a] Geometric means, calculated: $1/n\,(\sum_{i=1}^{n} \log \text{hydrolysis rate}_i)$ for isolates (i) of a given species. The antilog of each value has the units nanomoles of product per minute per ~5×10^7 cells. Rates indicating preferential activity of given substrates with particular species are shown in boldface.

[b] Ara, 4-MeU-α-L-arabinofuranoside; Xyl, 4-MeU-β-D-xylopyranoside; α-Gal, 4-MeU-α-D-galactoside; Phos, 4-MeU-phosphate; NAGlu, 4-MeU-*N*-acetyl-β-D-glucosaminide; β-Glu, 4-MeU-β-D-glucoside; β-Gal, 4-MeU-β-D-galactoside; NAGal, 4-Meu-*N*-acetyl-β-D-galactosaminide; Glucu, 4-MeU-β-D-glucuronide trihydrate; SO_4 = 4-MeU-sulfate; Pyrr = L-pyrrolidonyl-β-NA; Arg, *N*-benzoyl-DL-arginyl-β-NA; Pro, L-prolyl-β-NA; Tyr, L-tyrosyl-β-NA; Ala, L-alanyl-β-NA; Leu, L-leucyl-β-NA; Ser, L-seryl-β-NA.

react differentially within 30 min with 15 species of gram-negative bacilli. Eighteen of these substrates were selected for their potential use in distinguishing among these species (see Table 2). Further study resulted in the deletion of MeU-sulfate and the addition of hydroxyprolyl-β-NA to the list of "optimized" substrates.

Data base. Initial rate data from 539 isolates representing 10 species of *Enterobacteriaceae* were obtained with the profile substrates. When sufficient isolates were screened (≥25 per species), mean rates and standard deviations were calculated for each species-substrate pair.

Rate data for selected substrate-species combinations were found to follow a log normal distribution. Thus, the logarithms of the hydrolysis rates ("log rate") were used to form the data base for identification. The geometric mean rate (numerical average of log rates) for each substrate-species combination was calculated and stored in MASTERFILE. These values are summarized in Table 2. The unit of the antilog of each value in the table is "nanomoles of product per minute per ~5×10^7 cells."

Each substrate was evaluated as a potential species indicator. The complete set of profile substrates was also evaluated as the basis for species identification.

Four reactivity groups were found among the 18 substrates tested. Two groups were useful for species identification, differing only in their degrees of specificity. The third group exhibited no detectable species specificity and yet was highly reactive. These substrates are potentially interesting for microbial detection/enumeration and will be discussed elsewhere. A fourth group seemed to subdivide isolates within a species based on differential reactivity and may therefore have potential for biotype analysis.

Species-specific substrates. The following substrates reacted rapidly with only one or two species: urea, L-prolyl-β-NA, L-hydroxyprolyl-β-NA, 4-MeU-*N*-acetyl-β-D-galactosaminide, 4-MeU-*N*-acetyl-β-D-glucosaminide, 4-MeU-β-D-xylopyranoside, and 4-MeU-β-D-glucuronide. Data for these substrates are in boldface type in Table 2 (with the exception of hydroxyprolyl β-NA, which behaves like prolyl β-NA).

Table 3 quantitates the reactivity ranges (geometric mean ± 1 standard deviation) for some of these substrates, within and among species. The asymmetry of the standard deviations around the geometric mean reflects the skewed (log normal) rate distributions observed in these populations.

The rapid urease assay was specific for *Proteus* (*Morganella*) *morganii* (hereafter referred to as *M. morganii*). *Proteus mirabilis, Klebsi-*

