

Quantitative Analysis of *Actinomyces* Cell Walls

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Quantitative data on the amino acid composition of cell walls of five species of *Actinomyces* were obtained by using a Beckman-Spinco amino acid analyzer. The major amino acids in *A. israelii*, *A. naeslundii*, *A. eriksonii*, and *A. bovis* species included alanine, glutamic acid, lysine, aspartic acid, and ornithine, as reported by previous workers, whereas *A. propionicus* contained diaminopimelic acid. Other amino acids, including glycine, valine, leucine, proline, isoleucine, and threonine, were present in at least some of the walls in quantities equal to or slightly less than that of lysine. This raised the question of whether these may represent cross-links in the peptidoglycan or other cell wall structural components or whether the wall preparations contained nonpeptidoglycan material despite the use of electron microscopy as a standard of purity; further work is required to supply the answer. The quantitative data furnish relative molar concentrations of amino acids, which can provide definitive identification of some of the species and differentiation of *Actinomyces* from other members of the *Actinomycetales* and from morphologically similar genera such as *Corynebacterium* and *Propionibacterium*.

Over a decade ago, Cummins and Harris (7) recognized the value of cell wall composition in classifying gram-positive bacteria. When their studies were extended to the *Actinomyces*, they found that wall composition could be used to separate the genus *Actinomyces* from other *Actinomycetales* and, also within the genus, to separate *A. bovis* from *A. israelii* (5, 6, 7, 8). Other investigators (3, 10, 11, 12) studying the cell wall composition of these and other *Actinomyces* species have confirmed the value of cell wall analysis in classification. In contrast, Snyder et al. (15), who studied *Actinomyces* and other oral filamentous bacteria, concluded that cell wall composition was of little value for taxonomic purposes.

Most of the work on *Actinomyces* cell walls has been qualitative, and the relative proportions of the various compounds were judged on the basis of the size of chromatogram spots. Recently, Pine and Boone (12) reported quantitative data on *Actinomyces* walls based on the elution and quantitation of the spots obtained on paper chromatograms.

In our investigation, an amino acid analyzer was used to quantitate the amino acids in the cell walls of five *Actinomyces* species. It was hoped that the quantitative data could be used to differentiate these species on the basis of cell wall amino acids.

MATERIALS AND METHODS

Cultures. The 11 strains of *Actinomyces* sp. used in this study are listed in Table 1.

Growth and harvesting of cultures. The cultures were grown in 3-liter quantities of Actinomyces Broth (BBL). Each flask was flushed with a 95% N₂-5% CO₂ gas mixture (Puritan Compressed Gas Corp., Kansas City, Mo.), filtered through a sterile, plugged, capillary pipette, closed with a rubber stopper, and incubated at 37 C for 7 to 10 days. Formalin (0.5%) was then added to each flask and the cultures were held at room temperature for 24 hr. Before harvesting, the purity of the culture was checked with Gram stain and the fluorescent-antibody technique using specific antiserum (14).

Cells were harvested in a Sharples centrifuge, suspended and washed three times in formalinized saline, and packed by the use of an RC-2 Sorvall centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). All cultures were again checked for purity using the fluorescent-antibody technique.

Cell breakage. A dilute suspension of the cells was prepared in formalinized saline and passed twice through the Sorvall Ribi Cell Fractionator, model RF-1, first at 10,000 psi to break up the clumps of cells and then at 35,000 to 40,000 psi to break the cells. The suspension was examined with the phase-contrast microscope; if fewer than 50% of the cells appeared broken, the process was repeated at the higher pressures until breakage appeared adequate. The final suspension was centrifuged at 16,300 × g for 20 to 30 min. The sediment was washed three

