Use of the RapID-ANA System to Screen for Enzyme Activities That Differ Among Species of Bile-Inhibited *Bacteroides*

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The RapID-ANA System (Innovative Diagnostics Systems, Inc., Atlanta, Ga.) was used to test 102 strains of 14 species of phenotypically similar bile-inhibited *Bacteroides* from humans. *Bacteroides oris*, *Bacteroides veroralis*, *Bacteroides buccalis*, *Bacteroides melaninogenicus*, *Bacteroides loescheii*, and *Bacteroides denticola* had very similar enzyme activity profiles. Clear differentiation of these six species by the RapID-ANA System was not possible, but tests for arginine aminopeptidase and β -glucosidase were helpful. *Bacteroides oralis*, *Bacteroides intermedius*, *Bacteroides corporis*, *Bacteroides disiens*, *Bacteroides bivius*, *Bacteroides gingivalis*, *Bacteroides asaccharolyticus*, and *Bacteroides buccae* each had unique enzyme activity profiles. No consistent differences in enzyme activities were found between the two DNA homology groups within *Bacteroides melaninogenicus*, *Bacteroides loescheii*, or *Bacteroides intermedius*. Tests for glycine aminopeptidase, α -galactosidase, arginine aminopeptidase, α -fucosidase, *N*-acetylglucosaminidase, reduction of triphenyltetrazolium, and production of indole were helpful in the differentiation of the species studied.

In the last 10 years, DNA homology studies have guided descriptions of new species of *Bacteroides* and the emendation of the descriptions of existing species (1, 4-7, 9, 10, 13, 16). Some of the newly described species are difficult or impossible to differentiate by the usual phenotypic tests. There are particular problems in differentiating among some of the species of *Bacteroides* that do not grow well in bile. Many of these species were isolated from clinical samples from humans.

Strains of *Bacteroides oris* and *Bacteroides buccae* were identified as *Bacteroides ruminicola* subspecies *brevis* biovar 3 until they were shown to be of separate DNA homology groups (7). Although polyacrylamide gel electrophoresis patterns of soluble cellular proteins (12) of *B. oris* and *B. buccae* are different, there is no common morphological or biochemical characteristic that reliably differentiates these two species.

Bacteroides bivius and *Bacteroides disiens* are much like *Bacteroides intermedius* and *Bacteroides corporis* phenotypically (4, 6, 10). All are strongly proteolytic as well as saccharolytic. *B. bivius* and *B. disiens* do not produce pigmented colonies on blood agar. Strains of *B. corporis* and *B. intermedius* produce pigmented colonies, and both previously were classified as *Bacteroides melaninogenicus* subspecies *intermedius* (10).

As a result of DNA homology studies, strains previously identified as *B. melaninogenicus* subspecies *melaninogenicus* have been classified as *B. melaninogenicus*, *Bacteroides denticola*, and *Bacteroides loescheii* (5, 13). All three species usually produce pigmented colonies and ferment lactose.

B. melaninogenicus, *B. loescheii*, and *B. intermedius* each contain two DNA homology groups with only 26 to 50% intragroup homology (3, 5, 10). The second homology group in each species has not been named and usually is not recognized because no easily tested differential phenotypic characteristics have been reported.

The strains now identified as *Bacteroides buccalis* and

Bacteroides veroralis were considered to be strains of *Bacteroides oralis* until DNA homology studies indicated the need for separate species (13, 16). These three species usually do not produce pigmented colonies. Although there are helpful phenotypic differences between the species, *B. oralis*, *B. buccalis*, and *B. veroralis* are quite similar to *B. melaninogenicus*, *B. loescheii*, and *B. denticola* (6, 16).

The nonsaccharolytic pigmented strains of *Bacteroides* previously were designated *B. melaninogenicus* subspecies *asaccharolyticus*. The strains from human sources are now assigned to *Bacteroides asaccharolyticus* or *Bacteroides gingivalis* (1). The isolation site and production of phenylactic acid by *B. gingivalis* aid in their identification (1, 6).

At the research level, these phenotypically similar species usually can be differentiated by their polyacrylamide gel electrophoresis patterns. Because this technique is not available in many laboratories, additional differential tests would be helpful. The Innovative Diagnostics Systems, Inc., Atlanta, Ga. (IDS) RapID-ANA System tests for enzyme activities that commonly are not included in species characterization. The panel includes tests for aminopeptidases (17), glucosidases (11), and tetrazolium salt reduction (15) that previously have been recognized as potentially useful differential tests. The API ZYM System (Analytab Products, Plainview, N.Y.) includes tests for some of the same glucosidases as the IDS RapID-ANA System but does not include tests for the aminopeptidases and tetrazolium salt reduction (14). The IDS system is a 4-h micromethod that uses a suspension of bacteria to inoculate 10 wells that contain dehydrated substrates to test for 18 activities. In this study, we used the RapID-ANA System to screen for enzyme activities that might be helpful in the identification of some phenotypically similar *Bacteroides* species.