TABLE 3. *Hydrolysis rates of species-specific substrates*

Species	No. of isolates	Hydrolysis rate[a] on substrate:[b]				
		Urea	L-Pro-βNA	N-Ac-β-D-Glu	β-D-Xyl	β-D-Glucu
C. freundii	26	62 (115-34)	137 (248-76)	—[c]	—	
M. morganii	23	**800[d] (1,470-440)**	44 (101-19)	—	—	—
S. marcescens	61	21 (45-10)	**4,200 (5,900-3,000)**	**330 (640-170)**	—	—
S. typhimurium	46	59 (131-26)	40 (116-14)	—	—	—
E. cloacae	54	20 (45-9)	65 (108-39)	14 (42-5)	**134 (331-54)**	—
P. mirabilis	49	58 (146-23)	88 (315-25)	—	—	—
E. coli	69	27 (82-9)	78 (185-33)	—	—	**66 (156-28)**
K. pneumoniae	73	29 (74-11)	113 (176-73)	13 (31-5)	—	—
E. aerogenes	39	28 (84-9)	100 (210-50)	24 (52-11)	—	—
S. sonnei	30	27 (86-8)	100 (180-50)	—	—	**50 (98-25)**

[a] Rate (10^{-2} pmol ml^{-1} mm^{-1}, ~5×10^7 cells ml^{-1}) is the geometric mean, with the upper and lower boundaries (in parentheses) representing ±1 standard deviation.

[b] L-Pro-β-NA, L-prolyl-β-NA; N-Ac-β-D-Glu, 4-MeU-N-acetyl-β-D-glucosaminide; β-D-Xyl, 4-MeU-β-D-xylopyranoside; β-D-Glucu, 4-MeU-β-D-glucuronide.

[c] Less than 10^{-1} pmol ml^{-1} min^{-1} (for ~5×10^7 cells ml^{-1}).

[d] Rate given is not an initial rate, which would be 20 to 80 times greater. This reaction was no longer linear after ~6 min, but was measured between 5 and 25 min, as were the others, for convenience in data acquisition. The remaining 17 substrates gave initial rates of hydrolysis for the above 10 species which were constant during the 30-min test period.

ella pneumoniae, Proteus vulgaris, and *Proteus rettgeri* (only limited data for the latter two species) showed little reactivity in the rapid, no-growth urease assay.

Only *Serratia marcescens* could rapidly hydrolyze L-prolyl-β-NA and L-hydroxyprolyl-β-NA. Other species were capable of hydrolyzing these substrates at low levels, but the nearest average rate of hydrolysis was less than 30-fold that of *S. marcescens. S. marcescens* was also unique among the 10 data bank species for its reactivity toward 4-MeU-*N*-acetyl-β-D-galactosaminide and 4-MeU-*N*-acetyl-β-D-glucosaminide. However, limited data for *Providencia stuartii* and *P. rettgeri* (not shown) indicated that the enzyme *N*-acetyl-β-D-glucosaminidase may be active in these species as well.

Enterobacter cloacae could be separated from the other species belonging to the *Klebsiella-Enterobacter-Serratia* group by its ability to hydrolyze 4-MeU-β-xylopyranoside. None of the other nine species in the data bank showed any significant enzyme activity toward this substrate under the present assay conditions.

Escherichia coli and *Shigella sonnei* could be distinguished from other species by their ability to hydrolyze 4-MeU-β-D-glucuronide.

Clustering substrates. A second group of substrates clustered species with similar reactivities. 4-MeU-β-D-glucoside was hydrolyzed readily by all species in the data bank except *E. coli, M. morganii, P. mirabilis,* and *Salmonella typhimurium.*

L-Pyrrolidonyl-β-NA was rapidly hydrolyzed by only *Citrobacter freundii, K. pneumoniae, Enterobacter aerogenes,* and *S. marcescens. E. cloacae* demonstrated a reduced ability to cleave this substrate, whereas the remaining five species showed little or no ability to attack L-pyrrolidonyl-β-NA.

4-MeU-α-L-arabinofuranoside was consistently hydrolyzed by *E. coli, C. freundii, K. pneumoniae,* and *E. cloacae.* Other species reacted with this substrate to a lesser extent or not at all.

Highly reactive nonspecific substrates. The third group of substrates were highly reactive toward all species tested. Substrates in this group were (in order of decreasing reactivity) L-alanyl-β-NA, L-leucyl-4-methoxy-β-NA, L-seryl-β-NA, and L-tyrosyl-β-NA. These substrates were useful as a check on the overall reactivity of a given species, especially when variants were found which reacted at the outer limits of an expected range for a particular substrate-species combination.

