Distribution of 2,3-Diaminohexuronic Acid in Strains of *Propionibacterium* and Other Bacteria

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Trichloroacetic acid extracts of 119 strains of bacteria were examined by thin-layer chromatography for the presence of 2,3-diamino-2,3-dideoxyhexuronic acid. This compound was identified by thin-layer chromatography as material giving a distinctive blue spot with a ninhydrin-collidine reagent and moving to the same position as authentic 2,3-diamino-2,3-dideoxyglucuronic acid. Material of this kind was found in all strains of propionibacteria except *Propionibacterium freudenreichii* and also in some strains of *Clostridium*, *Actinomyces*, and *Arachnia*. It was not found in representatives of *Corynebacterium* (11 strains), *Bacillus* (4 strains), *Lactobacillus* (11 strains), *Staphylococcus* (3 strains), *Nocardia* (3 strains), *Streptomyces* (1 strain), *Actinoplanes* (1 strain), *Erysipelothrix* (1 strain), *Peptostreptococcus* (2 strains), and *Arcanobacterium* (1 strain), but was present in one of the four strains of *Eubacterium* examined and in one of two strains of *Bifidobacterium*. It was not present in whole cells or lipopolysaccharides of seven strains of *Bacteroides*, but was found in two of five *Pseudomonas aeruginosa* strains tested.

A new type of aminohexuronic acid, 2,3-dideoxy-2,3-diaminoglucuronic acid, was recently found in the cell wall polysaccharide of a strain of *Propionibacterium acnes* (6). An identical or very similar substance was also found in the cell wall polysaccharides of strains of *Propionibacterium avidum* and *Propionibacterium granulosum*. Other 2,3-diamino-2,3-dideoxyhexuronic acids have been reported from the polysaccharides of *Pseudomonas aeruginosa* strains (7, 10, 11). Since the substance from *Propionibacterium acnes* was easily recognized on thin-layer chromatograms because of the bright blue color which it gave with a ninhydrin-collidine reagent, it appeared to have the makings of a good taxonomic marker. Therefore, it seemed of interest to examine the distribution of this compound in strains of classical propionibacteria isolated from dairy products and in other bacteria.

MATERIALS AND METHODS

Strains of bacteria. The strains of bacteria used are listed in Table 1.

Conditions of growth. Most strains were grown for 48 h in Trypticase-yeast extract-glucose broth containing 0.1% Tween 80. However, for corynebacteria, 5% sterile rabbit serum was added to the medium. Strains of *Actinomyces israelii*, *Actinomyces bovis*, and *Actinomyces naeslundii* were also grown in 5% serum broth, usually for 5 to 6 days because of their slower rate of growth. The classical propionibacteria were grown at 30 to 33°C; the other organisms were grown at 35 to 36°C.

Pathogenic organisms, such as *Corynebacterium diphtheriae*, were killed by adding Formalin to a final concentration of 2%; other organisms were killed by heating at 56°C for 30 min (70°C in the case of staphylococci). All suspensions were washed three times in distilled water before extraction.

Cell wall fractions. Cell wall fractions were prepared as described previously by Cummins and Johnson (4).

TCA extraction. The material to be hydrolyzed was prepared by trichloroacetic acid (TCA) extraction of either whole cells or cell walls.

(i) Extraction of whole cells. Preliminary experiments with known positive strains established that 25 mg (dry weight) of lyophilized whole cells extracted three times with 1 ml of

10% TCA (56 to 60°C for 30 min) gave adequate amounts of material for examination. After each extraction, the mixture was centrifuged for 1 min at $13,000 \times g$ with a Fisher microfuge, the supernatant was removed, and the deposit was extracted with 1 ml of 10% TCA. Finally, the combined extracts were mixed with 5 volumes of acetone and a few drops of a 5% potassium acetate solution, and these preparations were allowed to stand overnight at 4°C. The precipitated material was recovered by centrifugation and dissolved in 1 ml of distilled water, and any insoluble material was removed by centrifugation at $13,000 \times g$. The supernatant was then hydrolyzed (see below).

