Analysis of the Cell Wall Constituents of *Corynebacterium genitalium*

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Cell walls of *Corynebacterium genitalium* (three strains) were analyzed for their content of neutral sugars, amino acids, amino sugars, and corynomycolates. Analyses of the walls of a strain of *C. xerosis*, a bona fide member of the genus, were effected for purposes of control. Walls of each of the four strains contained arabinose, galactose and alanine, glutamic acid, and diaminopimelic acid in similar molar ratios, as well as corynomycolic acids ranging from 32 to 36 in numbers of carbon atoms. This confirmed that they are *Corynebacterium* species. However, distinct differences in cell wall composition were found between *C. xerosis* and the strains of *C. genitalium*.

Among corynebacteria found in the male and female urogenital tracts (8), some have been associated with so-called nonspecific urethritis (NSU) (3, 7) and some have been associated with epididymitis (6, 9). Certain strains appear to be capable of initiating NSU (4), whereas others exist commensally with their hosts (5). These corynebacteria have been designated *Corynebacterium genitalium*, a species which encompasses five (I through V) biological varieties regarded as pathogenic and six (C-1 through C-6) biovars deemed nonpathogenic (G. Furness and A. T. Evangelista, Invest. Urol., in press). The bacteria assigned to the genus *Corynebacterium* are facultatively aerobic, gram-positive to gram-variable, nonmotile, rodlike, tapered bacteria, producing metachromatic granules and growing as black colonies on tellurite agar (1). *C. genitalium* not only has these characteristics, but also produces catalase and forms small circular colonies 1 to 2 mm in diameter on Tween 80 agar and NSU chocolate agar (Furness and Evangelista, in press). Moreover, biovars I through V are fructose negative and biovars C-1 through C-6 are fructose positive. They are differentiated by differences in their ability to ferment dextrose, fructose, and starch, to produce gelatinase, lipase, and urease, and in their requirement for O₂ only when grown on Tween 80 agar (Furness and Evangelista, in press). Members of the genus *Corynebacterium*, sensu stricto, possess a cell wall structure characterized by a peptidoglycan containing alanine, glutamic acid, and meso-diaminopimelic acid and an arabinogalactan polysaccharide (20) occur-

ring in conjunction with corynomycolic acids (12, 22, 24). Here we present data which show that representative strains of *C. genitalium* possess cell walls characteristic of the genus *Corynebacterium* (19).

MATERIALS AND METHODS

C. genitalium 392-1 (biovar I) and 321D (biovar IV) are pathogens which were cultured from patients with acute "coryneform urethritis" (Furness and Evangelista, in press), whereas strain 148A (biovar C-1) was isolated from the normal urogenital tract of a male control (4, 5; Furness and Evangelista, in press). *Corynebacterium xerosis* ATCC 373 was obtained from the American Type Culture Collection.

The bacteria for analysis were obtained by inoculating Tween 80 agar plates with a Tween 80 broth culture (8) and incubating the plates for 48 to 72 h when the growth was confluent. All cultures were incubated at 37°C. Thereafter, the organisms were washed off the agar with sterile distilled water and sedimented by centrifugation at 10,000 × g for 15 min, and the supernatant was discarded. When 15 g (wet weight) of each strain was obtained, 5 g was analyzed for the cell wall components and 10 g was analyzed for detecting the presence of the mycolates.

Preparation of cell walls. Cell wall preparations were obtained by a modification of the method of Kanetsuna (10). The 45% (wt/vol) buffered phenol and 90% (wt/vol) buffered phenol were prepared by adding appropriate quantities of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.2) to phenol, analytical-grade reagent (Fisher, Springfield, N.J.), and adjusting to pH 7.2 with 2 N NaOH. A 5-g (wet weight) amount of cells was suspended in 20 ml of 45% buffered phenol in a 30-ml Correx tube (Corning Glass, Corning, N.Y.) and disrupted by sonically extracting the suspension at 25°C by means of a Branson Sonifier (Heat Systems Co., New York). When the phenol started to boil after about 2 min of sonic extraction, the suspension was