MATERIALS AND METHODS

Strains. A total of 102 lyophilized strains of 14 species was used in this study. Sixty-two strains had 61 to 99% DNA homology with their respective type or reference strain as indicated below. Homology results were not available for the

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other strains. Homology values are given in parentheses, and the first strain listed is the DNA homology reference strain. With few exceptions, these strains were isolated from human sources. The *Bacteroides* species and strains tested are listed below. Unless otherwise indicated, strain numbers are those of the Virginia Polytechnic Institute Anaerobe Laboratory. Type strains are indicated with a superscript T.

B. asaccharolyticus 4198^T (ATCC 25260^T), 8945 (73%), 4199 (75%), and three other strains;

B. bivius 6822^T (ATCC 29303^T), 8587 (93%), 5966 (95%), 9498 (91%), 7962 (89%), and 6318 (83%) (4);

B. buccae D3A-6^T (ATCC 33574^T), 11107 (82%), 6404 (85%), D54C-6 (86%), D2B-29B (99%), and E1H-22 (88%) (7);

B. buccalis NCDO (National Collection of Dairy Organisms, National Institute for Research in Dairying, Reading, Berkshire, United Kingdom) 2354^T (13640^T), 8906D (96%) (16), and four other strains;

B. corporis 9342^T (ATCC 33547^T), 8667 (88%), 8558 (81%), 10443 (85%), 11329 (76%), and 6018 (94%) (10);

B. denticola 10043 (ATCC 33185), D19B-6B (85%), D2B-18 (79%), 11963 (72%), 9955 (86%), and 10099 (91%) (5);

B. disiens 8057^T (ATCC 29426^T), 7874 (89%), 10723 (98%), 9495 (82%), 8573 (80%), and 9061 (84%) (4);

B. gingivalis 12520, 12508 (78%), ATCC 33277^T (13207^T), and three other strains;

B. intermedius 4197^T (ATCC 25611^T), 13048 (80%), 12507 (68%), 6092 (72%), D12B-20 (66%), and D10A-24 (78%) (10);

B. intermedius 8944 homology group strains NCTC 9336 (8944), D25B-1 (87%), D27C-15 (81%), 12516 (90%), 11335 (80%), and 4203 (ATCC 25261) (85%) (10);

B. loescheii ATCC 15930^T (0037^T), D16B-5 (61%), D45C-10A (81%), E1X-22A (80%), 9621 (82%), and 12531 (86%) (5);

B. loescheii D1C-20 homology group strains D1C-20, D39D-9 (71%), D25B-5 (70%), D18A-29A (73%), D41A-24B (67%), and D26C-18 (73%) (5);

B. melaninogenicus 2381^T (ATCC 25845^T), 9847 (76%), 8227 (69%), 7465 (78%), 5693 (76%), and 7169A (71%) (5);

B. melaninogenicus 9343 homology group strains 9343, D13A-25 (82%), D45A-30 (78%), 7673A (74%), 13041 (66%), and 13040 (80%) (5);

B. oralis 5832, 9958 (107%), D27B-24^T (ATCC 33269^T) (90%) (16), and three other strains;

B. oris D1A-1A^T (ATCC 33573^T), D23B-27 (98%), E1H-3 (87%), D21B-16 (89%), D33A-30 (88%) (7), and one other strain;

B. veroralis D22A-7^T (ATCC 33779^T) and five other strains.

Identification of strains. Strains were characterized and identified by using prerduced media, gas chromatography, and polyacrylamide gel electrophoresis as previously described (2, 12). Polyacrylamide gel electrophoresis also was used for confirmation of identity and purity when cultures were brought out of lyophilized storage.