TABLE 1. Numbers, names, and sources of strains of *Actinomyces*

WVU no. ^a	Name	Source	Other numbers or designations	Isolated from
30	<i>A. israelii</i>	ATCC 10048 ^b	Emmons 1829	Pleural fluid
46	<i>A. israelii</i>	ATCC 12102	Howell 277	Brain abscess
390	<i>A. israelii</i>	Meyer, Chicago	Bradley	Pulmonary
45	<i>A. naeslundii</i>	ATCC 12104	Howell 279	Human sinus
158	<i>A. naeslundii</i>	Howell, NIH	286	
398A	<i>A. naeslundii</i>	Gerencser, WVU		Dental calculus
116	<i>A. bovis</i>	ATCC 13683	Pine P1S	Bovine lumpy jaw
351S	<i>A. bovis</i>	Slack, WVU		Bovine lumpy jaw
391	<i>A. bovis</i>	Meyer, Chicago	P114	
387	<i>A. eriksonii</i>	Georg, CDC X573	ATCC 15423	Lung abscess
471	<i>A. propionicus</i>	ATCC 14157	Pine 699	Lacrimal canaliculitis

^a West Virginia University.

^b American Type Culture Collection.

times in formalinized saline and the supernatant fluid was discarded.

Enzyme digestion and cell wall hydrolysis. The crude cell wall fractions were treated with trypsin and pepsin (three times crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio) by the procedure of Cummins and Harris (7).

After enzyme digestion, the cell walls were washed at least three times in saline and packed by centrifugation. These walls were then resuspended in saline by shaking and centrifuged at 1,000 × *g* to remove unbroken cells. To determine purity of the walls, the supernatant fluid containing the cell walls was negatively stained with phosphotungstic acid and examined in the electron microscope (model JEM-T6S). Pure wall preparations were lyophilized and stored at 4 C.

Cell walls were hydrolyzed in acid by a modification of the procedure of Cummins and Harris (7). A 10-mg amount of a lyophilized sample and 1 ml of 6 N HCl were placed in a 10-ml Florence flask. The sample was refluxed for 16 to 18 hr and the product was filtered through Whatman no. 2 filter paper. Each filtrate was evaporated under vacuum, redissolved in 1 ml of distilled water, and reevaporated three times. The final product was dissolved in 1 ml of a sodium citrate buffer (pH 2.2) and frozen until used.

Amino acid analysis. The amounts of amino acids and amino sugars in the hydrolyzed cell walls were determined in the Beckman-Spinco model 120C amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.). The procedure, including the reagents and buffers, was that given in the instruction manual for the amino acid analyzer. It was found that 0.1 ml of a hydrolysate containing 1 mg of cell walls/ml gave peaks of the desired height for calculation. Duplicates were run on both columns for each sample.

RESULTS

The results of amino acid analyses of walls of each of the strains are given in Tables 2-5, and a comparison of the 5 species studied is shown in Table 6. When more than one strain of a species was studied, the amount of an amino acid varied considerably from strain to strain; however, the

TABLE 2. Quantitative analysis of *A. israelii* cell walls

Amino acids and amino sugars	WVU 30	WVU 46	WVU 390
	μmole/mg	μmole/mg	μmole/mg
Alanine.....	0.407	0.337	0.297
Glutamic acid.....	0.382	0.327	0.263
Lysine.....	0.114	0.116	0.113
Diaminopimelic acid.....	0.000	0.000	0.000
Aspartic acid.....	0.091	0.106	0.117
Ornithine.....	0.125	0.115	0.112
Glycine.....	0.105	0.111	0.110
Valine.....	0.084	0.078	0.087
Leucine.....	0.090	0.096	0.103
Threonine.....	0.063	0.072	0.080
Serine.....	0.053	0.062	0.057
Proline.....	0.070	0.060	0.060
Arginine.....	0.051	0.073	0.079
Isoleucine.....	0.035	0.040	0.049
Phenylalanine.....	0.027	0.028	0.030
Histidine.....	0.011	0.017	0.018
Methionine.....	0.015	0.013	0.013
Muramic acid.....	0.072	0.064	0.031
Glucosamine.....	Present	Present	Present
Galactosamine ^a			
Ammonia.....	0.220	0.345	0.358

^a Unable to identify or calculate, if present.

mole ratios of most of the amino acids remained quite constant within a species. For example, *A. israelii* strain 30 contained 0.407 μmole of alanine/mg and 0.105 μmole of glycine/mg, whereas strain 390 contained 0.297 μmole of alanine/mg and 0.110 μmole of glycine/mg. However, in both cases, the relative molar concentration of alanine to glycine was approximately four to one.