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cooled in ice water. The process was repeated, and the resulting emulsion was separated into the two layers of phenol and buffer by centrifugation at $27,000 \times g$ for 30 min at 4°C in a Sorvall RC-5 refrigerated centrifuge (Dupont, Norwalk, Conn.). The pellet and the material at the phenol-buffer interface were pooled, and the supernatant was discarded. The 45% buffered phenol extraction, sonic extraction, and centrifugation procedures were repeated four additional times. Thereafter, it was confirmed that only a few of the cells in a Gram-stained smear of the residue were still intact. Phenol remaining in the cell wall fraction was removed by washing twice with acetone, once with ether, and twice with distilled water. At every stage, the cell wall fraction was sedimented by centrifugation at $27,000 \times g$ for 20 min at 4°C . The fraction was digested for 2 h with 0.1% (wt/vol) trypsin (Worthington Biochemicals Corp., Freehold, N.J.) in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.2) at 37°C with continuous agitation on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.). The undigested residue was removed by centrifugation at $27,000 \times g$ for 20 min at 4°C , the trypsin was decanted, and sonic extraction of the residue was repeated first in 45% buffered phenol and then in 90% buffered phenol. Only the material at the interface of the 90% phenol-buffer was collected, the supernatant fluid and the pellet being discarded. The phenol in this fraction was removed by washing with acetone followed by ether and distilled water. The residue was dried in vacuo over CaSO_4 at 25°C . About 60 mg of cell wall material containing the mycolic acid-arabinogalactan-peptidoglycan (MA-AG-PG) complex (11) was obtained from 5 g (wet weight) of bacteria.

Neutral sugar analysis. A 10-mg amount of the dried cell wall residue, which contained 24 to 30% neutral sugars as estimated by the anthrone (13) and orcinol (2) methods using galactose and arabinose, respectively, as standards, was hydrolyzed in vacuo in 1 ml of 2 N HCl in a sealed ampoule (Wheaton, Millville, N.J.) at 100°C for 6 h. The hydrolysate was dried in vacuo over NaOH pellets, suspended in 3 ml of 0.1 M sodium borate buffer (pH 8.00) (11), and filtered through a $0.45\text{-}\mu\text{m}$ membrane filter (Millipore Corp., Bedford, Mass.). Analysis of the neutral sugars was performed by a modification of the methods of Kesler (11) and Lee et al. (16) by use of a Technicon Autoanalyzer (Technicon Corp., N.Y.) equipped with a column (0.6 by 68 cm) packed with Chromobeads S resin (Technicon). The column was standardized by analyzing a calibration mixture of $0.1\text{ }\mu\text{mol}$ each of D-mannose, L-arabinose, D-galactose, D-glucose, L-rhamnose, and D-ribose (Calbiochem, Los Angeles, Calif.).

Amino acid and amino sugar analyses. A 1-mg amount of the dried MA-AG-PG material, which contained 12 to 18% protein as estimated by the method of Lowry et al. (17), was hydrolyzed at 100°C in sealed tubes either in 6 N HCl for 18 h for the amino acid determination or in 4 N HCl for 8 h for the amino sugar analysis. The hydrolysis tubes were intermittently evacuated by a vacuum pump and flushed with nitrogen and sealed in vacuo. After hydrolysis, HCl was removed by a rotary evaporator under reduced pressure, and the dried residue was dissolved in 0.35 M sodium citrate buffer (pH 2.2). Analyses of the

amino acids and amino sugars were performed by a modification of the method of Spackman et al. (23) on a Beckman 120C amino acid analyzer which was connected to a general-purpose, on-line laboratory computer (Infotronics CRS 1000, Columbia Scientific Instruments, Austin, Tex.). The buffer flow rate was 70 ml/h, and the ninhydrin flow rate was 35 ml/h.

The quantitation of the acidic and neutral amino acids and diaminopimelic acid (Dpm) and muramic acid (Mur) was performed on a UR 30 column (0.9 by 58 cm; Beckman). The analysis was carried out on this column at 53.5°C for a total of 140 min with 0.2 M sodium citrate buffer (pH 3.56) for 43 min followed by citrate buffer at pH 4.26. The Dpm determination was conducted at 53.5°C for 200 min with 0.2 M sodium citrate buffers (pH 3.44 and pH 4.26) with a buffer change at 107 min. The Dpm was eluted at 137 min. The Mur was analyzed on the same column with 0.2 M citrate buffer (pH 3.21) at 32.5°C for 110 min, with Mur emerging at 63 min.

The basic amino acids were quantitated by using a PA 35 column (0.9 by 7 cm; Beckman) and developed with 0.2 M sodium citrate buffer (pH 5.26) at 53.5°C for 65 min. The quantitation of glucosamine (GlcNH_2) and galactosamine (GalNH_2) was performed on a PA 35 column (0.9 by 15 cm; Beckman) with 0.2 M sodium citrate buffer (pH 5.21) at 53.5°C for 140 min.

A standard amino acid mixture (Beckman) containing 10 nmol of each amino acid in a $200\text{-}\mu\text{l}$ sample volume was used for calibration, as were 10-nmol standards of Dpm, Mur, GlcNH_2 , and GalNH_2 (Sigma).

