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Amino Acid Sequence of the Murein of *Planococcus* and Other *Micrococcaceae*

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The amino acid composition and amino acid sequence of the murein (peptidoglycan) of 10 strains of planococci were studied. It is shown that the peptide subunit consists of muramyl-L-alanyl-γ-D-glutamyl-L-lysyl-D-alanine. The cross-linking of two adjacent peptide subunits is mediated by D-glutamic acid which is bound to the ε-amino group of lysine by its γ-carboxyl group and to the carboxyl group of D-alanine of an adjacent peptide subunit by its amino group. About 20 to 25% of the peptide subunits are not cross-linked. The murein structure of the different species and strains of *Micrococcus*, *Staphylococcus*, and *Sarcina* are compared. It is evident that the murein structure is a very good criterion for grouping the micrococci. In addition, some of these groups are fairly well defined by physiological properties as well as by their guanine + cytosine content of the deoxyribonucleic acid e.g., *Micrococcus*, *Staphylococcus*, *Planococcus*, *Sarcina ureae*. Other groups, represented by a single or a few strains only, such as *M. varians* NTCC 7281, *M. radiodurans*, *M. freudenreichii* ATCC 407, and *M. luteus* ATCC 398, need further investigation.

The studies of Bohacek and co-workers (3, 4) have shown that the halophilic, gram-positive, and motile cocci differ from the majority of micrococci and staphylococci by their deoxyribonucleic acid base composition. The typical micrococci and staphylococci have a guanine plus cytosine (GC) content of about 70 or 32% respectively, and the halophilic strains contain 40 to 51%. Bohacek et al. (3, 4) therefore recommended that these strains be separated from the genera *Micrococcus* and *Staphylococcus* and that they be included in the genus *Planococcus* Migula.

Since the cell wall composition, and especially the amino acid sequence of the murein (peptidoglycan), in some cases is a valuable criterion to separate genera or species (6, 13, 15), we studied the cell wall composition of 10 strains of *Planococcus* and compared them to the cell walls of the other micrococci.

MATERIALS AND METHODS

The strains investigated were obtained through M. Kocur from the Czechoslovak Collection of Microorganisms, J. E. Purkyne University, Brno, Czechoslovakia. The strains are listed in Table 1.

The organisms were maintained by monthly transfer on yeast extract-glucose-sodium chloride-agar slants (5 g of yeast extract Cenovis; 10 g of peptone from casein, Merck; 5 g of glucose, 60 g of NaCl, 1 liter of tap water, 15 g of agar; *p*H 7.0 to 7.2). The incubation temperature was 30 C.

Mass cultures for the isolation of cell walls were performed in yeast extract-glucose-sodium chloride-broth under aerobic conditions (shaker) and harvested in the early stationary phase. Before harvesting, the cell suspension was heated to 100 C for 30 min to inactivate autolytic enzymes. Cell walls were prepared in the usual way by disintegrating a cell suspension with glass beads in a cell mill (Bühler, Tübingen). The cell walls were purified by digestion with trypsin. In one case the nonmurein components were removed by extraction with 10% trichloroacetic acid at 4 C for 2 days, followed by an extraction with hot formamide (7). Quantitative amino acid analysis was carried out on cell wall hydrolysates (4 N HCl, 16 hr, 100 C) with a Biocal autoanalyzer. The correction for destruction during hydrolysis was based on the analysis of mixtures of known amounts of authentic substance which were hydrolyzed under the same condition as the cell walls. The following solvent systems were used for the separation of amino acids, amino sugars, and peptides by paper chromatography: (i) isopropanol-acetic acid-water, 75:10:15; (ii) α-picoline-25% NH_4OH-water, 70:2:28.

Dinitrophenol (DNP)-amino acids were identified by thin-layer chromatography on silica gel in the following solvent systems: (i) chloroform-methanol-acetic acid, 95:5:1; (ii) chloroform-methanol-acetic acid-water, 65:25:13:8 (12).

Isolation and identification of the peptides in the partial hydrolysate of the cell walls were performed as described earlier (20). The configuration of glutamic acid was determined by measuring the optical rotatory dispersion of the DNP-derivative (14) or enzymatically (16).