Use of RapID panels. RapID-ANA panels, inoculation fluid, and reagents were a gift from IDS and were used in the manner recommended by the manufacturer. Cultures were grown for 48 h on supplemented brain heart infusion blood agar plates (2) in anaerobic jars. Plating medium contained 5% (vol/vol) sterile defibrinated rabbit blood. Although pigmented strains produced a tan-to-brown inoculation suspension, the bacteria settled during the incubation period, and interpretation of test results was not affected. Interpretation was as described by the manufacturer. In the *p*-nitrophenylglycosidase tests (wells 1 through 7; Table 1), a

TABLE 1. Enzyme activities detected by RapID-ANA panels

Well no. ^a	Test code	Enzyme activity
1	PO ₄	Alkaline phosphatase
1R	LGY	Leucylglycine aminopeptidase
2	ONPG	β-Galactosidase
2R	GLY	Glycine aminopeptidase
3	α-GLU	α-Glucosidase
3R	PRO	Proline aminopeptidase
4	β-GLU	β-Glucosidase
4R	PAL	Phenylalanine aminopeptidase
5	α-GAL	α-Galactosidase
5R	ARG	Arginine aminopeptidase
6	α-FUC	α-Fucosidase
6R	SER	Serine aminopeptidase
7	NAG	N-acetylglucosaminidase
7R	PYR	Pyrrolidone aminopeptidase
8	TTZ	Triphenyltetrazolium reduction
9	ADH	Arginine dihydrolase system
10	TRE	Trehalose fermentation
10R	IND	Tryptophanase (indole production)

^a R, Reagent was added to the well to read the reaction.

discernible yellow was reliably interpreted as positive. A strong yellow was not required to indicate a positive reaction. Visual horizontal examination through the panel walls toward a light source helped in the interpretation. The color reaction chart supplied by the manufacturer was used to interpret the β-naphthylamide hydrolysis tests (LGY, GLY, PRO, ARG, SER, and PYR; Table 1). Assays in the panel are listed in Table 1. In groups in which the identification by the RapID-ANA System did not agree with the identification by conventional tests or by DNA homology, each strain was tested at least two times in the RapID-ANA panels to verify the results.

RESULTS AND DISCUSSION

The enzyme activities detected for each species are listed in Table 2.

The results for *B. gingivalis*, *B. asaccharolyticus*, *B. disiens*, *B. bivius*, *B. intermedius*, *B. corporis*, and *B. oralis* differed sufficiently to allow these organisms to be separated from each other and from the other species tested.

N-acetylglucosaminidase (NAG) activity was consistently different between *B. gingivalis* and *B. asaccharolyticus*, but arginine aminopeptidase (ARG) and α-fucosidase (α-FUC) activities also differed sufficiently to be helpful. Because *B. disiens* and *B. bivius* differ in lactose fermentation, the difference in ONPG (β-galactosidase) results in the RapID System was expected. Reactions in α-FUC and NAG also were distinctive. In addition to the expected difference in indole production, *B. intermedius* and *B. corporis* differed in their ability to reduce triphenyltetrazolium and usually in their α-FUC reaction.

B. oralis and *B. buccalis*, which are difficult to differentiate by the usual phenotypic tests, were clearly separated in the RapID System by the production of glycine aminopeptidase by *B. oralis* strains. Shaw and Collins reported that α-FUC results differ between type strains of *B. oralis* and *B. buccalis* when tested by the API ZYM system (13). However, both species showed α-FUC activity when tested with the RapID-ANA System.

B. buccalis, *B. veroralis*, *B. denticola*, *B. melaninogenicus*, and *B. loescheii* had enzyme profiles with few differences. By the tests reported here, *B. veroralis* and *B. buccalis* could not be differentiated reliably from each other or from *B. melaninogenicus*, *B. loescheii*, or *B. denticola*.

TABLE 2. Enzyme activities of *Bacteroides* species observed in RapID-ANA System panels^a

Species ^b	Enzyme activity ^c									
	ONPG	GLY	α -GLU	β -GLU	α -GAL	ARG	α -FUC	NAG	TTZ	IND
<i>B. gingivalis</i>	—	—	—	—	—	83	—	+	33	+
<i>B. asaccharolyticus</i>	—	—	—	—	—	—	83	—	50	+
<i>B. disiens</i>	—	+	+	—	83	+	—	—	13	—
<i>B. bivius</i>	+	+	+	—	—	66	+	+	—	—
<i>B. intermedius</i>	—	—	+	—	—	+	66	—	—	+
<i>B. intermedius</i> VPI 8944 group	—	—	+	—	—	+	+	—	—	+
<i>B. corporis</i>	—	—	+	—	—	+	—	—	+	—
<i>B. oralis</i>	+	+	+	+	+	—	+	+	—	—
<i>B. buccalis</i>	+	—	+	83	83	50	+	+	13	—
<i>B. veroralis</i>	+	—	+	50	+	13	+	+	—	—
<i>B. denticola</i>	+	—	+	—	83	—	+	+	—	—
<i>B. melaninogenicus</i>	+	—	+	—	+	+	+	+	—	—
<i>B. melaninogenicus</i> VPI 9343 group	+	—	+	—	+	+	66	+	—	—
<i>B. loescheii</i>	+	—	+	66	+	—	83	+	—	—
<i>B. loescheii</i> VPI D1C-20 group	83	—	+	66	66	—	66	+	—	—
<i>B. oris</i>	+	—	+	+	83	—	+	+	—	—
<i>B. buccae</i>	+	—	+	+	+	—	—	—	13	—