The results obtained with *A. israelii* (Table 2) and *A. naeslundii* (Table 3) were very similar. Alanine and glutamic acid were present in approximately equal amounts and in three to four times

TABLE 3. Quantitative analysis of *A. naeslundii* cell walls

Amino acids and amino sugars	WVU 45	WVU 398A	WVU 158
	$\mu\text{mole/mg}$	$\mu\text{mole/mg}$	$\mu\text{mole/mg}$
Alanine.....	0.420	0.201	0.246
Glutamic acid.....	0.403	0.187	0.249
Lysine.....	0.083	0.056	0.069
Diaminopimelic acid.....	0.000	0.000	0.000
Aspartic acid.....	0.150	0.056	0.074
Ornithine.....	0.132	0.084	0.069
Glycine.....	0.134	0.060	0.074
Valine.....	0.057	0.043	0.065
Leucine.....	0.135	0.051	0.070
Threonine.....	0.100	0.040	0.053
Serine.....	0.078	0.041	0.042
Proline.....	0.082	0.036	0.048
Arginine.....	0.058	0.012	0.023
Isoleucine.....	0.068	0.021	0.034
Phenylalanine.....	0.041	0.015	0.022
Histidine ^a	0.017		0.004
Methionine.....	0.006	0.001	0.001
Muramic acid.....	0.080	0.055	0.033
Glucosamine.....	Present	Present	Present
Galactosamine ^b			
Ammonia.....	0.315	0.247	0.262

^a Either not present in WVU 398A or in too small a quantity to calculate.

^b Unable to identify or calculate, if present.

TABLE 4. Quantitative analysis of *A. bovis* cell walls

Amino acids and amino sugars	WVU 116	WVU 351S	WVU 391
	$\mu\text{mole/mg}$	$\mu\text{mole/mg}$	$\mu\text{mole/mg}$
Alanine.....	0.395	0.236	0.362
Glutamic acid.....	0.218	0.127	0.322
Lysine.....	0.276	0.151	0.201
Diaminopimelic acid.....	0.000	0.000	0.000
Aspartic acid.....	0.177	0.092	0.181
Ornithine.....	0.000	0.000	0.000
Glycine.....	0.078	0.037	0.143
Valine.....	0.051	0.026	0.136
Leucine.....	0.074	0.036	0.118
Threonine.....	0.052	0.023	0.104
Serine.....	0.044	0.023	0.081
Proline.....	0.046	0.019	0.072
Arginine.....	0.034	0.028	0.055
Isoleucine.....	0.032	0.007	0.093
Phenylalanine.....	0.021	0.010	0.062
Histidine ^a	0.012		0.026
Methionine.....	0.007	0.002	0.021
Muramic acid.....	0.073	0.050	0.015
Glucosamine.....	Present	Present	Present
Galactosamine ^b			
Ammonia.....	0.410	0.256	0.291

^a Either not present in WVU 351S or in too small a quantity to calculate.

^b Unable to identify or calculate, if present.