If freshly grown material was used, the washed cells from 25 to 100 ml of medium were extracted as described above.

(ii) Extraction of cell walls. A 10-mg portion of cell walls was extracted by the method described above for whole cells.

(iii) Extraction of lipopolysaccharide. The hot phenol method of Luderitz et al. (15) was used to extract lipopolysaccharide from lyophilized cell walls.

Hydrolysis of extracts. The clear extract (1 ml) after the final centrifugation was added to 1 ml of concentrated HCl, and the mixture was hydrolyzed for 18 h at 100°C in a sealed tube. If large amounts of humin formed, this was removed by centrifugation, and the supernatant was evaporated to dryness and taken up in about 0.2 ml of distilled water; if necessary, the solution was centrifuged again to remove insoluble material. For hydrolysis of lipopolysaccharide 10 mg of lipopolysaccharide was heated in 1 ml of 6 N HCl for 18 h at 100°C and then treated as described above.

Thin-layer chromatography. **(i) One-dimensional chromatography.** One-dimensional chromatography was performed on Avicel cellulose plates (20 by 20 cm; Fisher Scientific Co.) by using one or more of the following solvents: ethyl acetate-pyridine-acetic acid-Water (72:72:14:42) (solvent A); butanol-acetic acid-Water (160:40:67) (solvent B); and α -picoline-water-28% NH_4OH (140:56:4) (solvent C). In all cases plates were developed twice in the solvent and dried at room temperature in a hood for 30 min between ascents. Approximately 5 μl of each sample was applied on a line 3 cm from the bottom of the plate, and the solvent was normally run to within 2 cm of the top on each ascent. After the second development the plates were again dried, and the

TABLE 1. Occurrence of 2,3-diamino-hexuronic acids (2,3-DH) in TCA extracts^a

Organism	VPI	Culture number Other	Material examined (CW, WC, P. LPS)	Presence of 2,3-DH
<i>Propionibacterium</i>				
<i>P. acnes</i> type I	0009	Prevot 3109	CW, P	+
<i>P. acnes</i> type I	3706		CW	+
<i>P. acnes</i> type I	6636	Prevot 2444	CW	+
<i>P. acnes</i> type II	0162	Prevot 578	CW	+
<i>P. acnes</i> type II	0204	Prevot 329B _T	CW	+
<i>P. granulosum</i>	0507	ATCC 25564 _T	CW	+
<i>P. granulosum</i>	6500	Prevot 3085	CW	+
<i>P. avidum</i> type I	0575	CDC 71	CW	+
<i>P. avidum</i> type II	0589	CDC A518	CW	+
<i>P. avidum</i> type II		London Hospital 4982	CW	+
<i>P. lymphophilum</i>	0202	Prevot 1519F	CW, P	+
<i>P. lymphophilum</i>	0383	Prevot SB	CW	+
<i>P. lymphophilum</i>		BW 6294	WC	+
<i>P. lymphophilum</i>		BW 6295	WC	+
<i>P. jensenii</i>	0398	ATCC 4869	CW, P	+
<i>P. jensenii</i>	5166	ATCC ISL106	WC	+
<i>P. jensenii</i>	5169	ATCC 4870	WC	+
<i>P. jensenii</i>	5173	ATCC 4964 _T	WC	+
<i>P. thoenii</i>	0411	ATCC 4872 _T	CW, P	+
<i>P. thoenii</i>	5164	ATCC ISL104	WC	+
<i>P. thoenii</i>	5174	ATCC 4874 _T	WC	+
<i>P. acidi-propionici</i>	0399	ATCC 25562 _T	CW, P	+
<i>P. freudenreichii</i>	0392	IAM 1714	WC	—
<i>P. freudenreichii</i>	0404	ATCC 8262	WC	—
<i>P. freudenreichii</i>	0405	ATCC 9614	WC	—
<i>P. freudenreichii</i>	0406	ATCC 9614	WC	—
<i>P. freudenreichii</i>	0407	ATCC 9616	CW, P	—
<i>P. freudenreichii</i>	5165	ATCC ISL105	WC	—
<i>Arachnia</i>				
<i>A. propionica</i>	0026	ATCC 14157 _T	CW	+
<i>A. propionica</i>	5067	ATCC 29326 (WVU 346)	CW	+
<i>A. propionica</i>	5068	ATCC 29324 (WVU 427)	CW	+
<i>A. propionica</i>	5069	ATCC 29325 (WVA 439A)	CW	—
<i>A. propionica</i>	5070	WVU 445	CW	+
<i>A. propionica</i>	5074	WVU 572	CW	+
<i>Actinomyces</i>				
<i>A. israelii</i>		C153, ATCC 12104	CW	+
<i>A. israelii</i>		C276, ATCC 10048	CW	+
<i>A. israelii</i>		'Flynn' London Hosp.	CW	+
<i>A. bovis</i>		ATCC 13683 (Pine, P1) _T	CW	—
<i>A. bovis</i>		Pine A11	CW	—
<i>A. bovis</i>		Pine A12	CW	—
<i>Actino. sp.</i>		Holm 1452	CW	—
<i>A. pyogenes</i>		NCTC 5224	CW	—
<i>A. pyogenes</i>		ATCC 19411 _T	CW	—
<i>A. naeslundii</i>		ATCC 13683	CW	—
<i>Arcanobacterium</i>				
<i>A. hemolyticum</i>		C27	CW	—
<i>Bacillus</i>				
<i>B. sphaericus</i>		C1; NCTC 2608	WC	—
<i>B. licheniformis</i>	5970	ATCC 14580 _T	WC	—
<i>B. polymyxa</i>	5972	ATCC 842 _T	WC	—
<i>B. subtilis</i>	5974	ATCC 6051 _T	WC	—

