JOURNAL OF BACTERIOLOGY, Feb. 1984, p. 688–689
0021-9193/84/020688-02$02.00/0

Vol. 157, No. 2

Composition of the Peptidoglycan of Alkalophilic *Bacillus* spp.

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Received 15 July 1983/Accepted 1 November 1983

Peptidoglycans of 10 alkalophilic *Bacillus* strains were isolated as trichloroacetic acid-insoluble materials from cell walls prepared by treatment with sodium dodecyl sulfate, disruption with a sonic oscillator, and trypsin digestion. Major constituents detected commonly in hydrolysates of the peptidoglycans were glucosamine, muramic acid, D- and L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, and acetic acid. Ammonia derived from amide was found in a portion of the hydrolysates. The composition of peptidoglycan was not changed whether the strain was cultured at pH 7 or 10. All the peptidoglycan examined was of the A1γ type of peptidoglycan found in most strains of the genus *Bacillus*.

Certain microorganisms can grow in alkaline media inimical to most microbes. The intracellular pH of these alkalophilic bacteria is neutral (6). Therefore, only cell walls and membranes should make contact with an alkaline environment. Previously, we examined the chemical compositions of cell walls from a number of alkalophilic *Bacillus* strains and divided them into three groups on the basis of cell wall composition (2). Although their peptidoglycans appeared to be similar in composition to that of *Bacillus subtilis*, their composition was obscured by the excess of hexosamines and amino acids in the cell walls. This paper presents data concerning the composition of peptidoglycans of the alkalophilic *Bacillus* strains.

All strains used in this study (see Table 1) have been described previously (2). *B. subtilis* GSY1026 was used as a reference strain. Cell wall was prepared as described previously (2) and suspended in 5% trichloroacetic acid (TCA). The mixture was incubated at 60°C overnight and centrifuged at 7,000 × *g* for 30 min. This extraction was repeated for the precipitate until neutral sugars or uronic acids disappeared in the supernatant.

Amino acids and amino sugars released by hydrolysis (4 N HCl, 105°C, 15 h) were determined with an automatic amino acid analyzer after removal of HCl in vacuo over NaOH. L-Alanine and L-glutamic acid in the hydrolysates were determined by means of their respective dehydrogenases (3, 8). D-Alanine and D-glutamic acid contents were calculated by subtracting the enzymatically determined value from the total content obtained with the amino acid analyzer. The amide bond was cleaved with hydrolysis (2 N HCl, 100°C, 3 h). Ammonia was determined with the amino acid analyzer after neutralization of HCl with 4 N NaOH. The peptidoglycans were hydrolyzed in 2 N HCl in sealed tubes at 100°C for 5 h. HCl was neutralized with 4 N NaOH. Acetic acid released in the hydrolysates was determined by using a Boehringer acetic acid assay kit. Determination was uncorrected for destruction during acid hydrolysis or loss during the several treatments.

Peptidoglycans prepared as TCA-insoluble fractions were obtained in a 20 to 50% yield of the cell walls (dry weight). A small amount of diaminopimelic acid was found in the TCA-soluble fractions. It can be concluded that some loss of peptidoglycan occurred during the TCA extraction. Neutral sugars and uronic acids were almost completely extracted with TCA. These compounds remaining in the TCA-insoluble fractions were less than 3% of the contents in the whole cell walls.

Isomerism of diaminopimelic acid was determined by thin-layer chromatography of hydrolysates (4 N HCl, 105°C, 15 h) before and after denitrophenylation (4, 5). It was concluded that the diaminopimelic acid of alkalophilic *Bacillus* strains was a *meso* isomer. Isomerisms of alanine and glutamic acid were examined enzymatically.

Table 1 shows the molar ratios of the main components of the peptidoglycan. The contents of glucosamine, muramic acid, L-alanine, D-glutamic acid, and *meso*-diaminopimelic acid were almost equal to one another. The ratios of these substances were not affected by the culture pH. The peptide moieties of all the peptidoglycans examined should be composed of L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, and D-alanine. The peptides should be cross-linked directly between *meso*-diaminopimelic acid and D-alanine because of a lack of amino acids in the hydrolysates, which were known to be involved in an interpeptide bridge. It was concluded that all of the peptidoglycans of the alkalophilic *Bacillus* strains so far examined were of the A1γ type of peptidoglycan, which is found in the vast majority of strains of the genus *Bacillus* (7).