TABLE 5. Quantitative analysis of *A. eriksonii* and *A. propionicus* cell walls

Amino acids and amino sugars	<i>A. eriksonii</i>	<i>A. propionicus</i>
	$\mu\text{mole/mg}$	$\mu\text{mole/mg}$
Alanine.....	0.256	0.136
Glutamic acid.....	0.138	0.101
Lysine.....	0.028	0.025
Diaminopimelic acid.....	0.000	0.051
Aspartic acid.....	0.131	0.057
Ornithine.....	0.030	0.000
Glycine.....	0.075	0.130
Valine.....	0.105	0.048
Leucine.....	0.063	0.053
Threonine.....	0.064	0.039
Serine.....	0.044	0.030
Proline.....	0.035	0.025
Arginine.....	0.022	0.044
Isoleucine.....	0.040	0.026
Phenylalanine.....	0.027	0.017
Histidine.....	0.009	0.000
Methionine.....	0.018	0.051
Muramic acid.....	0.031	0.025
Glucosamine.....	Present	Present
Galactosamine ^a		
Ammonia.....	0.259	0.248

^a Unable to identify or calculate, if present.

the quantity of aspartic acid, lysine, glycine, leucine, ornithine, valine, and threonine. Serine and proline were present in slightly smaller quantities than was aspartic acid. Arginine was present in quantities equal to serine and proline in *A. israelii*, but in a smaller quantity in *A. naeslundii* where it showed considerable strain variation. Phenylalanine, histidine, isoleucine, and methionine were also present in all three strains of both species, but in very small amounts.

In *A. bovis* (Table 4), alanine, glutamic acid, aspartic acid, and lysine were again the major amino acids. Glycine, leucine, threonine, and valine were found in approximately equal amounts, but in smaller quantities than were the four major amino acids. Ornithine was not found in *A. bovis* cell walls. The three strains of this species showed more variation than did the strains of *A. israelii* and *A. naeslundii*. Strain 391 differed from the other 2 strains in that it contained larger relative amounts of glutamic acid, valine, and glycine and slightly larger amounts of leucine. In addition, the chromatograms of strains 116 and 351S showed an unidentified peak which eluted between histidine and arginine (250 to 260 min), which was not present in strain 391.

The single strain of *A. eriksonii* studied had alanine in quantities approximately twice those of glutamic acid and aspartic acid. Valine, leucine, and threonine were present in very large amounts approximately equal to that of glutamic acid. This strain had only a very small quantity of lysine and

TABLE 6. Amino acid composition of the cell wall of five *Actinomyces* species

Amino acids and amino sugars	<i>A. israelii</i> ^a		<i>A. naeslundii</i> ^a		<i>A. bovis</i> ^a		<i>A. eriksonii</i>		<i>A. propionicus</i>	
	Amt	Rel. ^b moles	Amt	Rel. moles	Amt	Rel. moles	Amt	Rel. moles	Amt	Rel. moles
	$\mu\text{mole}/\text{mg}$		$\mu\text{mole}/\text{mg}$		$\mu\text{mole}/\text{mg}$		$\mu\text{mole}/\text{mg}$		$\mu\text{mole}/\text{mg}$	
Alanine.....	0.347	4.0	0.289	4.0	0.331	4.0	0.256	4.0	0.136	4.0
Glutamic acid.....	0.324	3.7	0.280	3.9	0.222	2.7	0.138	2.2	0.101	2.9
Lysine.....	0.114	1.3	0.069	1.0	0.209	2.5	0.028	0.4	0.025	0.7
Diaminopimelic acid.....	0.000	0.0	0.000	0.0	0.000	0.0	0.000	0.0	0.051	1.5
Aspartic acid.....	0.105	1.2	0.093	1.4	0.150	1.8	0.131	2.0	0.057	1.7
Ornithine.....	0.117	1.4	0.095	1.3	0.000	0.0	0.030	0.5	0.000	0.0
Glycine.....	0.109	1.3	0.090	1.3	0.085	1.0	0.075	1.2	0.130	3.8
Valine.....	0.083	1.0	0.055	0.8	0.071	0.9	0.105	1.6	0.048	1.4
Leucine.....	0.096	1.1	0.085	1.2	0.076	0.9	0.063	1.0	0.053	1.5
Threonine.....	0.072	0.9	0.064	0.9	0.060	0.7	0.064	1.0	0.039	1.1
Serine.....	0.057	0.7	0.054	0.8	0.049	0.6	0.044	0.7	0.030	0.9
Proline.....	0.063	0.7	0.055	0.8	0.046	0.6	0.035	0.6	0.025	0.7
Arginine.....	0.068	0.8	0.031	0.4	0.039	0.5	0.022	0.3	0.044	1.3
Isoleucine.....	0.041	0.5	0.041	0.6	0.044	0.5	0.040	0.6	0.026	0.8
Phenylalanine.....	0.028	0.3	0.022	0.3	0.031	0.4	0.027	0.4	0.017	0.5
Histidine.....	0.015	0.2	0.007	0.1	0.013	0.2	0.009	0.1	0.000	0.0
Methionine.....	0.014	0.2	0.003	0.04	0.010	0.1	0.018	0.3	0.000	0.0

