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Cell Wall Modification Resulting from In Vitro Induction of L-Phase Variants of *Nocardia asteroides*

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The chemical composition of the cell walls of several L-form revertants derived from *Nocardia asteroides* 10905 was determined at different stages of growth. It was observed that each L-form revertant had a cell well that differed from that of the parental strain when grown under identical conditions. In some strains the peptidolipid and mycolic acid components were affected the most, whereas in other strains the fatty acid, sugar, and mycolic acid moieties were altered. Shifts in mycolic acid size were prominent, whereas the basic peptidoglycan structure appeared to be affected the least. Both the method used to induce the L-form of *N. asteroides* 10905 and the length of time these organisms were maintained in the wall-less state affected the degree of cell wall modification during the reversion process. Thus, removal of the cell wall appeared to potentiate and select for mutational alterations within the cell envelope of *N. asteroides*, and these changes resulted in altered cellular and colonial morphology.

In vitro-maintained peritoneal macrophages obtained from mice removed the walls from cells of *Nocardia asteroides* 10905 while at the same time protecting the resulting protoplasts from lysis. These wall-deficient forms persisted and ultimately grew within the macrophages (9). Occasionally the cell wall-deficient variants reverted to nocardial cells that once again possessed a cell wall. Because it was reported for other bacteria that passage of a cell through a cell wall-deficient state could result in phenotypic alterations (22), some of the L-form revertants of *N. asteroides* recovered from macrophages were selected for detailed study. Further, it was shown that protoplasts of *N. asteroides* 10905 could be induced in vitro by growth of the organisms in high concentrations of glycine and D-cycloserine, and L-forms could be isolated from these preparations (10). After several transfers of these in vitro-induced L-forms, occasional revertants were isolated when cultured on brain heart infusion agar. The colonial morphology of many of these revertants differed from that of the parental strain; therefore, some of these in vitro-induced L-form revertants were studied in more detail. It was shown that L-forms of *N. asteroides* 10905 induced by glycine and D-cycloserine could persist for several weeks when injected intraperitoneally into mice. These L-forms stimulated a purulent exudate within the peritoneal cavity of the animals, and usually abcesses were formed on the spleen. In most of the mice only L-forms could be recovered from the splenic lesions, but in one mouse nocardial revertants were isolated from the spleen 4 weeks after infection. The L-form revertants of *N. asteroides* 10905 isolated from this mouse were selected for further study.

Previous studies have established that the cell walls of *N. asteroides* are structurally and chemically complex and that a series of physical and biochemical processes occur within the wall during the growth cycle (4). Modification or alteration of any of these components by either mutation or environmental factors could potentially affect the total chemical, physical, and structural integrity of the wall which may result in alterations in colonial morphology, growth characteristics, cellular morphology, antigenic composition, and pathogenic capabilities.

To study modifications that occurred to the cell envelope as the result of passage through the cell wall-deficient state, several of the L-form revertants described above were studied. In addition, cell wall alterations of these strains during the logarithmic, early stationary, and late stationary phases of growth were determined to correlate specific structural and chemical shifts with variations in colonial morphology, pathogenicity, and taxonomic stability.

MATERIALS AND METHODS

Microorganisms. *N. asteroides* 10905-P was supplied by J. Rozanis, University of Western Ontario,

London, Canada. Stock cultures were maintained in brain heart infusion agar as previously described (4). *N. asteroides* 10905 MΦR was derived from a cell wall-deficient variant isolated from infected mouse peritoneal macrophages incubated on Barile-Yarguchi-Eveland broth containing 15% heat-inactivated fetal calf serum and 0.7% agar (9). *N. asteroides* 10905 R_4Gf was a spontaneous revertant derived from an in vitro-induced L-form of *N. asteroides* 10905 which had been transferred five times in vitro on Barile-Yarguchi-Eveland broth supplemented with 15% fetal calf serum, 0.35 M sucrose, 3% NaCl, and 0.7% agar (10). *N. asteroides* 10905 AniRev was a revertant isolated on BHI agar from the spleen of a mouse injected 4 weeks earlier with in vitro-induced L-forms of *N. asteroides* 10905. Before their injection intraperitoneally into mice, these L-forms had been passed five times in vitro without reversion.

Cell wall preparation. Cells of *N. asteroides* 10905-P, MΦR, R_4G, and AniRev were grown in brain heart infusion broth as described earlier, and their relative growth curves were established (7). Even though the strains differed in their growth in brain heart infusion broth, all strains were in the log phase of growth at 18 h of incubation, and all strains were in the late stationary phase of growth at 168 h of incubation at 37°C. At 18, 55, and 168 h, the cells were killed by adding Formalin to the culture (final concentration of 4% [vol/vol] formaldehyde) and allowed to stand overnight at 4°C.