Analysis of mycolates. A 10-g (wet weight) amount of each strain of corynebacteria was used for the extraction. Qualitative and quantitative analyses of mycolates in the extracts were performed by the methods of Lechevalier et al. (14, 15). The cells were boiled for 7 min in 50 ml of 2% methanolic KOH in a water bath at 100°C . The hot cell suspension was filtered through fluted Whatman no. 1 filter paper and rinsed twice with hot methanol, and the solvent was discarded. The cells were then extracted twice in succession for 2 min with boiling methylene chloride. The cells were removed by filtration using Whatman no. 1 filter paper, and the methylene chloride extracts were combined and reduced to 5 ml under reduced pressure at 60°C . A 5-ml volume of distilled water was added to the concentrated methylene chloride extract and acidified to pH 2.0 with 6 N HCl. The two phases were mixed thoroughly and then separated by centrifugation at $10,000 \times g$ for 15 min at 4°C , and the aqueous phase was discarded. The solvent was evaporated under reduced pressure at 60°C , and the resulting crude mycolate was desiccated in vacuo for 48 h at 25°C .

Samples were methylated using a modification of the procedure of Metcalfe and Schmitz (18). Approximately 100 mg of dried residue was dissolved in 3 ml of a 10% (wt/vol) solution of boron trichloride in methanol (Sigma) and boiled for 7 min in a 100°C water bath. To the boiled mixture was added 5 ml of methylene chloride and 5 ml of distilled water, and it was shaken vigorously. The mixture was separated into two phases by centrifugation at $10,000 \times g$ for 15 min at 4°C , and the aqueous-methanol phase was discarded. Solid materials formed at the interface were

retained along with the methylene chloride phase, and the solvent was evaporated in a 60°C water bath. The resultant residue was desiccated in vacuo and stored at -20°C.

The methylated residue, containing 10 to 20 mg of material, was purified by preparative thin-layer chromatography on a glass plate (10 by 20 cm) coated with Merck Silica Gel PF₂₅₄ (Brinkmann Instruments, Westbury, N.Y.) and developed with petroleum ether (bp 40 to 50°C)-diethyl ether (8:2). The plates were air dried, and the lipid-containing bands were revealed by spraying with rhodamine B, prepared by the method of Laneelle (13): 0.1% (wt/vol) rhodamine B in ethanol diluted 1:10 with 0.25 M KH₂PO₄. Compounds migrating the same distance as a methylated corynomycolate standard were scraped from the plate, dried at 25°C for 18 h, and eluted from the silica gel with distilled methylene chloride by filtering through glass wool. The rhodamine B remained absorbed to the silica gel.

Two to six micrograms of each dried eluate was redissolved in methylene chloride and injected into a Varian Aerograph 2800 Moduline dual-column gas-liquid chromatograph (Varian, Norwalk, Conn.) equipped with a hydrogen flame detector, a programmer, a flow controller, and two stainless-steel columns (1/8 inch by 6 feet; ca. 0.32 by 182.88 cm) containing Chromosorb W (Johns-Manville, Englewood Cliffs, N.J.), 100/120 mesh (AW-DMCS), with 10% OV-1 as liquid phase. Conditions for analysis were: detector at 300°C, injector at 300°C, oven at 185°C programmed to 300°C at 6°C/min. The carrier gas was prepurified nitrogen flowing at 30 ml/min. Pyrolysis fragments were identified by their retention times. Standards consisted of even, saturated, and unsaturated straight-chain methyl esters of fatty acids from C₁₂ to C₂₆ and the corresponding fatty aldehydes of C₁₄, C₁₆, and C₁₈ which were stabilized in CS₂.

RESULTS

Cell wall fractions containing MA-AG-PG complexes obtained from strains 392-1 (biovar I) and 321D (biovar IV), together with strain 148A (biovar C-1), of *C. genitalium* were compared with a fraction extracted similarly from *C. xerosis*.

Neutral sugar composition. The neutral sugar composition of the MA-AG-PG complex of the three strains of *C. genitalium* was compared quantitatively with that of *C. xerosis* (Table 1). The molar ratio of the sugars was expressed with respect to arabinose, which was found in the greatest amount. The cell wall fractions of all the strains contained sugars characteristic of the corynebacteria of human origin, namely, the pentose arabinose and the hexose galactose, with a molar ratio of more than 2 to 1 in the fractions from *C. genitalium* and *C. xerosis*. The main difference between *C. genitalium* and *C. xerosis* was in the molar ratios of mannose and glucose. The molar ratios for mannose and glucose in the cell wall fraction of *C. xerosis*, were 0.06 and 0.26, respectively, whereas