The molar ratio of D-alanine to *meso*-diaminopimelic acid varied between 0.37 and 0.86. Therefore, it is suggested that a C-terminal D-alanine of the peptide was split off when the peptide was cross-linked. Tripeptide as well as tetrapeptide should also occur. The frequency of cross-linking is still not known. The ammonia content also differed in different strains.

The molar ratio of ammonia to *meso*-diaminopimelic acid was comparatively high in the peptidoglycans of C-11, M-29, and 57-1 as well as in that of *B. subtilis*; it was low in the other strains. It is known that most of A1γ type of peptidoglycans contain one amidated carboxyl group, either D-glutamic acid in the case of *Bacillus licheniformis* or *meso*-diaminopimelic acid in the case of *B. subtilis* (7). The peptidoglycans of C-11, M-29, and 57-1 seemed to be similar to that of *B. subtilis*. In the other strains, carboxy groups of the peptidoglycans were not amidated. It is known that *Bacillus megaterium*, *Bacillus lentus*, and *Bacillus firmus* have no amide group at all (7).

The molar ratio of acetic acid to glucosamine plus muramic acid was approximately 1:1. All of the peptidoglycan could be digested with lysozyme (data not shown). It is known that lysozyme reaction requires *N*-acetyl substitution and no *O*-acetyl substitution of the glucosamine residues (1). There-

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TABLE 1. Composition of the peptidoglycan of alkalophilic *Bacillus* strains[a]

Strain	Culture pH	*meso*-DAP content (μmol/mg)	Molar ratio to *meso*-DAP						Molar ratio of GlcN + Mur to acetic acid
			GlcN	Mur	D-Ala	D-Glu	L-Ala	NH_3	
A-40-2	10	0.94	0.91	0.98	0.86	0.94	0.64	0.09	0.83
2B-2	10	0.82	0.86	0.99	0.82	1.0	0.37	0.04	0.91
C-11	10	1.1	0.84	0.93	0.81	0.93	0.52	0.43	0.94
C-125	7	0.90	0.83	0.99	0.88	1.0	0.70	0.02	0.72
	10	0.93	0.87	1.0	1.0	1.1	0.54	0.01	0.80
Y-25	7	0.84	0.99	1.1	1.0	1.0	0.60	0.06	0.72
	10	0.93	0.81	0.97	0.95	1.0	0.54	0.02	1.1
A-59	7	0.83	0.91	1.1	1.0	1.1	0.62	0.03	0.84
	10	0.87	0.89	1.1	1.0	1.1	0.64	0.01	0.87
C-3	7	1.1	0.87	0.97	0.86	1.0	0.74	0.05	0.80
	10	0.99	0.96	0.84	0.80	1.1	0.60	0.04	0.74
C-59-2	10	0.94	0.91	0.90	0.75	1.0	0.75	0.08	1.0
M-29	7	0.84	0.91	1.1	1.0	1.0	0.81	0.16	0.88
	10	0.94	0.83	1.0	0.97	1.0	0.86	0.33	0.98
57-1	7	0.81	0.93	1.1	1.0	0.98	0.45	0.31	0.84
	10	0.87	1.1	1.1	0.93	1.1	0.62	0.49	1.2
B. subtilis GSY 1026	7	0.97	0.80	0.86	0.83	0.87	0.59	0.43	0.82

[a] Abbreviations: Ala, alanine; GlcN, glucosamine; Glu, glutamic acid; *meso*-DAP, *meso*-diaminopimelic acid; Mur, muramic acid.

fore, glucosamine and muramic acid should be *N*-acetylated. The glycan moieties of peptidoglycans of the alkalophilic *Bacillus* strains should be the same as that of *B. subtilis*.

In all the strains so far examined, the peptidoglycan was of the A1γ type. The variation was found in the amide content and was similar to the variation which is known in neutrophilic *Bacillus* species. The culture pH did not affect the composition of the peptidoglycan. Therefore, the variations characteristic of the alkalophilic *Bacillus* strains were found in the TCA-extractable materials. Further studies are required for functions and structures of these non-peptidoglycan materials.

We thank M. Chijimatsu for excellent analyses of amino acids and amino sugars by the automatic amino acid analyzer.

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