The killed cells were harvested by centrifugation and washed twice in deionized water. Washed cells were resuspended in 50 ml of water by using a Virtis variable-speed blender. Samples (30 ml) of the bacterial suspension (5 to 10 g [wet weight] of cells) were combined with 40 g of 0.1-mm glass beads (Glasperlen) and were broken by using four 30-s bursts in a CO_2-cooled Braun tissue disintegrator. The extent of cell breakage was determined by phase-contrast microscopy. Crude cell walls were removed from whole cells and glass beads by differential centrifugation. The cell walls were washed four times in deionized water, and 100 to 200 mg (wet weight) of cell walls was suspended in 50 ml of trypsin (1 mg/ml in phosphate buffer at pH 8). After trypsin digestion (37°C for 12 h), the cell walls were washed four times and suspended in 50 ml of pepsin (2× crystallized; 1 mg/ml in 0.2 N HCl) and incubated at 37°C for 4 h. The trypsin-pepsin-treated walls were washed four times in deionized water and lyophilized as previously described (4). The cell walls were considered pure only when the following criteria were met: very few or no gram-positive cells were found; homogeneous fragments lacking filled walls were observed under phase contrast microscopy; no ribose was detectable; there was no UV absorption at 260 nm; and only empty walls were observed in negative-stained, thin-sectioned preparations by electron microscopy (4). Further, the cell walls were shown to be free of cytoplasmic proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; only one band stained with Coomassie blue was observed in the cell wall extracts. Similar results were obtained with lipase-treated cell walls of *N. asteroides* GUH-2, indicating that the cell wall peptide is resistant to the action of trypsin and pepsin.

Amino acid analysis. Purified unextracted and extracted cell walls were hydrolyzed in 4 N HCl in evacuated tubes for 18 h at 110°C as previously described. It was shown that 4 N HCl resulted in complete hydrolysis of the cell wall peptide, whereas unlike 6 N HCl, there was less destruction of the amino sugars (4). All samples were analyzed on a Beckman 120C amino acid analyzer, and the amino acids were quantitated by the standard height-times-width method. All samples were analyzed in duplicate, and each experiment was repeated. The amino sugars were corrected for hydrolytic loss as described previously (4).

Fatty acid analysis by gas-liquid chromatography. The fatty acids were quantitated by using either a Beckman GC 65 or a Hewlett-Packard 5880 gas chromatograph by methods described in detail previously (4).

Isolation and analysis of mycolic acids. (i) Alkaline hydrolysis of cell walls. Lyophilized cell walls (5 μg) were weighed and placed in culture tubes capped with Teflon-lined screw caps. To the tubes, 3 ml of toluene-saturated 10% methanolic KOH was added. The tubes were purged with nitrogen or argon, sealed, and heated in an oven for 18 to 24 h at 110°C. The tubes were removed from the oven, allowed to cool, and then acidified with 10 to 30 drops of concentrated HCl to make the final pH <1.0. The hydrolysates were then extracted in the following manner. An equal volume of petroleum ether was added and shaken vigorously for a few seconds. After separation of the solvent layers had occurred, the upper layer was removed and placed in a clean tube. The lower phase was then extracted once more with an equal volume of petroleum ether, and the upper layer was pooled with the first. The lower phase was extracted twice with an equal volume of petroleum ether diethyl ether (1:1). The upper phases were then pooled and concentrated to dryness under nitrogen.

(ii) Methyl-esterification of mycolic and fatty acid residues. The isolated mycolic and fatty acid material was methylated by reacting the samples with 3.0 ml of a solution of freshly prepared HCl-methanol (Applied Science Kit) and toluene (2:1). The samples were purged with nitrogen or argon, sealed, and then heated to 110°C for 16 to 18 h. After the samples were cooled, the solvent was evaporated to dryness under nitrogen.

(iii) Trimethyl silylation of mycolic acid methyl ester derivatives. Before derivatization, an internal standard of C-30 methyl ester fatty acid (50 μg) was added. The samples were then derivatized by adding 100 to 200 μl of SiL-Prep (Applied Science Kit) to the sample tubes, and the tubes were sealed to prevent contact with water vapor. The tubes were agitated in a Vortex mixer, heated for 30 s under hot tap water while agitating gently, and allowed to stand at room temperature for at least 30 min. The pyridine solvent in the Sil-Prep was evaporated to dryness under nitrogen, and 1.0 ml of iso-octane solvent was added (24).

(iv) Gas-liquid chromatography. Analysis of the mycolic acids was carried out by using short packed glass columns at high operating temperatures. The best results were obtained with 3% OV-17 on Gas

Chrom Q 100/120 mesh in an 18-in. (ca. 45-cm) glass column (2-mm inner diameter). The following oven temperature program was used on a Hewlett-Packard 5880 gas chromatograph: 250°C 1-min isothermal hold followed by a 4°C/min linear gradient to 330°C with a 40-min isothermal hold. The inlet and the flame ionization detector temperature was set at 350°C (24).