TABLE 1. *List of organisms studied and their DNA base composition*[a]

Species	CCM no.[b]	% GC[c]
Micrococcus aquivivus	316	51.2
M. eucinetus	2389	50.3
M. eucinetus	2388	48.0
M. eucinetus	2387	40.0
Micrococcus sp	2069	42.2
Micrococcus sp	2104	42.4
Micrococcus sp	1849	39.6
Planococcus sp	2414	—[d]
Planococcus sp	2416	—
P. citreus	2415	—

[a] According to Auletta and Kennedy (1), Bohacek et al. (3), and M. Kocur (*personal communication*).

[b] Numbers of the Czechoslovakia Collection of Microorganisms, University J. E. Purkyne, Brno, Czechoslovakia.

[c] Per cent guanine plus cytosine.

[d] DNA base composition not determined.

RESULTS

Amino acid composition and N-terminal amino acids of the murein. The quantitative amino acid and amino sugar composition of cell walls (purified by digestion with trypsin) of the various strains are shown in Table 2. In contrast to most of the other mureins known, the ratio of lysine to glutamic acid is 1:2 instead of 1:1. A similar excess of glutamic acid was recently found in the cell walls of *M. luteus* and *M. freudenreichii* (16). In these organisms either glycine or a 3rd mole of alanine was present in addition to the usual components of the peptide subunit. This is not the case in the murein of planococci.

The glucosamine content of the cell walls varied considerably. The cell walls of *M. aquivivus* CCM 316, *M. eucinetus* CCM 2387, 2388, and 2389, and *P. citreus* CCM 2416 contained more than 2 moles of glucosamine per mole of lysine. The other strains (*Micrococcus* species 2069, 2104, and 1849 and *Planococcus* species 2414 and 2416) showed a lower content of glucosamine (1 to 1.5 mole). They contained galactosamine in addition.

Cell walls of strain CCM 2416 were extracted with 10% trichloroacetic acid and subsequently with hot formamide. By this procedure, galactosamine was completely removed from the cell walls and was found in the polysaccharide fraction (formamide extract). Teichoic acid was not detected.

In all strains, less than 1 mole of ammonia per mole of lysine was found. To check whether this ammonia arose from the destruction of amino sugars or from the hydrolysis of amides (isoglutamine), we hydrolyzed the cell walls with 4 N HCl for 4 hr (9). About 0.2 mole of ammonia was found. This small amount is to be expected from the destruction of amino sugars and is no indication of the presence of amides.

The hydrolysis of dinitrophenylated cell walls yielded DNP-glutamic acid and traces of ε-DNP-lysine as the only DNP-derivatives. The quantity of *N*-terminal amino acids was determined by comparison of the amino acid content of the dinitrophenylated with that of the unreacted cell walls as well as by the photometric determination of the DNP derivatives. In the walls of *P. citreus* CCM 2415, about 10% of the total glutamic acid and only traces of lysine are *N*-terminal. In the walls of strain CCM 2416, about 12% of the

TABLE 2. *Quantitative amino acid and amino sugar composition of cell walls of different planococci*