^a Average of three strains, as shown in Tables 2, 3, and 4.

^b Molar concentration relative to alanine with a value of four.

an approximately equal amount of ornithine. Serine, proline, and isoleucine were present in quantities equal to or slightly greater than that of lysine.

The single strain of *A. propionicus* studied contained glycine in about as large a quantity as that of alanine. The next most abundant amino acid found was glutamic acid, followed by aspartic acid, leucine, valine, diaminopimelic acid (DAP), threonine, and arginine. Serine, proline, and lysine were present in smaller quantities. No ornithine was found in this strain.

The only amino sugar which could be quantitated was muramic acid, which was found in all strains studied. The glucosamine peaks could not be calculated since they formed a broad peak which overlapped that of methionine; however, glucosamine was present in all cell walls. Known samples of galactosamine eluted a few minutes after the acidic and neutral amino acids on the basic column and could not be identified with certainty. Therefore, it was not possible to identify this amino sugar in the cell walls studied. In addition to the amino acids and amino sugars, ammonia was present in large amounts in all of the cell walls studied.

DISCUSSION

In this study, a greater number of amino acids was found in larger quantities than had been

previously reported in *Actinomyces* cell walls (3, 5, 6, 8, 9, 11-13), although the major amino acids which included alanine, glutamic acid, lysine, aspartic acid, and ornithine were the same as those found by previous workers.

In addition to the major amino acids indicated above, glycine, valine, leucine, isoleucine, threonine, serine, proline, and sometimes arginine were present in quantities equal to or slightly less than that of lysine. The presence of such a large number of amino acids in the walls of gram-positive bacteria is not expected and requires an explanation. The first factor to be considered is that the walls were contaminated with cytoplasmic material. The wall fractions were treated with pepsin and trypsin to remove cytoplasmic material, and they appeared in electron micrographs to be clean. Also, the relative molar concentrations of these amino acids (except arginine) were consistent within a species. Thus, both of these facts suggest that more than accidental cytoplasmic contamination is involved. Snyder et al. (15) also reported a number of amino acids in *Actinomyces* cell walls in addition to the generally recognized major components. Pine and Boone (12) considered aspartic acid to be a major component in *A. israelii* and *A. naeslundii*, as well as in *A. bovis*, and they considered valine and glycine to be consistent parts of the cell wall mucopeptide. They also found considerable amounts of the combination leucine,

isoleucine, methionine, and phenylalanine. Therefore, it is possible that all or some of these "extra" amino acids represent cross-links in the peptidoglycan, or that they are in some way closely associated with the cell wall structure.