TABLE 1. Neutral sugar composition of the mycolic acid-arabinogalactan-peptidoglycan complex in the cell walls of *Corynebacterium genitalium* and *Corynebacterium xerosis*

Neutral sugar	Molar ratio ^a			
	<i>C. xerosis</i> ATCC 373	<i>C. genitalium</i> 392-1 (biovar I)	<i>C. genitalium</i> 321D (biovar IV)	<i>C. genitalium</i> 148A (biovar C-1)
Arabinose	1.00	1.00	1.00	1.00
Galactose	0.41	0.45	0.36	0.48
Mannose	0.06	0.32	0.24	0.45
Glucose	0.26	0.09	0.08	0.06
Rhamnose	0.13	0.13	0.03	0.06
Ribose	0.07	0.02	0.08	0.09

^a Molar ratios are expressed with respect to arabinose.

the average molar ratios in *C. genitalium* were 0.33 and 0.08, respectively. The remaining detectable sugars of the MA-AG-PG complexes were rhamnose and ribose. Rhamnose occurred at a slightly higher molar ratio in *C. xerosis* and *C. genitalium* 392-1 (biovar I) than in *C. genitalium* 321D (biovar IV) and 148A (biovar C-1), but the difference was not significant. The ratios of ribose in the cell wall fractions of both *C. xerosis* and *C. genitalium* were uniformly very low, with ratios of 0.02 to 0.09.

Amino acid and amino sugar composition. The quantitative amino acid and amino sugar composition of the MA-AG-PG complexes in the cell walls of *C. genitalium* and *C. xerosis* are given in Table 2. The values are expressed as molar ratios to glutamic acid even though alanine was present in greater concentrations. In all the cell wall fractions the major amino acids were glutamic acid, alanine, and diaminopimelic acid which occurred in similar molar ratios. The relative proportions of the remaining minor amino acids of the MA-AG-PG complex also were similar in these four strains of corynebacteria. They were aspartic acid, threonine, serine, proline, valine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine. No component corresponding to the methionine standard was detected in any fraction. The amino sugars muramic acid and glucosamine were present in the four cell wall fractions, but the molar ratios of the two amino sugars were slightly higher in *C. xerosis*. A significant variation in the MA-AG-PG complexes was noted only with respect to galactosamine. *C. xerosis* contained galactosamine in a molar ratio of 0.62 in contrast to *C. genitalium* 392-1 (biovar I), which contained a smaller molar ratio of 0.27, whereas no galactosamine was detected in either *C. genitalium* 321D (biovar IV) or *C. genitalium*

148A (biovar C-1).

Mycolic acids. On analysis of *C. xerosis* and the three strains of *C. genitalium*, corynomycolates were detected in all the strains. A characteristic band which is typical of corynomycolates was observed on thin-layer chromatography plates of all four strains. Each of the bands was eluted and examined by gas-liquid chromatography. The fatty acid ester and aldehyde pyrolysis fragments of the corynomycolates were different in *C. xerosis* and the strains of *C. genitalium*. *C. xerosis* corynomycolates gave rise to saturated fatty acid and aldehyde fragments, whereas the strains of *C. genitalium* 392-1 (biovar I) and 321D (biovar IV) yielded mono-unsaturated fragments. In addition, traces of as-yet-unidentified pyrolysis fragments were found in the three strains of *C. genitalium*.

DISCUSSION

The coryneform group of bacteria together with genera of the *Actinomycetales* including *Actinomyces*, *Bacterionema*, *Bifidobacterium*, *Mycobacterium*, *Nocardia*, and *Rothia* present a number of unresolved problems in taxonomy and classification. Members of the various genera cannot be differentiated from the corynebacteria on the basis of cellular morphology alone since, at some stage in their growth cycle on artificial media, they all produce "straight to slightly curved rods with irregularly staining segments and sometimes granules" (19). Certain other morphological and physiological characteristics do contribute towards separating the corynebacteria from the other similar organisms, such as acid fastness, the presence of branching forms and filamentous or myelium-like growth, and the requirement for anaerobic conditions for growth. However, the mycobacteria and nocardia are strict aerobes which is characteristic of the pathogenic *C. genitalium* when grown on Tween 80 agar, and even though strains of *C. genitalium* are not acid fast this property is not a conclusive criterion for their identification since some strains of mycobacteria and all of the nocardia are at best weakly acid fast.