Species	CCM no.	Cell wall prepn.[a]	Amino acid or amino sugar (μmole/mg of cell wall)						Amino acid or amino sugar (Molar ratio Lys = 1)			
			Lys	Glu	Ala	Mur	$GlcNH_2$	$GalNH_2$	Glu	Ala	Mur	$GlcNH_2$
Micrococcus aquivivus	316	CW-Tryp	0.288	0.614	0.524	0.253	0.859	—	2.13	1.82	0.88	2.98
M. eucinetus	2389	CW-Tryp	0.287	0.620	0.563	0.300	0.860	—	2.16	1.96	1.04	3.00
M. eucinetus	2388	CW-Tryp	0.286	0.616	0.569	0.288	0.884	—	2.15	1.98	1.00	3.09
M. eucinetus	2387	CW-Tryp	0.316	0.706	0.646	0.348	0.908	—	2.24	2.04	1.10	2.87
Micrococcus sp	2069	CW-Tryp	0.298	0.582	0.521	0.226	0.451	+	2.00	1.81	0.76	1.51
Micrococcus sp	2104	CW-Tryp	0.374	0.708	0.613	0.322	0.471	—	1.89	1.64	0.86	1.26
Micrococcus sp	1849	CW-Tryp	0.377	0.755	0.645	0.332	0.581	+	2.00	1.71	0.88	1.54
Planococcus sp	2414	CW-Tryp	0.452	0.881	0.755	0.488	0.594	+	1.95	1.67	1.08	1.31
Planococcus sp	2416	CW-Tryp	0.456	0.903	0.777	0.474	0.566	+	1.98	1.70	1.04	1.24
Planococcus sp	2416	CW-TCA	0.517	1.030	0.882	0.532	0.646	+	1.99	1.70	1.02	1.25
Planococcus sp	2416	CW-FA	0.633	1.369	1.265	0.750	0.716	+	2.06	1.91	1.13	1.08
P. citreus	2415	CW-Tryp	0.366	0.685	0.650	0.333	0.901	—	1.87	1.78	0.91	2.46

[a] CW-Tryp, cell walls purified by digestion with trypsin; CW-TCA, cell walls additionally extracted with trichloroacetic acid; CW-FA, cell walls extracted with formamide.

total gultamic acid and 2% of the lysine (ε-amino group) were found to be *N*-terminal.

To determine the configuration of glutamic acid, the ORD was measured either of the dinitrophenylated glutamic acid isolated from the total hydrolysate of cell walls, or of the DNP-glutamic acid isolated from the hydrolysate of DNP-cell walls. In both cases, only D-glutamic acid was found. This result was confirmed by the fact that glutamic acid isolated from the total hydrolysate of the cell wall is not attacked by L-glutamic acid dehydrogenase (strains CCM 2415 and 2416 investigated).

The enzymatic determination of the configuration of alanine in the total hydrolysate yielded a ratio of L-ala/D-ala of 1:0.8.

Determination of the amino acid sequence of the murein. Cell walls of strains CCM 2104, 2415 and 2416 were hydrolyzed in 4 N HCl for 30 or 120 min at 100 C. The various peptides were isolated from the partial hydrolysates by repeated one-dimensional or two-dimensional paper chromatography. They were analyzed by determining the quantitative amino acid composition and the *N*-terminal amino acid. Figure 1 shows the scheme of a two-dimensional paper chromatogram and the various peptides identified. The occurrence of the peptides Mur-L-Ala (no. 13), Mur-L-Ala-D-Glu (no. 12), L-Ala-D-Glu (no. 10), γ-D-Glu-L-Lys (no. 6) [γ-bond present in this peptide demonstrated previously (16)], and L-Lys-D-Ala (no. 9) indicates that the usual subunit muramyl-L-alanyl-γ-D-glutamyl-L-lysyl-D-alanine is present. In contrast to the subunit of the murein of *Staphylococcus aureus* and other mureins (8), the α-carboxyl group of the glutamic acid is probably not amidated.

It must be assumed that the 2nd mole of D-glutamic acid found in the total hydrolysate contributes to the cross-linkage. This assumption was confirmed by isolating the peptides N^{ϵ}-D-Glu-L-Lys (no. 7), D-Ala-D-Glu (no. 11), and L-Lys-D-Ala-D-Glu (no. 8). To demonstrate which carboxyl group of the glutamic acid is bound to the ε-amino group of lysine, the peptide N^{ϵ}(DNP-D-Glu)-L-Lys was isolated from a partial hydrolysate (4 N HCl, 15 min, 100 C) of dinitrophenylated cell walls and irradiated by ultraviolet light. The photolysis was followed by measuring the increase of absorption at 284 nm and the decrease of absorption at 348 nm (17). The photolysis was completed within 10 min. This indicated that the α-carboxyl group of glutamic acid was free and that the γ-carboxyl group was involved in the peptide bond.