Analysis of cell wall sugars. Cell walls (5 mg) were weighed into dried, cleaned, and preweighed Pyrex tubes. A solution of toluene and freshly prepared HCl-methanol (1:2) was added to the tubes, purged with nitrogen or argon, and sealed with a Teflon-lined cap. The samples were hydrolyzed for 16 h at 110°C, cooled, and combined with an equal volume of petroleum ether. Two to four drops of water was added to the samples until separation of the solvent phases occurred. The upper phase was removed and used for fatty acid analysis. The methanol phase was extracted with an equal volume of petroleum ether. The remaining methanol layer was neutralized with 50 mg of Ag_2CO_3, and the salt residue was allowed to settle. The supernatant was collected and transferred to clean tubes, the Ag_2CO_3 residue was washed twice, and the washes were pooled with the first supernatant. The samples were concentrated to dryness under nitrogen. The sugar residues were silylated with Sil-Prep reagent by first purging the sample tubes with nitrogen by hand for 30 s to ensure that all traces of solvent and water were removed and then adding 200 μl of reagent. The samples were incubated for 30 min at room temperature, and the pyridine solvent was removed under nitrogen with care so that no water vapor came in contact with the sample. The silyl-sugar derivatives were dissolved in a known volume of iso-octane, transferred to injection vials, and analyzed by gas-liquid chromatography in a 6-ft (ca. 180-cm) glass column packed with 3% SE-30 on Chromosorb WHP 100/120 mesh. The temperature programming consisted of a 1-min isothermal hold at 120°C with a 4°C/min linear program to 200°C followed by a 5-min isothermal hold. The monosaccharide composition was determined by mathematical interpolation from a standard curve which was programmed into the HP 5880 by external standard integration methods. Further, the sugar content of the 10905 L-form revertants was determined at least three times by two different investigators using two different gas chromatographic facilities (Beckman GC-65 and Hewlett Packard 5880). In addition, the total sugar values were determined by the phenol method (4).

RESULTS

Peptidoglycan and cell wall amino acids. The total peptidoglycan amino acid and amino sugar composition of the cell walls of the parent *N. asteroides* 10905 (1095-P) and the L-form revertants are shown in Table 1. From these data it is evident that quantitative shifts occurred in the composition of the cell wall during growth. The non-peptidoglycan amino acids of unextracted cell walls are also presented in Table 1. The percentage of the cell wall composed

TABLE 1. *Amino acid composition of whole cell walls of N. asteroides at different stages of growth*[a]

Amino acid	10905-P			10905 MΦR			10905 R$_4$G			10905 AniRev		
	18 h	55 h	168 h	18 h	55 h	168 h	18 h	55 h	168 h	18 h	55 h	168 h
Peptidoglycan amino acids												
Muramic acid[b]	8.8	4.1	4.6	6.5	5.6	5.7	6.2	5.5	4.0	7.9	9.7	6.4
Glucosamine[b]	6.0	3.5	3.8	3.8	3.8	3.9	3.6	2.6	3.8	3.7	3.2	4.5
Alanine	4.0	6.0	7.6	4.6	7.3	6.9	11.0	8.1	13.0	3.3	6.1	9.9
Glutamic acid	3.0	3.3	2.0	3.0	3.2	2.9	5.5	3.0	5.7	2.1	3.3	4.1
Meso-diaminopimelic acid	2.1	3.1	1.9	2.2	2.1	2.5	3.1	1.7	2.1	1.5	2.5	3.6
Total (μg/100 μg)	23.9	20.0	19.9	20.1	22.0	21.9	29.4	20.9	28.6	18.5	24.8	28.5
Non-peptidoglycan amino acids												
Aspartic acid	0.5	0.6	0.3	0.3	0.5	0.7	0.6	0.4	0.8	0.9	1.3	0.8
Threonine	0.9	2.2	4.5	1.3	4.6	4.8	0.9	0.8	1.6	1.7	1.9	2.1
Serine	0.6	1.7	2.4	1.1	2.6	2.5	1.2	1.0	2.9	1.9	3.3	3.9
Glycine	0.6	1.1	1.0	0.6	2.3	1.8	9.8	6.1	17.0	1.4	2.6	4.3
Valine	0.5	1.1	1.9	0.7	1.9	2.3	0.5	0.5	0.8	1.5	2.6	2.7
Isoleucine	0.3	0.9	0.5	0.2	0.6	0.4	0.4	0.4	0.5	0.7	1.2	1.0
Leucine	0.7	3.4	2.3	1.5	2.4	2.5	4.9	4.7	7.1	1.5	2.8	2.9
Phenylalanine	T[c]	0.3	0.5	0.4	1.4	0.5	1.5	1.2	2.3	0.9	1.5	2.0
Other amino acids	T	0.5	0.5	0.2	T	0.4	T	T	0.5	0.4	0.6	0.4
Total (μg/100 μg)	4.1	11.3	13.9	6.3	16.3	15.9	19.8	15.1	33.5	10.9	17.8	20.1

[a] Results are expressed as percentages of total cell wall weight. The values represent a mean of duplicate determinations. Analyses of amino acids were repeated with identical results.

[b] Corrected for hydrolytic loss.

[c] T, Trace amount detected.

of peptidoglycan and non-peptidoglycan amino acids generally increased with culture age; however, *N. asteroides* 10905 R_4G was an exception in that the total amino acid composition of the cell wall initially decreased before a significant increase (49.3 to 36 to 62%). The peptidoglycan and peptide composition of the cell walls of *N. asteroides* 10905 MΦR most nearly resembled that of the parent strain throughout the growth cycle. In contrast, the cell wall composition of both *N. asteroides* 10905 R_4G and *N. asteroides* 10905 AniRev differed significantly from that of the parental strain depending upon the phase of growth.