In the most recent edition of *Bergey's Manual* (19), distinctive cell wall components are considered essential taxonomic criteria for a species to be a member of the genus *Corynebacterium* and specifically of the human corynebacteria. Previous studies of the cell walls of the parasitic and pathogenic corynebacteria of human origin have detected the presence of an arabinogalactan polymer and the amino acids alanine, glutamic acid, and diaminopimelic acid (1, 19). Organisms having these major cell wall components include the bacterionema, corynebacteria, mycobacteria, and nocardiae (19).

TABLE 2. Amino acid and amino sugar composition of the mycolic acid-arabinogalactan-peptidoglycan complex in the cell walls of *Corynebacterium genitalium* and *Corynebacterium xerosis*

Amino acids and amino sugars	Molar ratios ^a			
	<i>C. xerosis</i> ATCC 373	<i>C. genitalium</i> 392-1 (biovar I)	<i>C. genitalium</i> 321D (biovar IV)	<i>C. genitalium</i> 148A (biovar C-1)
Glutamic acid	1.00	1.00	1.00	1.00
Alanine	1.21	1.29	1.24	1.29
Diaminopimelic acid	0.92	0.96	0.78	0.76
Muramic acid ^b	1.27	0.92	0.94	1.00
Glucosamine ^b	0.86	0.60	0.77	0.71
Galactosamine ^b	0.62	0.27	ND ^c	ND
Aspartic acid	0.10	0.10	0.13	0.15
Threonine	0.06	0.07	0.08	0.09
Serine	0.07	0.07	0.09	0.08
Proline	0.06	0.06	0.09	0.09
Glycine	0.08	0.12	0.15	0.14
Valine	0.05	0.05	0.08	0.09
Methionine	ND	ND	ND	ND
Isoleucine	0.07	0.03	0.06	0.06
Leucine	0.09	0.10	0.10	0.11
Tyrosine	0.02	0.02	0.03	0.03
Phenylalanine	0.03	0.03	0.07	0.03
Lysine	0.08	0.08	0.07	0.05
Histidine	0.02	0.02	0.02	0.03
Arginine	0.06	0.06	0.04	0.05

^a Amino acid and amino sugar composition was based on a calibration mixture, containing 10 nmol of each component. Molar ratios were then calculated with respect to glutamic acid.

^b Hydrolysis performed in 4 N HCl at 100°C for 8 h.

^c ND, Not detected.

On the basis of cell wall analysis, the three strains of *C. genitalium* examined could be assigned to one of these three genera. However, the results of the neutral sugar analysis of the cell wall of the three strains of *C. genitalium* showed the presence of the characteristic arabinogalactan in which the pentose "arabinose" and the hexose "galactose" were detected in equimolar ratios when compared to the reference strain *C. xerosis*, a recognized member of the genus *Corynebacterium* (19). This confirmed that they are *Corynebacterium* species. This analysis also showed that *C. genitalium* contained a larger amount of an additional component mannose, whereas a larger proportion of glucose was found in *C. xerosis*. Differences in these two sugars can be indicative of different species (19).

The amino acid and amino sugar compositions of the cell wall preparations of the three strains of *C. genitalium* also supported the evidence derived from the sugar analysis, namely, that the strains belonged to one of the genera of the *Corynebacterium* - *Mycobacterium* - *Nocardia* group. Of the major components the molar ratios of glutamic acid, alanine, diaminopimelic acid, muramic acid, and glucosamine were similar in all strains of *C. genitalium* and also in *C. xerosis*. The lack of galactosamine in *C. genitalium* biovar IV and biovar C-1 suggests that *C. genitalium* might be composed of more than one species.

The sensitivities of the amino acid and amino sugar analyses were at extended levels, enabling the quantitation of minor amino acids in 0.2-nmol quantities per sample. Whether the minor amino acid components are part of the cell wall complex proper, or cytoplasmic remnants, or whether they represent the remains of surface protein layers as exemplified by the M antigens of streptococci, remains to be determined.

The mycolic acid found in corynebacteria is designated corynomycolic acid and contains 32 to 36 carbon atoms, in contrast to those of nocardiae and mycobacteria which contain, respectively, 48 to 58 and 78 to 85 carbon atoms (19). The three strains of *C. genitalium* and the control *C. xerosis* contained mycolates of 32 to 36 carbon atoms which confirmed that they belonged to the genus *Corynebacterium*. Therefore, the previous assignment of *C. genitalium* to the genus *Corynebacterium* on the basis of biological characteristics has been confirmed by the cell wall analysis of their neutral sugars, amino acids, amino sugars, and mycolates. Moreover, some differences in the cell wall constituents suggest that these corynebacteria may consist of more than one species.

ACKNOWLEDGMENT

This research was supported in part by the Foundation of the College of Medicine and Dentistry of New Jersey.

REPRINT REQUESTS

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