The glycan moiety of the murein was not studied in detail. The sensitivity of the cell walls to lysozyme, together with the absence of DNP-muramic acid and DNP-glucosamine in the hydrolysate of DNP cell walls, indicate that the glycan is linked in the usual way and is *N*-acetylated.

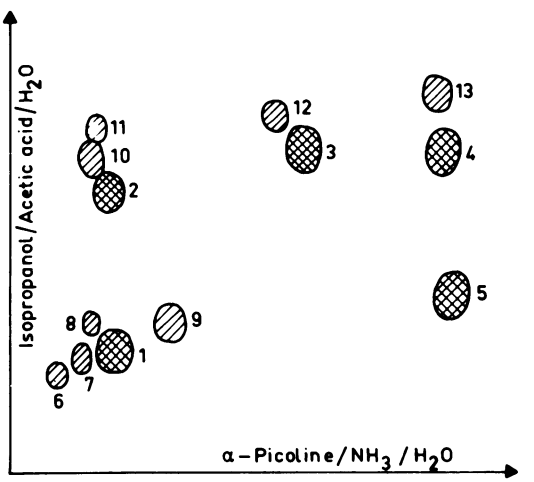

FIG. 1. *Paper chromatogram of an acid partial hydrolysate of the murein of Planococcus. 1, Lysine; 2, glutamic acid; 3, alanine; 4, muramic acid; 5, glucosamine; 6, γ-D-Glu-L-Lys; 7, N^{6}-γ-D-Glu-L-Lys; 8, L-Lys-D-Ala-D-Glu; 9, L-Lys-D-Ala; 10, L-Ala-D-Glu; 11, D-Ala-D-Glu; 12, Mur-L-Ala-D-Glu; 13, Mur-L-Ala. Cross hatch, amino acids or amino sugars; single hatch, peptides.*

The interpretation of the results of the partial hydrolysis is based on the usual assumption that the murein consists of identical subunits. The fragment of the primary structure of the murein of planococci shown in Fig. 2 is the most common sequence in the murein of planococci. The murein as it occurs in the cell walls shows some deviations from this scheme. As mentioned before, 10 to 12.5% of the glutamic acid of the cell wall is *N*-terminal. This means that 20 to 25% of the possible cross-linkages do not occur. In addition, a small percentage of lysine is not substituted by D-glutamic acid since 2% of the lysine can be dinitrophenylated at the ε-amino group. Another deviation from the scheme given in Fig. 2 is the absence of some of the D-alanine. Instead of 2 moles of alanine per mole of lysine, about 1.8 moles was found. The ratio of L-alanine to D-alanine of 1:0.8 indicates that about 20% of the peptide subunits consist of tripeptides instead of tetrapeptides with lysine being C-terminal in these cases. Such tripeptides also exist in other mureins (8, 25). They arise probably by the action of a carboxypeptidase which splits off the C-terminal D-alanine from subunits which are not cross-linked. The molar ratios of the amino acids of the total hydrolysate as well as the peptide pat-

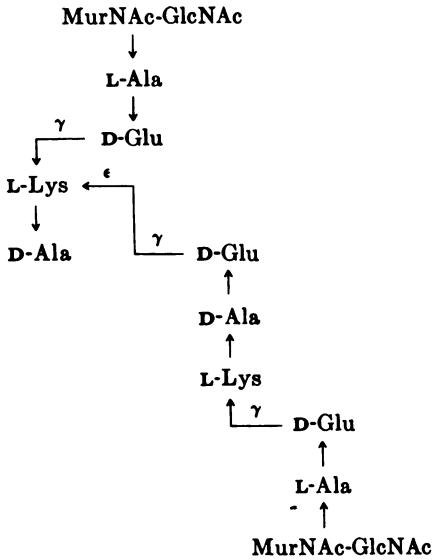

FIG. 2. *Fragment of the primary structure of the murein of planococci.*

tern of the partial hydrolysate of the other seven strains proved to be identical. This shows that all 10 strains investigated belong to the same murein type.

DISCUSSION

The results described here support the proposal of Bohacek et al. (3, 4) to separate the motile, halophilic cocci (*Planococcus*) from the micrococci and staphylococci. The planococci are obviously uniform with respect to their murein type which is clearly different from all the other mureins found within the genera *Micrococcus* and *Staphylococcus*.