Although the peptidoglycan amino acids of the revertants varied during the growth cycle and differed slightly from those of the parental strain, the most pronounced alterations occurred in the non-peptidoglycan amino acids which represented a major constituent of the cell wall peptidolipids. *N. asteroides* 10905 R_4G had significantly increased amounts of glycine, leucine, and alanine as compared with the other isolates. Thus, these three amino acids represented between 19% and 37% of the total cell wall weight depending upon the stage of growth (Table 1). In contrast, the combined glycine, leucine, and alanine composition of *N. asteroides* 10905-P, MΦR, and AniRev never exceeded 10.9, 12, and 17.1%, respectively (Table 1). Further, the data suggested that the increased glycine, leucine, and alanine found in the cell walls of *N. asteroides* 10905 R_4G did not represent altered incorporation of these amino acids into the peptidoglycan structure because the KOH-methanol-extracted wall did not contain abnormally increased amounts of glycine, alanine, and leucine (Table 2).

Sugar composition of the cell wall. In *N. asteroides* carbohydrate is linked to the peptidoglycan and interspersed within the cell wall (4). Arabinose, galactose, mannose, glucose, and inositol were the only sugars identified within the walls of *N. asteroides* 10905 and its L-form revertants (Table 3). In all strains the amount of carbohydrate was greatest in the log phase of growth and decreased in the stationary phase. Arabinose and galactose were the major sugars detected, and the ratios of arabinose to galactose varied slightly during the growth cycle. Cells of *N. asteroides* 10905 R_4G had increased levels of arabinose in the wall, whereas log-phase cells of 10905 AniRev had dramatically increased amounts of galactose and decreased levels of arabinose. Mannose was slightly more prevalent in both 10905-P and 10905 MΦR than in 10905 R_4G and 10905 AniRev. Both glucose and inositol were relatively constant in all strains of *N. asteroides* 10905. The arabinose and galactose composition of the cell walls of *N. asteroides* 10905 AniRev during the log phase of growth was difficult to interpret; however, repeated analysis of duplicate preparations indicated that these values were accurate (Table 3). Further, the phenol method for determining total sugar composition (10905 AniRev at 18 h, 28.4% of the cell wall; at 55 h, 12.1% of the cell wall; and at 168 h, 12.4% of the cell wall) gave results similar to the totals obtained with gas chromatography (Table 3).

Mycolic acid composition. The mycolic acid composition of the cell walls of *N. asteroides* 10905 and its L-form revertants represented between 15 and 21% of the total wall mass. The percentage of mycolic acids within the cell wall increased slightly with culture age in all strains. However, there were major shifts in the size and molecular structure of the individual mycolic acids that were dependent upon both culture age and the specific strain of *N. asteroides* 10905 (Fig. 1). The relative distribution of the mycolic acids is presented in Table 4. Mycolic acid with a total carbon chain length of C_{54} was a major constituent in the cell walls of all revertants of *N. asteroides* 10905, and it represented about 30% of the total mycolates in cells in the log phase. This mycolic acid moiety decreased significantly in cells during the sta-

TABLE 2. *Peptidoglycan composition of KOH-methanol-extracted cell walls in N. asteroides 10905 R_4G at different stages of growth*[a]

Amino acid	μg/100 μg of cell wall			Molar ratios[b]		
	18 h	55 h	168 h	18 h	55 h	168 h
Muramic acid	14.7	8.7	9.4	1.7	2.7	1.2
Glucosamine	7.4	6.4	6.4	1.1	2.4	0.9
Alanine	4.8	3.4	5.9	1.8	3.1	2.2
Glutamic acid	4.4	2.6	4.4	1.0	1.0	1.0
Meso-diaminopimelic acid	4.7	3.6	4.6	1.1	1.5	0.8
Glycine	0	0	0			
Other amino acids	0	0	1.3			

[a] The values represent means of duplicate determinations.
[b] Molar ratios with respect to glutamic acid.

TABLE 3. *Sugar composition of unextracted cell walls of N. asteroides 10905 L-form revertants at different stages of growth*[a]

Sugar	10905 MΦR[b]			10905 R_4G			10905 AniRev		
	18 h	55 h	168 h	18 h	55 h	168	18 h	55 h	168 h
Arabinose	7.5	7.4	7.3	9.5	9.9	7.4	2.5	5.3	6.8
Galactose	8.3	6.3	5.5	6.0	4.9	4.4	18.9	4.7	5.9
Mannose	2.9	2.3	1.5	0.6	0.5	0.8	0.5	1.8	1.2
Glucose	2.3	1.7	1.7	2.0	1.4	1.5	1.4	1.4	1.8
Inositol	0.5	0.6	0.2	0.2	0.2	0.4	0.4	0.5	0.3
Total (μg)[c]	21.5	18.3	16.2	18.3	16.9	14.5	26.2 (±2.7)	12.3 (±1)	14.1 (±2.1)

[a] Results are expressed as percentages of total cell wall weight. The values represent means of duplicate determinations repeated twice. The percentages and standard deviations of arabinose and galactose in cell walls of 10905 AniRev at 18 h are 2.5 ± 0.6 and 18.9 ± 0.7 for arabinose and galactose, respectively.

[b] The sugar composition of *N. asteroides* 10905-P was approximately the same as 10905 MΦR; therefore, only 10905 MΦR is included to compare with R_4G and AniRev.

[c] Micrograms per 100 μg of cell wall. The total sugar content as determined by the phenol method gave similar values.