Biotyping substrates. The fourth group divided certain species into biotypes. The substrates 4-MeU-α-D-galactoside and 4-MeU-β-D-galactoside each divided the isolates of *K. pneumoniae* (83) and *E. aerogenes* (39) into two distinct subpopulations (Fig. 1).

Identification scheme. The probability algorithm was used to test the overall effectiveness of the 18-test substrate battery. It assumed the likelihood of occurrence of a particular species to be equal to that of other species. This method identified an unknown isolate from its enzyme activity profile as that species in the data base most likely to have generated the unknown profile. When an unknown rate profile was entered into the time-share system, the rate for each substrate was converted to a log rate. The probability of each species in the data base generating each test result in the unknown profile was then calculated. The product of these individual

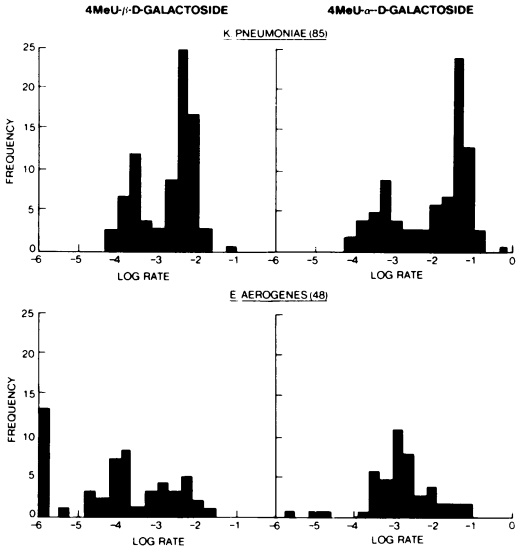

FIG. 1. *Frequency distributions of the reactivity of K. pneumoniae and E. aerogenes isolates toward 4-MeU-α-D-galactoside and 4-MeU-β-D-galactoside. Reaction conditions included 10^{-4} M substrate in 0.1 M HEPES (pH 8), 37°C, ~5 × 10^7 cells ml^{-1}. Rate units are nanomoles of fluorescent product per milliliter per minute.*

test probabilities was determined for each species. The absolute value of the profile probability indicated its reliability. To obtain a positive identification rather than a probability score, a "relative" probability was calculated as follows: (profile probability for species i)/($\sum_i$ profile probability).

Thus, each species in the data base was assigned a number from 0 (no probability) to 1 (only possible identification) according to its likelihood of producing the unknown enzyme activity profile. In this program, positive identification was limited to the species in the data base, with no "rule out" capability. This scheme was useful for demonstrating the effectiveness of the profile method of microbial identification but must be modified further for clinical laboratory application.

Each of the 539 enzyme activity profiles in the data base was treated as an unknown and identified using the above method. A threshold of 0.95 (95%) was used as the working confidence level to establish agreement between isolate identification based on conventional methods and that generated by the probability-based method. Results indicated that >95% of isolates of five species could be correctly identified at this level of confidence and that the majority of the remaining species were also correctly identified (Table 4).

TABLE 4. *Probability-based identification*

Species	No. of isolates profiled[a]	% "Correct" ID[b]
S. marcescens	62	98
M. morganii	24	96
P. mirabilis	55	93
S. typhimurium	51	90
E. coli	77	90
K. pneumoniae	83	88[c]
E. aerogenes	46	85[d]
E. cloacae	65	83
S. sonnei	40	75
C. freundii	36	72
Other species	49	—[e]

[a] Total of 588.

[b] Probability-based identification (ID) agrees with confirmed source identification to 95% confidence.

[c] Identified with >95% confidence as being *K. pneumoniae* or *K. pneumoniae* + *E. aerogenes*.

[d] Identified with >95% confidence as being *E. aerogenes* or *E. aerogenes* + *K. pneumoniae*.

[e] —, Not significant.