^a All species tested showed phosphatase and leucylglycine aminopeptidase activity but lacked proline, phenylalanine, serine, and pyrrolidone aminopeptidases and arginine dihydrolase activity. None produced acid from trehalose.

^b Six strains of each species or DNA homology group were tested.

^c +, All strains were positive; —, all strains were negative. The numbers are percentages of strains positive for this activity. ONPG, β -Galactosidase; GLY, glycine aminopeptidase; α -GLU, α -glucosidase; β -GLU, β -glucosidase; α -GAL, α -galactosidase; ARG, arginine aminopeptidase; α -FUC, α -fucosidase; NAG, *N*-acetylglucosaminidase; TTZ, triphenyltetrazolium reduction; IND, tryptophanase (indole production).

ARG activity was a consistent difference between the *B. melaninogenicus* and the *B. loescheii*-*B. denticola* profiles but did not help in separating *B. melaninogenicus* from *B. buccalis* and *B. veroralis*.

B. oris differed from *B. buccae* in the α -FUC and NAG reactions. These two species cannot be distinguished by common phenotypic tests, so this difference is an aid to identification. However, *B. oris* could not be differentiated from *B. loescheii*, *B. buccalis* or *B. veroralis* by RapID-ANA System results. Cellobiose fermentation (by *B. loescheii* and *B. oris* strains) and xylose fermentation (by *B. oris* strains) would be useful supplementary tests to differentiate among these species. *B. denticola* does not ferment either of these two sugars.

The *B. oris* strains were identified as *B. loescheii* or *B. denticola* by the RapID-ANA System (Table 3) because leucylglycine aminopeptidase (LGY) activity was observed in all of our strains. The discrepancy in results for the LGY test for *B. oris* may relate to a difference we observed between the prototype and marketed panels. When *B. oris* was tested with prototype panels, the LGY test was negative. However, with the later panels this test consistently was positive. It is possible that the manufacturer's data base includes results obtained with the prototype panels. Recently, IDS also has observed LGY activity in *B. oris* strains (N. Hodinka, personal communication).

B. melaninogenicus, *B. loescheii*, and *B. intermedius* each include two DNA homology groups. The RapID-ANA System results provided no clear means to distinguish between homology groups within these species.

The results listed in the manufacturer's differential charts

(8) differ from those reported here primarily in the β -naphthylamide hydrolysis tests (LGY, GLY, PRO, ARG, SER, and PYR; Table 1). They report positive results as possible for species for which we observed no positive results (GLY, PRO, ARG, SER, and PYR; Table 1) and report *B. oris* as negative for LGY. Overall, the β -naphthylamide tests were difficult to interpret. The color developed over a period of 2 min and had to be fairly intense to be positive. Because negative tests often produced some color change, discernment between positive and negative tests was a matter of judgment developed by experience. This is a probable source for some of the difference between our results and those reported by the manufacturer. Another difference was that we observed no triphenyltetrazolium reduction by *B. bivius*, *B. intermedius*, *B. corporis*, *B. loescheii*, *B. denticola*, *B. melaninogenicus*, *B. oralis*, and *B. oris*. Overall, our results showed clearer differences between species than those reported by the manufacturer, possibly because many of the strains we used were known to belong to their respective species as determined by DNA homology studies.

Because all of our *B. denticola* strains were β -glucosidase (β -GLU) negative and the manufacturer's differential charts indicate that the *B. denticola*-*B. loescheii* group is 99% positive for this activity, our *B. denticola* strains were identified as most probably being *B. melaninogenicus* or *B. bivius*. *B. denticola* also fails to demonstrate β -GLU activity in the API ZYM system (13).