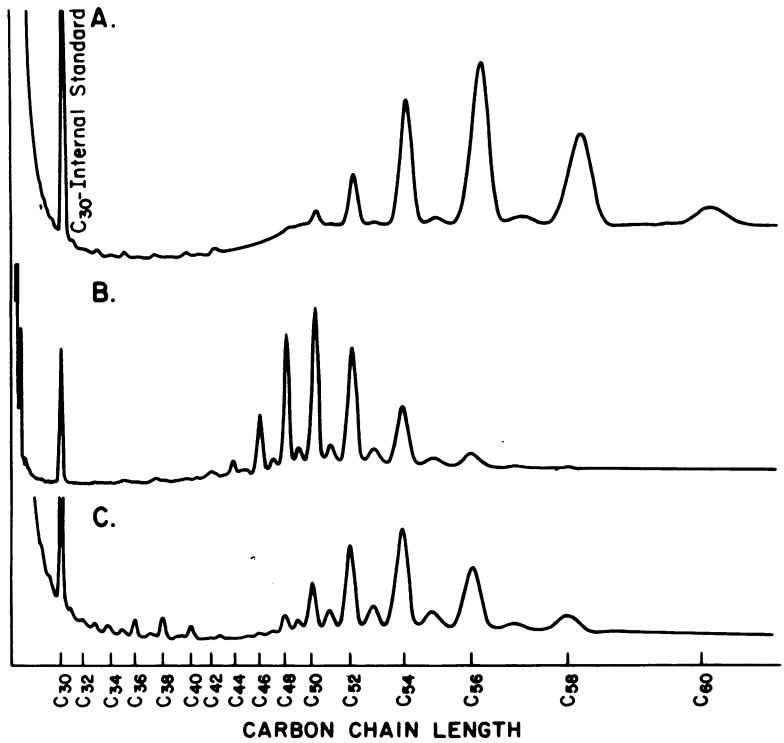


FIG. 1. *Trimethyl-silyl-mycolic acid profiles of the cell walls of N. asteroides 10905 during the stationary phase of growth. A, Mycolic acids of N. asteroides 10905 R_4G. B, Mycolic acids of N. asteroides 10905-AniRev. C, Mycolic acids of N. asteroides 10905-P. The small peaks in the C_{34}-to-C_{40} region represent trace amounts of aldehydes as the result of pyrolysis of nonsilylated mycolic acids. The mycolic acid profiles of 10905 MΦR are essentially the same as those of 10905-P.*

tionary phase of growth (Table 4). The total mycolic acid distribution of *N. asteroides* 10905-P and 10905 MΦR ranged from C_{46} to C_{58} with approximately 75 to 80% of the mycolates composed of C_{52}, C_{54}, and C_{56}. The L-form revertant isolated from the animal (AniRev), on the other hand, had cell walls with a shift in mycolates to a smaller size, and the mycolic acid distribution ranged from C_{44} to C_{58} with approximately 57 to 70% of the mycolates composed of C_{46}, C_{48}, C_{50},

TABLE 4. *Relative distribution of mycolic acids in the cell walls of L-form revertants of N. asteroides 10905 at different stages of growth*[a]

Carbon chain length[b]	10905 MΦR		10905-P	10905 R_4G		10905 AniRev		
	18 h	55 h	55 h	18 h	168 h	18 h	55 h	168 h
C_{44}	0	0	0	0	0	0	0.6	0.5
C_{45}	0	T[c]	T	0	0	0	T	T
C_{46}	T	0.4	0.3	0	0	3.1	6.7	6.7
C_{47}	0	T	T	0	0	0	T	0.1
C_{48}	1.9	5.6	5.4	0	T	11.6	15.1	14.6
C_{49}	T	0.1	0.9	T	T	0	0.7	1.3
C_{50}	7.6	11.0	13.2	2.5	3.7	19.0	23.1	22.1
C_{51}	T	4.7	1.0	0	T	0	1.0	2.0
C_{52}	21.0	20.9	23.1	11.1	5.4	23.2	24.2	23.1
C_{53}	1.9	1.4	0.5	0.3	0.2	0	1.3	2.7
C_{54}	32.5	28.9	30.3	32.6	19.2	32.5	17.9	16.8
C_{55}	5.1	1.9	1.6	1.3	0.6	0	1.3	2.2
C_{56}	23.6	21.5	21.2	37.9	35.4	8.3	6.6	6.2
C_{57}	3.2	1.2	0.5	1.7	1.0	0	0.8	1.0
C_{58}	3.2	1.6	1.8	12.4	27.5	2.3	0.6	0.8
C_{59}	0	0	0	0	0	0	0	0
C_{60}	0	0	0	0	6.7	0	0	0

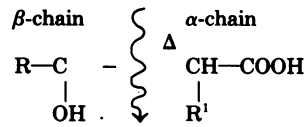
[a] Results are expressed as percentages of total mycolic acids detected. The mycolic acid composition of *N. asteroides* 10905-P is essentially the same as that of 10905 MΦR, and 55-h data are similar to 168-h data; therefore, these data are not shown.

[b] The carbon chain length of the mycolic acids was determined both by comparing fatty acid and aldehyde pyrolysis products with internal standards and by relative retention times on an OV-17 column as defined by Yano et al. (24).

