PRESENCE OF DIAMINOPIMELIC ACID IN PROPIONATE-NEGATIVE BACTEROIDES SPECIES AND IN SOME BUTYRIC ACID-PRODUCING STRAINS

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SUMMARY. The presence of diaminopimelic acid (m-DAP) in strains of *Bacteroides melaninogenicus*, *B. bivius* and other species as well as in unidentified strains of *Bacteroides* was investigated by thin-layer chromatography. Strains of *B. bivius* and *B. disiens* all contained m-DAP as did the subspecies *intermedius* and *melaninogenicus* of *B. melaninogenicus*. Strains of *B. asaccharolyticus* and similar black pigment-producing butyrate-positive isolates showed heterogeneity. Asaccharolytic strains were DAP negative, whereas two strains fermenting glucose were positive. Some of the non-pigmented propionate-negative and butyrate-negative unidentified strains also contained DAP. The consistent finding of m-DAP in strains of *B. bivius*, *B. disiens*, and *B. melaninogenicus* indicates that DAP detection might be of value in the identification of these species.

INTRODUCTION

Weakly saccharolytic, pigmented and non-pigmented and asaccharolytic pigmented bacteroides strains have been isolated as normal constituents of the oral, vaginal, and urethral flora (Loesche, Socransky and Gibbons, 1964; Hurley *et al.*, 1974; Werner, 1977; Holbrook, Ogston and Ross, 1978; Marrie, Harding and Ronald, 1978; Russell and Melville, 1978; Duerden, 1980). Involvement of these organisms in periodontitis, vaginitis, urethritis, and several other infections has been discussed by several study groups (Loesche, 1980; Sundqvist, 1976; Hammann and Werner, 1980; Takazoe, Tanaka and Homma, 1971; Werner *et al.*, 1979; Socransky and Gibbons, 1965).

In 1964, Loesche *et al.* described the species *Bacteroides oralis* as propionate-negative, saccharolytic, non-pigmented, non-sporing anaerobic gram-negative rods, but their strains have been lost. The only extant collection strain of *B. oralis*, AICC15930, has been shown to produce black pigment (Holbrook and Duerden, 1974). However, *B. melaninogenicus* and *B. asaccharolyticus* are at present recognised as the taxa comprising black pigmented bacteroides (Finegold and Barnes, 1977; Holdeman, Cato and Moore, 1977). *B. bivius* and *B. disiens* described by Holdeman and Johnson (1977) include non-pigmented strains that are weak acid producers.

Cell-wall constituents such as diaminopimelic acid (DAP) have been used for

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taxonomic purposes in the classification of many gram-positive bacteria (Becker *et al.*, 1964; Schleifer and Kandler, 1972; Kroppenstedt and Kutzner, 1976). Williams and coworkers (1975) reported the presence of m-DAP in the cell wall of *Bacteroides melaninogenicus* ssp. *intermedius*. These authors did not find m-DAP in *B. melaninogenicus* ssp. *melaninogenicus* or *B. melaninogenicus* ssp. *asaccharolyticus* (now *B. asaccharolyticus*), but only one strain of *B. melaninogenicus* ssp. *melaninogenicus* was included in their study. In 1976, Shah *et al.* tested numerous strains of *B. melaninogenicus* ssp. *melaninogenicus* for the presence of DAP and found two groups, one with lysine and one with DAP, whereas strains of the subspecies *intermedius* were all DAP positive and strains of *B. asaccharolyticus* were always negative.

The present paper is concerned with a study of DAP detection in bacteroides strains of several species not belonging to the *B. fragilis* group, with special reference to the taxonomy of the genus *Bacteroides*.

MATERIALS AND METHODS

Test strains. The origin of the strains, most of which were clinical isolates, is shown in table I. Strain M95 isolated from the protozoan *Paramecium caudatum* was supplied by Dr Carmen Martínez Medina, Servicio de Análisis Clínicos, Hospital Provincial, Valencia, Spain. Strain M88 is an isolate from the sewage plant of a village near Bonn; it was isolated from sewage sludge by the same methods as described below for the clinical isolates. The type strains of *B. bivia* (ATCC29303), *B. disiens* (ATCC29426), *B. melaninogenicus* ssp. *levii* (VPI3300), as well as the neotype strains (Cato, Holdeman and Moore, 1979) of *B. capillosus* (ATCC29799), *B. praeacutus* (ATCC25539), and *B. furcosus* (ATCC25662) were kindly provided by Dr L. V. Holdeman, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA, and were included for reference purposes.