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TABLE 1. Continued

Organism	Culture number		Material examined (CW, WC, P, LPS)	Presence of 2,3-DH
	VPI	Other		
<i>Lactobacillus</i>				
<i>L. cateniforme</i>	0118	PIP 3016C	CW	—
<i>L. cateniforme</i>	2933	ATCC 25536 ^T	CW	—
<i>L. cateniforme</i>	3091	PIP 1792	CW	—
<i>L. crispatus</i>	0253	PIP 1409	CW	—
<i>L. acidophilus</i>	1294	ATCC 33199	CW	—
<i>L. acidophilus</i>	1754	ATCC 33198	CW	—
<i>L. acidophilus</i>	1784		CW	—
<i>L. acidophilus</i>	6032	ATCC 4356 ^T	CW	—
<i>L. acidophilus</i>	6033	ATCC 19992	CW	—
<i>L. acidophilus</i>	7960	ATCC 33200	CW	—
<i>L. fermentum</i>	8153		WC	—
<i>Clostridium</i>				
<i>C. botulinum</i> A	3800		CW	+
<i>C. botulinum</i> B	1624	ATCC 7948	CW	+
<i>C. botulinum</i> B	2129	PIP 29	CW	+
<i>C. botulinum</i> C	1615	ATCC 9633	CW	±
<i>C. botulinum</i> C	3802		CW	±
<i>C. botulinum</i> C	3804		CW	+
<i>C. botulinum</i> D	1543	CDC KA 39	CW	+
<i>C. botulinum</i> D	3805		CW	±
<i>C. sporogenes</i>	2737		CW	+
<i>C. tertium</i>	1565	ATCC 14573	CW	+
<i>C. tertium</i>	2033	CDC 481	CW	+
<i>C. novyi</i>	1617	ATCC 898	CW	Tr
<i>C. novyi</i>	4258	McClung 117	CW	Tr
<i>C. perfringens</i>	1537	CDC KA 3	CW	—
<i>C. perfringens</i>	5694	ATCC 13124 ^T	CW	—
<i>C. butyricum</i>	1718	ATCC 860	CW	+
<i>C. butyricum</i>	2983	Prevot A9E1	CW	+
<i>C. beijerinckii</i>	4420	ATCC 17778	CW	+
<i>C. acetobutylicum</i>	3163	ATCC 824 ^T	CW	—
<i>C. innocuum</i>	2581		CW	—
<i>C. putrificum</i>	4440	ATCC 25784 ^T	CW	—
<i>C. paraputrificum</i>	1584	ATCC 25780 ^T	CW	—
<i>C. paraputrificum</i>	1586	CDC 391	CW	—
<i>C. tetani</i>	1609	ATCC 8033	CW	—
<i>C. pasteurianum</i>	4215	ATCC 6013 ^T	CW	—
<i>C. limosum</i>	1748	PIP 666	CW	+
<i>C. limosum</i>	1950	CDC 1218	CW	+
<i>C. limosum</i>	1740	CDC 1402	CW	—
<i>C. limosum</i>	1928	CDC 719	CW	—
<i>C. limosum</i>	2755	PIP 278	CW	+
<i>Clostridium</i> sp.	3160	FDA S9	CW	+
<i>Clostridium</i> sp.	2808A		CW	—
<i>Staphylococcus</i>				
<i>S. aureus</i>	19a		CW	—
<i>S. aureus</i>	2a		WC	—
<i>S. epidermidis</i>		Biology Dept., VPI & SU	WC	—
<i>Peptostreptococcus</i>				
<i>P. magnus</i>	5467	PIP 1469B	WC	—
<i>P. glycinophilus</i>	5664	ATCC 23195 ^T	WC	—
<i>Bifidobacterium</i>				
<i>B. asteroides</i>	5636	ATCC 25910 ^T	WC	+
<i>B. adolescentis</i>	5640	Scardovi C410	WC	—