[c] T, Trace amounts detected.

and C_{52} (Table 4). In sharp contrast, the mycolic acids in the cell walls of *N. asteroides* 10905 R_4G were significantly larger than in either the parental strain or the other L-form revertants (Table 4). The mycolic acids of 10905 R_4G ranged in size from C_{50} to C_{60} with approximately 83 to 89% of the mycolates composed of C_{54}, C_{56}, C_{58}, and C_{60} (Table 4).

The mycolic acids are α-branched, β-hydroxylated, long chain fatty acids with the general formula (3, 14, 24):



Upon pyrolysis at temperatures greater than 300°C, methyl esters of mycolic acids yield a fatty acid methyl ester moiety from the α-chain (R^1 fragment) and an aldehyde group from the β-chain (R portion) of the molecule. These products of pyrolysis are characteristic of a specific mycolic acid and they are used to taxonomically distinguish *Corynebacterium* spp., *Mycobacterium* spp. and *Nocardia* spp. (3, 5, 8, 14, 21). The pyrolysis of the mycolic acids of *N. asteroides* 10905 and its L-form revertants clearly distinguished R_4G from AniRev (Table 5). Further, the recognition of an unsaturated fatty acid in the α-chain of mycolic acids from R_4G is unusual since these moieties are normally straight-chained, even-numbered, and saturated fatty acids (3, 8, 20, 24). The fact that these mycolic acids differed significantly from those of the parental strain is important in understanding their effects on the cell wall structure and function as well as in the biosynthesis of mycolates. In addition, mycolic acid diversity emphasizes the importance of L-phase variation to cellular heterogeneity among *N. asteroides* (Tables 4 and 5).

TABLE 5. *Relative distribution of fatty acid methyl esters released during pyrolysis of methyl esters of nocardomycolic acids of N. asteroides 10905 and its L-form revertants*[a]

Carbon chain length of fatty acid	10905-P	10905 MΦR	10905 R_4G	10905 AniRev
C_{12}	6.2	6.4	0	13.7
C_{14}	30.5	31.3	0	40.5
C_{16}	63.3	62.3	38.6	45.8
C_{18}	0	0	48.4	0
$C_{18:1}$	0	0	13.0	0

[a] Results are expressed as percentages of total detected fatty acids derived from mycolic acids in cell walls at the stationary phase of growth.

Fatty acid composition of cell walls. Within the cell walls of *N. asteroides* are a few loosely associated and firmly bound compounds

that contain fatty acid moieties. These include the peptidolipid in the outer portion of the wall, the loosely associated pigment, some glycolipid, and other lipoidal components (1, 4, 6, 23). The complete fatty acid composition of the cell walls of *N. asteroides* 10905 and its L-form revertants was determined by using nonpyrolytic temperatures (less than 250°C) during gas chromatographic analysis of digested and methylated wall fractions (Table 6). The fatty acids within the cell walls (not derived from pyrolytic cleavage of mycolic acids) represented approximately 7 to 8% of the wall in *N. asteroides* 10905-P and 10905 MΦR and from 4 to 7.5% of the wall in both 10905 R_4G and 10905 AniRev. There was little change in the amount of fatty acids in the wall during the growth cycle of these organisms; however, there were dramatic and major shifts in the specific kinds of fatty acids depending entirely upon the stage of growth (Table 6). Further, the fatty acid composition of the cell walls of the L-form revertants, especially 10905 R_4G and 10905 AniRev, differed significantly from that of the parental strain (Table 6).

During the log phase of growth palmitic and oleic (vaccenic) acids were the major fatty acids in all strains of *N. asteroides* 10905. These compounds decreased with culture age with a concomitant relative increase in stearic and tuberculostearic acids (Table 6). Further, there was a general increase in the larger fatty acids as the cultures went from the log phase to the stationary phase of growth. *N. asteroides* 10905 R_4G and 10905 AniRev were very different from *N. asteroides* 10905-P and 10905 MΦR in both the types and the relative amounts of fatty acids that were present (Table 6). *N. asteroides* 10905 AniRev had cell walls rich in unsaturated and branched-chain fatty acids that varied from C_{20} to C_{24}, whereas the parental strain of *N. asteroides* 10905 had few or none of these fatty acids present in detectable quantities (Table 6). In addition, *N. asteroides* 10905 R_4G had significantly less palmitic and oleic (vaccenic) acid during the log phase than did the parental strain, whereas larger amounts of eicosenoic ($C_{20:1}$), arachidic (C_{20}), erucic ($C_{22:1}$), nervonic ($C_{24:1}$), and cerotic (C_{26}) acids were prominent in the cell walls of 10905 R_4G (Table 6).