Culture. The strains were grown on peptone yeast-extract glucose sheep-blood agar or on Schaedler agar containing kanamycin 75 µg/ml and vancomycin 7.5 µg/ml and 8% of sheep blood. The plates were incubated in GasPak jars (BBL) for at least 3 days at 37°C. The strains were identified by physiological tests and gas chromatography of the acid end-products. The methods have been described in detail by Werner and Rintelen (1968), Werner (1972) and Hammann and Werner (1980). The strains were kept in Rosenow broth tubes under oxygen-free CO₂ and were capped with black rubber stoppers.

Cell-wall analysis for DAP. Strains were grown in 100-ml flasks containing 90 ml of peptone yeast-extract glucose medium, heavily inoculated with Rosenow culture. As soon as sufficient growth was apparent, usually after 2 days, cells were harvested by centrifugation and washed twice with distilled water. Appropriate controls for purity of cultures were done in the usual manner. One 100-ml flask yielded 1.5–2.0 g wet weight of cell deposit which was directly used for the following analysis. Cells were disrupted by ultrasonic treatment (MSE sonicator) for 5–10 min at maximum amplitude. After this time, 80–100% of the cells were disrupted as shown by gram-stained smears of some of the cultures. The sonicated cell walls were centrifuged at about 5000 *g* for 30 min; the supernate was discarded and cell fragments were incubated overnight at 37 °C with pepsin (1 mg/ml in 0.02N HCl, containing streptomycin 0.1 mg/ml to prevent bacterial growth). The incubated cell-wall fragments were centrifuged, washed with distilled water and centrifuged again. They were hydrolysed with 6N HCl at 100 °C for 16 h in sealed screw-capped tubes. The hydrolysate was filtered through Schleicher and Schüll paper (No. 595) and dried by evaporation. The dried brownish residues were resuspended in 0.2 ml of distilled water and 5–8 µL amounts were applied to cellulose thin-layer plates (Merck No. 5716). The plates were developed in methanol:distilled H₂O:10N HCl:pyridine (80:17.5:2.5:10) and sprayed with ninhydrin. DL-DAP (Serva, Heidelberg), L-lysine (Merck), and LL-DAP from whole-cell hydrolysates of *Streptomyces griseus* or *Propionibacterium acnes* were included as standards. DAP was detected by its greenish coloration, turning yellow after 1 day, and by comparison of the R_F-values. All strains were analysed twice at different times.

TABLE I
Origin of strains of bacteroides

Species and strain no.	Source	Species and strain no.	Source
<i>B. bivius</i>	M21 Vaginal swab	<i>B. melaninogenicus</i>	
	M22 Vaginal swab	ssp. <i>melaninogenicus</i>	
	M23 Ear of newborn infant	M6 Human (unknown)	
	M24 Skin of newborn infant	M47 Oral mucosa, normal flora	
	M25 Vaginal swab	M48 Oral mucosa, normal flora	
	M26 Skin of newborn infant	M51 Fistula, gunshot wound	
	M28 Vaginal swab	M54 Oral mucosa, cancer patient	
	M35 Vaginal swab	<i>B. melaninogenicus</i>	
	M36 Vaginal swab	ssp. <i>melaninogenicus</i>	
	M41 Vaginal swab	M55 Oral mucosa, cancer patient	
	M43 Vaginal swab	M59 Oral mucosa, normal flora	
	M44 Cervical swab	M81 Oral mucosa, normal flora	
	M45 Vaginal swab	M83 Oral mucosa, normal flora	
	M56 Throat, cancer patient	M95 Isolate from <i>Paramecium</i>	
	M57 Throat, cancer patient	<i>B. melaninogenicus</i>	
	M58 Throat, cancer patient	ssp. <i>intermedius</i>	
	M74 Vaginal swab	M3 Vaginal swab	
	M79 Vagina, leukorrhoea	M17 Bartholinitis	
	M80 Human (unknown)	M27 Operation wound, gynaecology	
	M86 Vaginal swab	M34 Oral mucosa, normal flora	
	M87 Seminal fluid	M63 Oral mucosa, normal flora	
	M91 Ear of newborn infant	M64 Oral mucosa, normal flora	
	ATCC29303 VPI	M65 Vaginal swab	
<i>B. disiens</i>	M27 Operation wound, gynaecology	M76 Tracheal secretions	
	M38 Vaginal swab	M78 Oral mucosa, normal flora	
	ATCC29426 VPI	<i>B. melaninogenicus</i>	
<i>Bacteroides</i> spp.	M4 Vaginal swab	ssp. <i>levii</i>	
	M42 Oral mucosa, normal flora	VPI3300 VPI	
	M46 Vaginal swab	<i>B. asaccharolyticus</i>	
	M52 Oral mucosa, cancer patient	and related strains	
	M53 Oral mucosa, cancer patient	M2 Vaginal swab	
	M67 Prepuce	M7 3280 (Dr W. E. C. Moore)	
	M68 Abdominal drain	NCTC 9337 NCTC	
	M75 Pus, ear abscess	M14 Vaginal swab	
	M85 Vaginal swab	M16 Human (unknown)	
	M88 Sewage sludge	M18 Human (unknown)	
	M93 Pus	M29 Urethra, male	
	M104 Abdominal drain	M62 Throat, cancer patient	
<i>B. capillosus</i>	ATCC29799 VPI	M72 Urethra, female	
<i>B. praeacutus</i>	ATCC25539 VPI	M73 Operation wound	
<i>B. furcosus</i>	ATCC25662 VPI		