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TABLE 1. Continued

Organism	Culture number		Material examined (CW, WC, P, LPS)	Presence of 2,3-DH
	VPI	Other		
<i>Eubacterium</i>				
<i>E. limosum</i>	5167	ATCC 8486 ^T	WC	—
<i>E. lentum</i>	0255	ATCC 25559 ^T	WC	—
<i>E. lentum</i>	8251		WC	+
<i>E. lentum</i>	10290		WC	—
<i>Erysipelothrix</i>				
<i>E. rhusiopathiae</i>		Coll. of Vet. Med., VPI & SU	WC	—
<i>Corynebacterium</i>				
<i>C. diphtheriae</i>		C122	WC	—
<i>C. diphtheriae</i>		C123	WC	—
" <i>C. ulcerans</i> "		C102, NCTC 7910	WC	—
" <i>C. ovis</i> "		C103, NCTC 3450, ATCC 19410	WC	—
<i>C. xerosis</i>		C105, NCTC 9735	WC	—
<i>C. renale</i>		C104, NCTC 7448, ATCC 19412 ^T	WC	—
" <i>C. equi</i> "		C101, NCTC 1621	WC	—
<i>C. bovis</i>		ATCC 7715 ^T	WC	—
<i>C. paurometabolum</i>		ATCC 8368 ^T	WC	—
<i>C. minutissimum</i>		1130	WC	—
<i>C. minutissimum</i>		771	WC	—
<i>Nocardias</i> and <i>Streptomyces</i>				
<i>Nocardia</i> sp.		C132, Rutgers 736B	WC	—
<i>N. autotrophica</i>		6031	WC	—
<i>N. mediterranei</i>		6030	WC	—
<i>S. coelicolor</i>		186	WC	—
<i>Actinoplanes missouriensis</i>		5375	WC	—
<i>Bacteroides</i>				
<i>B. fragilis</i>	2553	ATCC 25285 ^T	WC	—
<i>B. fragilis</i>	2552	NCTC 8560	WC	—
<i>B. vulgatus</i>	4245	ATCC 8482 ^T	WC	—
<i>B. ovatus</i>	0038	ATCC 8483 ^T	WC	—
"3452-A" group	3452		WC	—
<i>B. fragilis</i>	2393	PIP Beerens 12	P, LPS	—
<i>B. fragilis</i>	5482	NCTC 10582	LPS	—
<i>Pseudomonas</i>				
<i>P. aeruginosa</i>		ATCC 15152 (NCTC 1999)	LPS	—
		ATCC 27314 (Habs Type 3)	WC	—
		ATCC 27315 (Habs Type 4)	WC	++
		ATCC 27578 (Habs Type 5)	WC	—
		ATCC 27586 (Habs Type 1)	WC	++