DISCUSSION

The data presented above show that the chemical composition of the cell walls of *N. asteroides* 10905 were different from that of its L-form revertants. Further, the changes that occurred in the walls of these organisms during their growth cycle were not the same as those observed previously in *N. asteroides* 14759 (4) or in *N. asteroides* GUH-2 (unpublished data). The results indicated that removal of the cell wall with subsequent growth of the wall-deficient forms of *N. asteroides* could result in modification of phenotypic expression once reversion to an organism with a cell wall had occurred. It appeared that both the length of time that the cells were maintained in the wall-less state and

TABLE 6. *Fatty acid methyl esters obtained from unextracted cell walls of N. asteroides 10905 and its L-form revertants at different stages of growth*[a]

Fatty acid	10905-P			10905 MΦR			10905 R_4G			10905 AniRev		
	18 h	55 h	168 h	18 h	55 h	168 h	18 h	55 h	168 h	18 h	55 h	168 h
Lauric acid (C_{12})	0	0	0	0	0	0	0	0	0	1.2	0.8	0.8
Myristic acid (C_{14})	T[b]	2.0	T	1.6	2.2	T	0.3	T	0.4	4.3	5.2	5.1
Palmitoleic acid ($C_{16:1}$)	8.4	2.2	2.1	3.0	3.6	4.1	3.0	3.3	2.8	2.9	4.3	3.1
Palmitic acid (C_{16})	38.7	25.5	14.4	39.5	24.1	16.1	20.8	18.4	16.1	36.0	27.3	19.5
Iso-heptadecanoic acid (iC_{17})	T	0.7	2.7	0.8	0.7	1.5	0.7	T	0.5	1.0	0.4	0.5
Oleic (vaccenic) acid ($C_{18:1}$)	32.0	8.1	5.6	19.3	9.1	4.5	22.2	17.1	13.7	19.1	13.7	6.4
Stearic acid (C_{18})	10.9	35.8	58.9	15.7	34.8	51.1	11.6	7.3	9.6	5.4	3.0	2.1
Tuberculostearic ($10CH_3$-C_{18})	8.7	15.8	11.2	10.8	15.7	17.4	9.5	10.2	12.9	11.3	15.3	11.7
Eicosenoic acid ($C_{20:1}$)	T	0.3	T	0.7	0.5	T	4.5	9.8	8.1	1.9	3.4	2.8
Arachidic acid (C_{20})	T	1.0	1.7	0.4	1.1	T	5.5	5.9	4.0	0.9	0.6	0.5
Docosadienoic ($C_{22:2}$)	0	0	0	0	0	0	0	0	0	0	2.6	11.2
Erucic acid ($C_{22:1}$)	T	0.5	T	0.4	0.5	T	8.5	18.2	15.0	4.9	8.8	9.4
Behenic acid (C_{22})	1.1	0.8	2.2	1.2	0.8	T	1.3	2.5	0.6	1.4	0.5	1.4
Lignoceric acid (C_{24})	0	0	0	0	0	0	0	0	0	0	1.2	3.2
Nervonic acid ($C_{24:1}$)	0	2.1	1.6	1.9	1.7	T	3.3	3.2	3.7	2.3	5.5	3.1
Cerotic acid (C_{26})	0	0.4	T	0.3	0.3	T	2.0	2.0	2.0	0.6	1.7	3.4
Unknown (>C_{19})[c]	T	4.8	T	6.0	4.9	5.3	6.8	2.1	10.6	6.8	6.8	15.8

[a] Results are expressed as percentages of fatty acids detected.

[b] T, Trace amount detected.

[c] Most of the unknown peaks appear to be polyunsaturated or branched-chain C_{20}, C_{21}, and C_{22} fatty acids.

the method used initially to remove the wall potentiated cell wall modification during the reversion process. Concomitant with cell wall modification was an alteration in colonial morphology, pigmentation, pathogenicity for mice, and some metabolic capacities. These expressions of modified cellular characteristics appeared to be the result of mutational events since the observed changes were constant over a period of 6 years within our laboratory (4a). Interestingly, all of the L-form revertants had the same sensitivity to the cell wall inhibitors (penicillin G, D-cycloserine, and bacitracin) as did the parent. Similarly, Landman observed that revertant strains of *Salmonella* and *Escherichia* recovered from L-forms did not differ in their antibiotic sensitivities (19).

The structure of the peptidoglycan of *N. asteroides* has been shown to consist of β-*N*-acetylglucosaminyl-1,4-*N*-glycolylmuramic acid with L-alanine-Dα-glutamine-meso-diaminopimelic acid-D-alanine tetrapeptide linked to the carboxyl group of the *N*-glycolylmuramic acid (2). Further, the tetrapeptides are cross-linked by D-alanine to meso-diaminopimelic acid linkages (1, 23). During the log phase of growth the cell walls of *N. asteroides* 14759 had approximately twice the amount of muramic acid than glucosamine; however, at the stationary phase, the ratio of muramic acid to glucosamine was about 1:1 (4). The peptidoglycan of *N. asteroides* 14759 represented 25% of the cell wall mass during the log phase, and it increased to about 43% of the wall mass at the stationary phase of growth (4). During the early stages of growth, *N. asteroides* 10905 and its L-form revertants had more muramic acid than glucosamine; as observed in *N. asteroides* 14759, the ratios of these compounds approached 1:1 as the cultures reached the stationary phase (4). In contrast to *N. asteroides* 14759, the total peptidoglycan portion of the cell walls of the strains of *N. asteroides* 10905 increased only slightly with culture age, and it never exceeded 29% of the wall.