The staphylococci are known to contain mureins which are rich in glycine (Fig. 3). Most of the strains usually named *Staphylococcus aureus* contain a murein cross-linked by pentaglycine bridges (11). Only when the medium contains an unusually high level of serine (*unpublished results*), then serine is incorporated in the interpeptide bridge in significant amounts.

The murein of most strains of *S. epidermidis* contains also a pentaglycine, but ⅕ to 1/10 of the glycine is replaced by serine (23, 24).

A few strains contain a murein in which a tetraglycyl-L-alanine peptide mediates the cross-linkage (22).

Two types of murein are predominant among the micrococci. (i) The L-Lys-L-Ala_3-type was found in 25 strains studied in our laboratory (Fig. 4). This murein type was described in detail in *M. roseus* (18) and in *Streptococcus thermophilus* and *S. faecalis* (20). (ii) An unusual murein type was first described in *M. lysodeikticus* (10, 21). The interpeptide bridge of this murein consists of a complete peptide subunit (Fig. 5). According to Ghuysen et al. (10), the interpeptide bridges may contain even more than one (up to four) peptide subunit. Consequently, several adjacent muramic acid molecules are then unsubstituted.

Other murein types are found in a few odd strains only. In *M. luteus* ATCC 398 and *M. freudenreichii* ATCC 407, the cross-linkage is mediated by the peptides γ-L-glutamyl-glycine or γ-L-glutamyl-L-alanine, respectively (Fig. 6; *see* reference 16). The taxonomic position of these species has to be reconsidered. The GC content of the DNA of *M. luteus* (66%) and *M. freudenreichii* (59%) is significantly lower than that of the other micrococci (1). A murein almost identical to one found in *M. luteus* was found in *Sarcina ureae* (ATCC 6473, CCM 1732, 1743, 752, 380, 981, 860), but L-glutamic acid is replaced by D-glutamic acid (*unpublished data*). The interpeptide bridge is fomed by γ-D-glutamyl-glycine.

S. ureae should be transferred to the separate genus (*Sporosarcina*), as pointed out by Bohacek

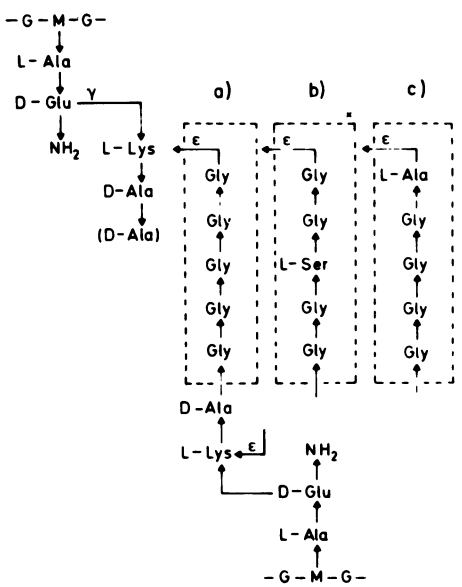

FIG. 3. *Mureins found in the following staphylococci: a, S. aureus ATCC 12600, 14458, 15234, own isolates: 1, 2, 3, 4, 5, 6, 7, 9; S. lactis ATCC 13517, CCM 1400, 2210. b, S. epidermidis ATCC 14990, 155, 12228, own isolates: 24, 26, 44, 45, 50, 51, 71, 83, 92; S. aureus ATCC 151, 8094, 8095 (no orange pigment, coagulase-negative); S. muscae ATCC 12162; S. saprophyticus ATCC 13518, CCM 2204. c, S. aureus ATCC 6601 (no orange pigment, coagulase-negative); Staphylococcus sp., own isolates: 66, 79.*