VPI = strain obtained from Dr L. V. Holdeman, Virginia Polytechnic Institute, Blacksburg, Virginia, USA.

RESULTS

The results of the DAP thin-layer chromatography study of the strains are shown in table II. The 22 strains previously identified as *Bacteroides bivius* and the type strain of this species, ATCC29303, as well as two isolates of *Bacteroides disiens* and the type strain of this species, ATCC29426, contained m-DAP in their peptidoglycan. All strains of *B. melaninogenicus* tested, no matter which subspecies they belonged to, were positive for m-DAP. In some strains of *B. melaninogenicus* ssp. *melaninogenicus*, the m-DAP was difficult to detect and the samples had to be concentrated before clearly visible spots could be found.

The black pigmented, butyric and propionic acid-positive strains of *B.*

TABLE II
Presence of diaminopimelic acid in the strains tested

Bacteroides species and strain no.	m-DAP present (+) or absent (-)
<i>B. bivius</i> (23 strains) *	+
<i>B. disiens</i> (3 strains) *	+
<i>Bacteroides</i> spp.	
M4	-
M42	-
M46	-
M52	-
M53	-
M67	+
M68	+
M75	+
M85	-
M88	-
M93	+
M104	+
<i>B. capillosus</i>	ATCC29799
<i>B. praeacutus</i>	ATCC25539
<i>B. furcosus</i>	ATCC25662
<i>B. melaninogenicus</i> ssp. <i>melaninogenicus</i> (10 strains)	
*	+
<i>B. melaninogenicus</i> ssp. <i>intermedius</i> (9 strains)	
*	+
<i>B. melaninogenicus</i> ssp. <i>levii</i>	VPI3300
<i>B. asaccharolyticus</i> and related strains	
M2	-
M7	-
M14	-
M16	+
M18	-
M29	-
M62	+
M72	-
M73	-
NCTC9337	-

* See table I for strain numbers.

asaccharolyticus and the butyric and propionic acid-forming strains fermenting glucose as the only carbohydrate, and the type strain of *B. melaninogenicus* spp. *levii*, showed some heterogeneity. Besides differences in DAP content, the strains differed in their fermentative abilities towards glucose, lactose, and galactose; *B. melaninogenicus* spp. *levii* fermented glucose, lactose, and galactose weakly. Some of the butyrate-positive, black pigmented strains fermented glucose, whereas others did not; 26 other carbohydrates tested were not affected. In table III the fermentation results are compared with the DAP content of the cell walls. As can be seen, several groups were found. The first group (five strains) consisted of DAP-negative, glucose-positive strains; the second (three strains) neither contained DAP nor fermented glucose or any other carbohydrate tested (= *B. asaccharolyticus* sensu strictiore) and the third (two strains) contained DAP and fermented glucose. *B. melaninogenicus* spp. *levii* was found to contain DAP.

Some of the non-pigmented strains that could not be assigned to any of the species *B. bivius*, *B. disiens*, *B. melaninogenicus*, or *B. asaccharolyticus*, showed a positive DAP reaction whilst others did not, according to their different physiological abilities. Some of them (strains M42, M46, M52, M53 and M67) resembled *B. oralis*; others (M4, M68, M75, M85, M88, M93 and M104) showed some physiological similarities with *B. capillosus*. Most of the strains resembling *B. oralis* did not contain DAP, whereas most of the strains conforming to the physiological description of *B. capillosus* were positive. In the neotype strain of *B. capillosus* and *B. furcosus* DAP could not be detected. *B. praeacutus* ATCC25539 had m-DAP in its peptidoglycan, but none of the aforementioned strains showed physiological similarities with *B. praeacutus*.