The molar ratios of the components (muramic acid-glucosamine-diaminopimelic acid-glutamic acid-alanine) in the normal peptidoglycan structure of *N. asteroides* should approach 1:1:1:1:2 (1, 4, 23). The peptidoglycan of the cell walls of the stationary phase (168 h) of *N. asteroides* 10905-P, AniRev, and R_4G have molar ratios that are approximately 1:1:1:1:2 (Table 2). However, at other stages of growth the molar ratios of the amino acids and amino sugars of 10905 R_4G vary significantly, with the greatest alteration observed at the early stationary phase (55 h). There is an increased amount of glucosamine and muramic acid in the walls which may represent an amino sugar backbone that lacks the normal peptide side chains (Table 2).

Amino acid analysis of the cell walls of *N. asteroides* 10905 and its L-form revertants demonstrated that all of these organisms had the same amino acids within the wall; however, *N. asteroides* 10905 R_4G and AniRev contained significantly more glycine and alanine in their walls than did the other organisms. In stationary-phase cells of 10905 R_4G, about 37% of the cell wall was composed of alanine, glycine, and leucine, whereas in contrast these three amino acids represented only about 10% of the cell walls of the parental strain. It is tempting to speculate that this change in the cell wall amino acids resulted from the method used for L-form induction. Both 10905 R_4G and AniRev were derived from L-forms that were induced in vitro by serially passing *N. asteroides* 10905 (parent) in medium containing glycine and D-cycloserine (10). Both of these substances are known to affect the incorporation of D-alanine into the peptidoglycan (12, 15). Further, D-cycloserine was shown to inhibit the synthesis of the wax D peptidoglycolipid of *Mycobacterium tuberculosis* (13). A wax D-like compound containing D-alanine has been isolated from the cell walls of *Nocardia brasiliensis* (21); therefore, D-cycloserine may also affect the synthesis of either this or related compounds within the nocardial cell wall.

Based on the known effects of glycine and D-cycloserine on cell wall synthesis, it seems likely that serial passage of *N. asteroides* 10905 in a medium containing these two compounds would select for organisms with altered mechanisms controlling the incorporation of both alanine and glycine into the various structural components of these cells. Analysis of alkaline methanol-extracted cell walls of 10905 R_4G and 10905 AniRev indicated that the majority of the glycine and alanine was in the peptides associated with the lipoidal material in the outer layer of the wall. These peptidolipids (and possibly proteins), in addition to the peptidoglycan, might contribute to the cellular morphology of *N. asteroides*. Similar functions have been suggested for proteins in the cell envelope of some gram-negative bacilli (16, 17). Thus, in *Nocardia* spp., the association between cell wall peptides and peptidoglycan may be important in determining cell shape. In *N. asteroides* 10905 R_4G the substitution of glycine for alanine in these wall-associated peptides may have significantly altered their association with the peptidoglycan and, as a result, affected the shape-determining ability of the cell wall as well as the overall interrelationships of the numerous components that make up the cell envelope.

In *N. asteroides* it has been shown that an

arabinogalactan consisting of a 1-5 linked arabinofuranosyl, 1-4 linked galactopyranosyl, and some 1-2 linked arabinofuranosyl is attached to a mycolic acid moiety through the arabinose and not the galactose portion of the complex (1, 14, 23). This arabinogalactan-mycolate is covalently bound to the muramic acid portion of the peptidoglycan forming a large, complex polymer (3).

In addition to the murein-arabinogalactan-mycolates there are complexes of arabinogalactomannans, glucose-containing polymers (18), mannose, and inositol within the wall (11). Most of these sugar moieties are linked to either fatty acids or mycolic acids (3, 14, 18). The specific ratios and the quantitative amounts of these compounds probably reflect both their function and location within the cell wall as the organisms develop through their growth cycle. There is an overall decrease in the amount of these sugars as the cultures go from the log phase to the stationary phase of growth. Specific changes within the walls of each strain are difficult to interpret because these changes probably reflect the different types of mycolic and fatty acids that predominate at each state of growth within each L-form revertant.

Both the mycolic acid and fatty acid composition of 10905 R_4G and 10905 AniRev cell walls were different from either the parental strain or 10905 MΦR. In all of the isolates these components represented between 20 and 30% of the cell wall; however, there was an increase in relative size and unsaturation of mycolic acids in 10905 R_4G and a decrease in the relative size of the mycolic acids of 10905 AniRev. Also in 10905 AniRev there was an increase in unsaturated and branched-chain fatty acids with a carbon chain length greater than C_{20}. The parental strain of *N. asteroides* 10905 was intermediate between 10905 R_4G and 10905 AniRev with respect to both mycolic acid and fatty acid composition. However, it should be noted that 10905 R_4G differed significantly from 10905 AniRev. These changes in mycolic acid structure and the increased amounts of unsaturated long-chained fatty acids should alter the charge and lipophilic nature of the surface of the bacterial cell. Such alterations would affect the organism's hydrophobicity as well as the cell-to-cell interaction within the culture. These alterations in cell interactions would result in a modification of the colonial morphology when the organisms are grown on agar media. Further, these kinds of alterations in the cell envelope would probably affect the overall interaction of the various components that make up the complex structure of the wall which in turn would be expected to alter uptake of nutrients. Therefore, 10905 R_4G and 10905 AniRev were different from the parental strain of *N. asteroides* 10905 with respect to their colonial and cellular morphology, in some of their metabolic capacities, and in their relative pathogenicity for mice (4a). These observations have basic implications in understanding cell wall biosynthesis of *N. asteroides* and the relationship of the cell envelope to the growth characteristics of the organism.

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