^aAbbreviations used:—TCA = Trichloroacetic acid; CW = cell walls; WC = whole cells; P = cell wall polysaccharide; LPS = Lipopolysaccharide; 2,3-DG = 2,3-diamino-2,3-dideoxyglucuronic acid; VPI & SU = Virginia Polytechnic Institute and State University; BW = Burroughs Wellcome; PIP = Pasteur Institute Paris; ATCC = American Type Culture Collection; CDC = Communicable Disease Center, Atlanta, Ga.; NCTC = National Collection of Type Cultures, Colindale, England. For *Pseudomonas aeruginosa* types, see references 1, 8, and 14.

spots were visualized by spraying with the following mixture: 50 mg of ninhydrin in 50 ml of 95% ethanol containing 10 ml of glacial acetic acid and 1 ml of collidine (2,4,6-trimethylpyridine) (formula slightly modified from that of Cowgill and Pardee [3]). To develop the spots, the plates were heated in an oven at 90°C for exactly 3 min. This temperature and time were adhered to because overheating was found to obscure the distinctive bright blue color of 2,3-diaminoglucuronic acid.

(ii) **Two-dimensional chromatography.** Two-dimensional chromatography was performed on Avicel cellulose plates by using a single ascent in solvent B in the first dimension, followed by two ascents in solvent C in the second dimension. The plates were dried overnight after the first solvent, and for 1 h between the two ascents in the second solvent.

The spots were visualized by the ninhydrin-collidine reagent described above for one-dimensional chromatography.

FIG. 1. Separation of 2,3-diamino-2,3-dideoxyglucuronic acid from other amino sugars and peptidoglycan components. Abbreviations: Ala, alanine; DAP, diaminopimelic acid; GalNH₂, galactosamine; GluNH₂, glucosamine; Glu, glutamic acid; Gly, glycine; Mur, muramic acid; 2,3-DG, 2,3-diamino-2,3-dideoxyglucuronic acid; HAc, acetic acid.

RESULTS AND DISCUSSION

The primary purpose of this investigation was to establish whether 2,3-diamino-2,3-dideoxyglucuronic acid is confined to *Propionibacterium acnes* and other cutaneous propionibacteria or whether it also occurs in the classical propionibacteria from cheese and dairy products. Since a variety of cell wall preparations were available from previous investigations on members of other genera, these were also examined. In addition, extracts of whole cells of a limited number of other species were analyzed. Since 2,3-diaminoglucuronic acid was originally described in polysaccharides extracted from the cell walls of *Propionibacterium acnes* by using 10% TCA at 56 to 60°C, my investigations were essentially limited to material extracted from other organisms by a similar method, except in three cases where lipopolysaccharide was used. Whenever TCA extracts of whole cells and of cell walls from the same organism were compared, the results were identical, suggesting that if the substance is present in the wall polysaccharide, it can readily be detected also in TCA extracts of whole cells. Long storage (up to 12 years) did not appear to affect the amount of 2,3-diamino-2,3-dideoxyhexuronic acid that could be detected when extracts of old samples were compared with extracts of fresh cell walls.

At the time that 2,3-diamino-2,3-dideoxyhexuronic acid was first isolated and identified in the cell wall polysaccharide of *Propionibacterium acnes* (6), I was not aware that an apparently identical substance had been described by Knirel et al. (10) from the lipopolysaccharide of *Pseudomonas aeruginosa*. Subsequently, Knirel et al. (11), Dmitriev et al. (7), and Okuda and Suzuki (16) described other strains of *Pseudomonas* which contained the acetyl derivatives of 2,3-diamino-2,3-dideoxymannuronic acid and 2,3-diamino-

2,3-dideoxyglucuronic acid or 2,3-diamino-2,3-dideoxyglucofuranurono-6,3-lactam (16, 17). In the present investigations five strains of *Pseudomonas aeruginosa* were examined, four of them as TCA extracts of whole cells and one as lipopolysaccharide. Two of these strains were found to contain large amounts of material giving the same color and running to the same position as 2,3-diamino-2,3-dideoxyglucuronic acid from *Propionibacterium acnes*.