Unresolved species. *Citrobacter-Enterobacter* and *Citrobacter-Shigella* could not always be separated with the probability-based scheme. In addition, the present substrate battery could not separate *K. pneumoniae* from *E. aerogenes*, and these were always identified as a pair (Table 4).

The confusion of *K. pneumoniae* and *E. aerogenes* was traced to the bimodal frequency distributions of 4-MeU-α-D-galactoside and 4-MeU-β-D-galactoside reactivity toward both species. The patterns of the two species overlapped for each substrate. There was more similarity between high reactors of the two species than between high- and low-reacting isolates within a species. All correctly identified *K. pneumoniae* isolates belonged to the high-reactivity biotype (≥4 × 10^{-3} nmol min^{-1} of 4-MeU-β-D-galactoside and ≥2 × 10^{-3} nmol min^{-1} of 4-MeU-α-D-galactoside), whereas 96% of all *K. pneumoniae* misidentified as *E. aerogenes* belonged to the low-reactivity biotype (<4 × 10^{-3} nmol min^{-1} of 4-MeU-β-D-galactoside and <2 × 10^{-3} nmol min^{-1} of 4-MeU-α-D-galactoside).

Matrix cluster analysis. Compared to the probability-based identification scheme, matrix cluster analysis had much less resolving power. It identified only those species for which species-specific substrates were available.

AMC derivatives. The known carcinogenic nature of β-NA made it desirable to find an alternate fluor for the aminopeptidase substrates. Preference was given to compounds optically similar to 4-MeU, to simplify the analysis procedure. Hydroxyproline, L-alanine, L-arginine, L-leucine, and glycine derivatives of AMC

and their β-NA analogs were tested with 10 isolates of each of the 10 major data bank species. In most cases, the AMC derivatives reacted similarly to their β-NA counterparts, including the hydroxyproline derivative, which maintained its specificity for *S. marcescens*.

DISCUSSION

The trend in microbial identification is to decrease analysis time while maintaining or improving one's ability to identify the unknown microorganism. Identification based on microbial enzyme activity profiles provides the basis for a truly rapid system. Since enzymes are catalysts, the method has its own internal amplification system. Since the enzymes are already present in the cells at the time of analysis (i.e., do not require induction), the time-consuming dependence on microbial growth is eliminated. The method is limited by enzyme availability to the substrates, substrate turnover rate, and the sensitivity of the detection system, all of which can be addressed experimentally. Substrates can be nonclassical or adapted from classical tests to accommodate the faster analysis time.

We used enzyme activity profiling to identify *Enterobacteriaceae* species within 30 min of test inoculation. Sterile inoculation conditions were not required because of the inoculum density ($\sim 5 \times 10^7$ cells per ml) and short assay incubation time. Using fluorogenic substrates and an automated fluorescence detection system, we routinely measured ≥100 pmol of product over the 25-min analysis period. This contrasts with the 5 to 50 nmol of product required for a manual chromogenic system with a 4-h incubation time (5).

Thirty-five fluorogenic substrates for peptidases, glycosidases, and esterases were screened with 10 species of *Enterobacteriaceae*. Eighteen of these substrates reacted detectably within the 30-min incubation period with one or more of the test species. Detailed analysis of the 18 profile substrates with more than 500 isolates revealed four substrate reactivity groups with the *Enterobacteriaceae* species tested.

One group of substrates was highly reactive with only one or two species, under our test conditions. Urea was specific for *M. morganii*. Other species known to be urease positive in growth-dependent methods (*P. mirabilis*, *P. vulgaris*, *P. rettgeri*) were negative in our system and probably require induction. This conclusion was also reached by Bascomb and Grantham (2), who developed a rapid colorimetric assay for urease and other ammonia-releasing enzymes.