TABLE III
Some differences between *B. melaninogenicus* spp. *levii*, *B. asaccharolyticus*, and related strains*

Species and strain no.	DAP present (+) or absent (-)	pH value† recorded in tests with		
		glucose	galactose	lactose
<i>B. melaninogenicus</i> spp. <i>levii</i> VPI3300	+	5.9	5.8-6.0	5.9
<i>B. asaccharolyticus</i> M7	-	—	—	—
M72	-	—	—	—
NCTC 9337	-	—	—	—
Related strains M2	-	5.4	—	—
M14	-	5.8-5.9	—	—
M16	+	5.3	—	—
M18	-	5.0	—	—
M29	-	5.9	—	—
M62	+	5.5-5.6	—	—
M73	-	5.9	—	—

* All strains produced acetic, propionic, isobutyric, butyric and isovaleric acids; small amounts of lactic and succinic acids were also detected. The strains were more or less brown or black pigmented.

† pH values ≤ 6.2-7.0 are recorded as —.

DISCUSSION

The results show that DAP as a cross-linking amino acid of the peptidoglycan is not limited to various gram-positive bacteria such as clostridia, *Eubacterium* spp. or aerobic actinomycetes, but may be also a constituent of many anaerobic gram-negative bacilli. This has already been demonstrated by Williams *et al.* (1975) and Shah *et al.* (1976) for some of the black pigmented bacteroides species. The occurrence of m-DAP in the species *B. bivius*, *B. disiens* and other vaginal and oral isolates of the genus *Bacteroides* has, to our knowledge, not previously been recorded. The inclusion of strains of the same species isolated from different ecosystems (e.g., *B. melaninogenicus* from human sources and from *Paramecium*) or from different sites of the human body was meant to test the consistency of certain biochemical markers within a well defined species and, on the other hand, to detect differences within a "bad" species. The presence of m-DAP in all 23 strains of *B. bivius* and three strains of *B. disiens* possibly provides a new reliable basis for identification. Strains of non-pigmented propionate-negative bacteroides isolates not containing m-DAP should probably be assigned to other species. This holds true for our strains M42, M46, M52, and M53 which are very similar to *B. oralis*. They should, however, not be assigned to this species until a valid neotype strain of this species is available (Shah and Hardie, 1979). The proper taxonomic assignment of the remaining unspecified strains must await future studies.

The black pigmented, butyrate-negative strains of *B. melaninogenicus* all contained DAP. DAP-negative strains of *B. melaninogenicus* ssp. *melaninogenicus* were not observed in our studies but, as was reported above, we had some difficulty in detecting the amino acid in some strains of this subspecies, e.g., strain M81. According to Shah *et al.* (1976), within *B. melaninogenicus* spp. *melaninogenicus* there exist strains with lysine in their peptidoglycan. The differences that can be demonstrated among butyrate-positive, black pigmented strains in relation to DAP content and glucose fermentation reflect the non-homogeneity of this group, which was also demonstrated by van Steenberg, de Soet and de Graaf (1979). It is obvious from the species description (Finegold and Barnes, 1977; Holdeman *et al.*, 1977) that only those of our strains that do not ferment glucose should be assigned to *B. asaccharolyticus*. The three non-fermenters of glucose that we encountered did not contain DAP and this is in accordance with the results of Shah *et al.* (1976). The assignment of the glucose-fermenting, black pigmented, butyrate-positive strains to their proper taxon now awaits further studies.

Recently, Mansheim and Coleman (1980) showed that even oral and non-oral strains of *B. asaccharolyticus* are serologically different, have different membrane structures, and have different sugars in their surface layers. According to Slots and Genco (1980) the newly described subspecies *macacae* of *B. melaninogenicus* consists of black pigmented strains that ferment glucose, galactose, lactose and mannose weakly, produce acetic, propionic, *i*-butyric, butyric, *i*-valeric and succinic acids. Biochemically, they are very similar to *B. melaninogenicus* spp. *levii*, which also ferments glucose, galactose and lactose

and produces the same acids (Holdeman *et al.*, 1977), but ssp. *macacae* is serologically different from all previously described species and sub-species.

Thin-layer chromatography has proved to be very useful and reliable for the detection of DAP, not only in gram-positive but also in gram-negative bacteria. A review of our own data and those of other groups shows that DAP occurs consistently in strains of *B. bivius* and *B. disiens*. Our DAP-detection studies indicate that *B. asaccharolyticus*, *B. melaninogenicus* and *B. oralis* form a rather heterogeneous group of organisms.

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