So far, it has not been possible to obtain and examine the strains investigated by Knirel et al. (7, 10–13) and to compare directly the chromatographic behaviors of the different diaminohexuronic acids. Knirel et al. (7, 10–13) reported 2,3-diaminoglucuronic acid in a strain of *Pseudomonas aeruginosa* belonging to Habs serotype 5, but the strain of Habs group 5 examined in the present investigation (strain ATCC 27578) contained no blue-spot material of any kind.

Chemical identity of the blue-spot material. With the solvents used, especially in two-dimensional thin-layer chromatography, the substance giving the distinctive blue spot was well separated from other amino acids and amino sugars (Fig. 1). However, except in the case of *Propionibacterium acnes* VPI 0009, from which it was originally isolated (6), the material was not separated and identified chemically. In all strains in which it was found, the compound ran to the same position as authentic 2,3-diamino-2,3-dideoxyglucuronic acid. This may mean either that the same 2,3-diamino-2,3-dideoxyhexuronic acid occurs in all cases or that all of the diaminohexuronic acids that occur in these strains have identical chromatographic properties in the solvents.

As noted above, various other 2,3-diamino-2,3-dideoxyhexuronic acids have been identified by ¹³C nuclear magnetic resonance spectroscopy in the polysaccharides of *Pseudomonas aeruginosa* (7, 10–13, 16) and may well occur in the polysaccharides of other organisms as well.

It seems reasonable to assume that they have very similar chromatographic properties, but this has not yet been proven.

Distribution of 2,3-diamino-2,3-dideoxyhexuronic acids. In all, extracts of 119 strains of bacteria were examined. It appears that 2,3-diamino-2,3-dideoxyhexuronic acid is a rather characteristic feature of the cell wall polysaccharides of propionibacteria, with the striking (and perhaps unfortunate) exception of the type species, *Propionibacterium freudenreichii*. This is another illustration of the fact that *Propionibacterium freudenreichii* is a somewhat atypical species in the genus (5). Apparently identical material was also found in seven of eight strains of *Arachnia propionica*, which appear to have a metabolic pattern similar to that of propionibacteria, although they show no relationship to propionibacteria by deoxyribonucleic acid homology (9).

In *Clostridium* the material was present in a number of species. In *Clostridium botulinum* (types A, B, C, and D) the amount present varied considerably although the same weight of cell walls was extracted in each case; this may have been due to its being part of a polymer which occurs in different amounts in different strains. The occurrence of 2,3-diamino-2,3-dideoxyhexuronic acids in such diverse groups as propionibacteria, actinomyces, pseudomonads, and clostridia suggests that the presence of these compounds is not associated with any particular type of metabolism. Preliminary experiments with a few strains of capsulated propionibacteria (*Propionibacterium jensenii* and *Propionibacterium thoenii*) showed that diaminohehexuronic acids are absent from the capsular material even though they are present in the cell wall polysaccharides of the same strains. They were not found in purified peptidoglycan from propionibacteria (i.e., cell walls after extraction with TCA and hot formamide) (Cummins, unpublished data). Therefore, in bacteria these substances appear to be particularly associated with cell wall polysaccharides. Their patchy distribution makes it unlikely that they play a general role in cell wall structure as, for example, muramic acid does in peptidoglycan. They could possibly act as linking agents between polysaccharide and peptidoglycan in cases where the polysaccharide lacks other suitable groups, such as phosphate.

The limited distribution and unusual color reaction of 2,3-diamino-2,3-dideoxyhexuronic acids with ninhydrin suggest that they may be of value in systematic surveys of chemical composition as applied to taxonomy.

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