On the other hand, the consistent presence of nonpeptidoglycan amino acids in our wall preparations must be considered. The presence of such material is suggested by the *A. propionicus* results in which lysine occurred in approximately one-half the amount of diaminopimelic acid. Since diaminopimelic acid is a major component, lysine is unlikely to be present in the peptidoglycan. If one nonpeptidoglycan amino acid is present in the *A. propionicus* walls, others may be present in any of the preparations. Further studies are needed of the same walls, including quantitative analysis before and after extraction with solvents such as formamide, which does not affect the basic peptidoglycan structures. However, our results and those of Snyder et al. (15) and Pine and Boone (12) do suggest that some amino acids, in addition to alanine, glutamic acid, lysine, aspartic acid, and ornithine, may play important roles in the cell wall structure of *Actinomyces*.

A. israelii and *A. naeslundii* were indistinguishable on the basis of their cell wall amino acids because the same major amino acids were present in the same relative molar concentrations (Table 6). Alanine, glutamic acid, lysine, and ornithine were the major components, and they were present in ratios of 4:4:1:1. In addition to these, aspartic acid, glycine, valine, and leucine were present in a ratio to alanine of 1:4. Aspartic acid has not been uniformly reported (3, 13, 15) in these species, but it was demonstrated in all of our preparations.

A. bovis could be distinguished from *A. israelii* and *A. naeslundii* by the high ratio of aspartic acid to alanine (2:4) and by its relatively large lysine content. None of the *A. bovis* strains contained ornithine. The relative molar concentrations of the major amino acids in *A. bovis* were alanine, 4; glutamic acid, 3; lysine, 3; and aspartic acid, 2. The cell wall of *A. bovis* is generally distinguished from that of *A. israelii* by the presence of aspartic acid as a major component and by the lack of ornithine. Despite the presence of aspartic acid in *A. israelii* in this study, these criteria could still be used because of the high mole ratio of aspartic acid to alanine.

A. eriksonii walls resemble those of *A. bovis* more closely than they do those of *A. israelii*. They could be distinguished from *A. bovis* by the presence of ornithine and by the small amount of lysine. The relative molar concentrations of the major amino acids in *A. eriksonii* were alanine, 4;

glutamic acid, 2; lysine, 0.5; aspartic acid, 2; and ornithine 0.5. Valine was present in a very large amount with a mole ratio of two. These results are based on the study of only one strain of *A. eriksonii*; thus, strain variation may occur.

A. propionicus differed significantly from the other *Actinomyces* species in that it contained diaminopimelic acid as a major wall component. Since diaminopimelic acid eluted with methionine, the values given for it in Table 5 may include a small amount of methionine. *A. propionicus* was also unique in that it contained glycine in quantities equal to those of alanine and glutamic acid. Again, the results for *A. propionicus* are based on one strain and the high value for glycine might be strain variable. The presence of LL-diaminopimelic acid in major quantities is characteristic for this species and has been reported in the cell wall (3, 4) and from whole cell hydrolysates (9). This cell wall type is characteristic of *Corynebacterium* (7, 11, 13) and *Propionibacterium* (1, 8, 13), but not of other *Actinomyces*, and it is a major reason for recommending the removal of this organism from the genus *Actinomyces*.

The amino acid and amino sugar composition of the cell wall is useful but cannot be used as the sole criterion for species differentiation in the genus *Actinomyces*. By including the sugars in the cell wall analysis, all the species can be separated on the basis of cell wall composition (3, 13). In routine use, most *Actinomyces* cultures can be identified by the use of morphological, biochemical, and serological tests. However, some strains have unusual morphological and biochemical characteristics and fail to react in available antiserum. For example, rough strains of *A. bovis* may fail to react in antiserum to smooth strains, and they may closely resemble *A. israelii* in other characteristics. In such cases, quantitative cell wall analysis provides a tool for definitive identification of the species. Such data are especially helpful for species identification since the relative molar concentration of the amino acids can be used instead of having to rely on the presence or absence of the amino acid.

Perhaps the greatest value of cell wall analysis is in differentiating *Actinomyces* from other members of the *Actinomycetales* (2, 3, 5, 8, 16) and from morphologically similar genera such as *Corynebacterium* and *Propionibacterium*.

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