Despite the differences noted, the manufacturer's compendium (8) of coded reaction patterns lists the correct species as the most probable identification for most of the

TABLE 3. Identities assigned to strains of bile-inhibited *Bacteroides* by the RapID-ANA Code Compendium by using results given in Table 2

Species	Encoded results	Most probable identification	No. of strains	Confidence level	Correct identification by IDS ^a
<i>B. asaccharolyticus</i>	300004	<i>B. asaccharolyticus</i>	1	Implicit	Yes
	300204	<i>B. asaccharolyticus</i>	2	Implicit	Yes
	300244	<i>B. asaccharolyticus</i>	3	Implicit	Yes
<i>B. bivius</i>	730210	<i>B. bivius</i>	2	Satisfactory	Yes
	730310	<i>B. bivius</i>	4	Inadequate	(Yes)
<i>B. buccae</i>	725000	<i>B. buccae</i>	5	Implicit	Yes
	725040	<i>B. buccae</i>	1	Implicit	Yes
<i>B. buccalis</i> ^b	725250	<i>B. loescheii/B. denticola</i>	1	Satisfactory	No
	725310	<i>B. loescheii/B. denticola</i>	2	Inadequate	(No)
	724310	<i>B. melaninogenicus</i>	1	Satisfactory	No
	725210	<i>B. loescheii/B. denticola</i>	2	Inadequate	(No)
<i>B. corporis</i>	320140	<i>B. corporis</i>	6	Satisfactory	Yes
<i>B. denticola</i>	724210	<i>B. melaninogenicus</i>	5	Inadequate	(No)
	720210	<i>B. bivius</i>	1	Inadequate	(No)
<i>B. disiens</i>	330100	<i>B. disiens</i>	3	Implicit	Yes
	334140 ^c	<i>B. disiens</i>	1	Implicit	Yes
	334100	<i>B. disiens</i>	2	Implicit	Yes
<i>B. gingivalis</i>	300014 ^c	<i>B. gingivalis</i>	5	Inadequate	(Yes)
	300114	<i>B. gingivalis</i>	4	Implicit	Yes
	300154	<i>B. gingivalis</i>	1	Implicit	Yes
<i>B. intermedius</i>	320304	<i>B. intermedius</i>	11	Implicit	Yes
	320104	<i>B. intermedius</i>	1	Implicit	Yes
<i>B. loescheii</i>	725210	<i>B. loescheii/B. denticola</i>	4	Inadequate	(Yes)
	725010	<i>B. oralis</i>	3	Implicit	No
	724210	<i>B. melaninogenicus</i>	3	Inadequate	(No)
	721210	<i>B. loescheii/B. denticola</i>	1	Inadequate	(Yes)
	320010	<i>B. oris</i>	1	Implicit	No
<i>B. melaninogenicus</i>	724310	<i>B. melaninogenicus</i>	10	Satisfactory	Yes
	724110	<i>B. melaninogenicus</i>	2	Inadequate	(Yes)
<i>B. oralis</i>	735210	<i>B. oralis</i>	6	Satisfactory	Yes
<i>B. oris</i>	725210	<i>B. loescheii/B. denticola</i>	5	Inadequate	(No)
	721210	<i>B. loescheii/B. denticola</i>	1	Inadequate	(No)
<i>B. veroralis</i> ^b	724210	<i>B. melaninogenicus</i>	2	Inadequate	(Yes)
	725210	<i>B. loescheii/B. denticola</i>	3	Inadequate	(No)
	724310	<i>B. melaninogenicus</i>	1	Satisfactory	No

^a (Yes), (No), Accuracy of the most probable interpretation if pigment production, when recommended by the manufacturer, is considered.

^b Codes for *B. buccalis* and *B. veroralis* are not in the manufacturer's data base.

^c Code sequence not reported in compendium. Results derived from computer data base by N. Hodinka, IDS.

strains we tested (Table 3). When the confidence level of the most probable species identification is termed "inadequate," the manufacturer usually recommends using pigment production as a key test to choose between two possible species. If pigment production was considered when indicated, 72% of these strains would have been correctly identified, either as the correct species or as a species not listed in the data base (*Bacteroides* species). However, we hold cultures on hemolyzed rabbit blood agar for up to 3 weeks to determine whether pigment is produced. This is not possible in clinical situations and may affect the accuracy of identification. The incorrect identification of 28% of the strains included six strains each of *B. buccalis* and *B. veroralis* that should have been identified as "*Bacteroides* species"; these species are not included in the manufacturer's data base.

ACKNOWLEDGMENTS

Included above in the list of strains are DNA homology results for which no literature citations are given. These are unpublished data from J. L. Johnson, whom we thank for their use. We also thank Innovative Diagnostic Systems, Inc., for supplying panels, reagents,

and the compendium. We thank N. Hodinka of IDS for advice about their product and comments on this manuscript.

This research was supported by Public Health Service grants DE 05054 and DE 05139 from the National Institute of Dental Research and by project 2025790 from the Commonwealth of Virginia.

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