The β-xylosidase substrate, 4-MeU-β-xylopyranoside, was reactive with *E. cloacae*, but not the closely related *E. aerogenes* and *K. pneumoniae*. Using the nitrophenyl analog and a much longer incubation period, Kilian and Bülow (9) detected β-xylosidase activity in all three of these species. The kinetic (as opposed to endpoint) nature of our system may be an important factor in the increased specificity of the β-xyloside in our system compared to that of Kilian and Bülow. In addition, the authors indicated tris(hydroxymethyl)aminomethane-hydrochloride (but not phosphate) buffer inhibition of β-xylosidase. Our use of HEPES buffer may therefore be a factor in the apparent discrepancy from the findings of Kilian and Bülow. Finally, sterochemical differences between the xyloside derivatives used (4-MeU versus *p*-nitrophenol) may influence reactivity.

4-MeU-β-glucuronide reactivity with *E. coli* and *S. sonnei* detected by our system is in agreement with the findings of Kilian and Bülow using colorimetric substrates and the longer incubation period.

L-Prolyl and L-hydroxyprolyl-β-NA were hydrolyzed by *S. marcescens* at rates 30- to 100-fold greater than by the other nine species. The hydroxyproline derivative of AMC was also diagnostic for this species. Prolyl derivatives are also reported to be useful in discriminating among *Neisseria* species (5).

Like the prolyl and hydroxyprolyl derivatives, *N*-acetyl-β-glucosaminide and *N*-acetyl-β-galactosaminide derivatives of 4-MeU were uniquely reactive with *S. marcescens* among the 10 data base species. More limited data, however, suggested reactivity of these sugar derivatives with *P. rettgeri* and *P. stuartii* strains.

The second group of substrates was less selective than the first and separated the data base species into two or more clusters. 4-MeU-β-D-glucoside closely resembled the classical substrate, esculin (7,6-dihydroxycoumarin-β-D-glucoside), in this respect. The non-reactivity of *E. coli* toward 4-MeU-β-D-glucoside was probably an important factor in its distinction from *S. sonnei*.

L-Pyrrolidonyl-β-NA was useful in differentiating among the *Enterobacteriaceae*, as first suggested by Mulczyk and Szewczuk (12). This substrate divided the data base species into three clusters in our system.

4-MeU-α-L-arabinopyranoside divided the data base species into two reactivity clusters, although the boundary between these clusters tended to be less well defined than for the other clustering substrates (Table 2).

The third group of substrates were highly

reactive with *Enterobacteriaceae* tested in an identification scheme. Their inclusion is useful as a check on overall reactivity of the unknown organism. The remarkable invariance of reaction rate of these substrates toward the *Enterobacteriaceae* may be important for detection, enumeration, and antibiotic susceptibility applications.

Substrates in the first three groups exhibited little intra-species variation in reaction rate for the isolates tested. In contrast, the reaction rates of the fourth group of substrates sharply divided *K. pneumoniae* and *E. aerogenes* into subspecies, or biotypes. These may prove useful in the future in tracing nosocomial infections.

Of the three remaining substrates, benzoyl-arginine-β-NA and 4-MeU-phosphate were non-differentiating substrates which reacted at a relatively low level, and 4-MeU-sulfate was generally unreactive despite its promise in the initial screen.

We tested two approaches for generating an identification scheme from the mean rates stored in the data base. Bayesian probability analysis provided much greater resolution than matrix cluster analysis and seemed better able to accommodate the biological variability in microorganisms. Improvements needed in the probability algorithm used here would involve adding an "exclusivity" step to the present program. Thus, profiles of bacteria not found in the data base would be granted "no identification" rather than being forced into a "best fit" identification as is now the case.

Much work remains in the development of an optimized substrate battery for the identification of all clinically important *Enterobacteriaceae*. Additional substrates could include those specific for antibiotic resistance enzymes such as β-lactamases and cephalosporinases, both for identification purposes and for antibiotic susceptibility screening.

The work presented here demonstrates the potential of enzyme activity profiling for rapid microbial analysis. It is simple to use and can be applied to many microorganisms. It represents an important tool in the development of new, rapid, and automated procedures for microbial identification, detection, and antibiotic susceptibility testing.

ACKNOWLEDGMENTS

We acknowledge the excellent technical contributions of K. Dunlevy and L. Otto in the acquisition of profile data, and of A. Shatz in the synthesis of the coumarin 120 analogs.

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