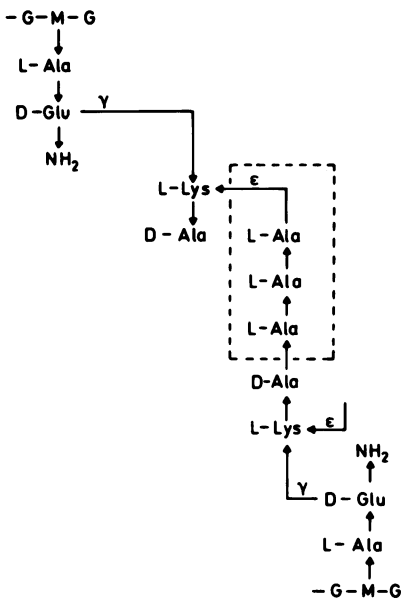

FIG. 4. *Murein of the Lys-Ala$_3$-type found in the following micrococci: M. roseus ATCC 144, 177, 178, 179, 185, 412, 416, 418, 516, 534, 9815; M. varians ATCC 399, 19099, 19100; M. conglomeratus ATCC 401, CCM 825, 884; M. salivarius ATCC 14344; M. pulcher ATCC 15936; Sarcina lutea ATCC 9341, 383, 533; S. aurantiaca ATCC 146; S. erythromyxa 187; Micrococcus sp. CCM 740.*

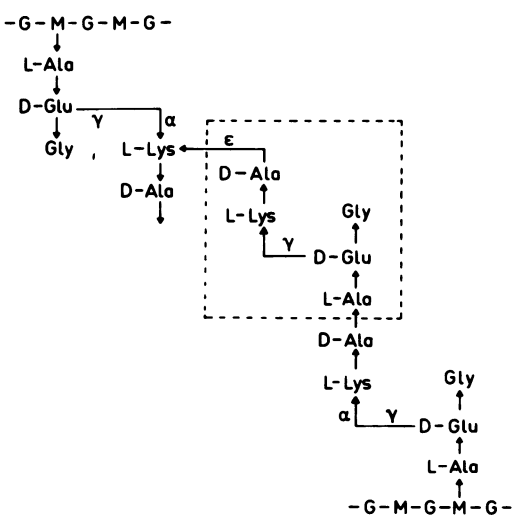

FIG. 5. *The murein found in M. lysodeikticus and the following micrococci: M. flavus ATCC 400, 10240; M. flavoroseus ATCC 397; M. lysodeikticus ATCC 12698; M. sodonensis ATCC 11880; Micrococcus sp. 2, 6; Sarcina flava ATCC 147, 540; S. lutea ATCC 381, 382, 272, 9622, 10054, 10773, 15220; S. pelagio ATCC 14408; S. subflava ATCC 7468.*

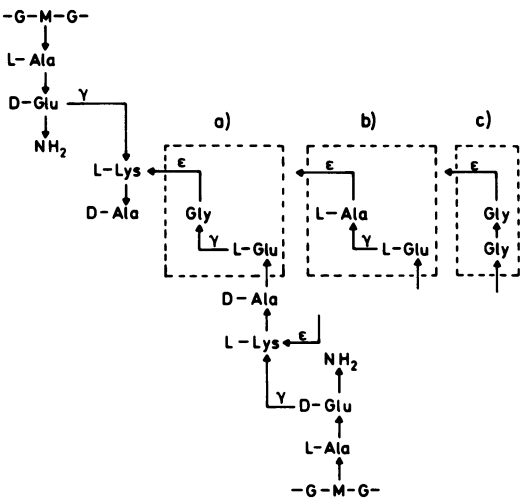

FIG. 6. *The mureins of M. luteus ATCC 389 (a), M. freundenreichii ATCC 407 (b), and M. radiodurans ATCC 13939 (c). In the latter case* L*-lysine is replaced by* L*-ornithine.*

et al. (5). These authors found a GC content of about 40% in 11 strains. *S. ureae* is, on the one hand, related to the *Planococcus* (similar shape of cells, presence of flagella, similar GC content) and, on the other hand, to *Bacillus pasteurii* (presence of spores, similar GC content, similar physiological properties).

A murein with a diglycine as the interpeptide bridge and with ornithine as the dibasic amino acid was found in *M. radiodurans* (8). This organism, however, differs from the majority of the other micrococci by the occurrence of significant amounts of lipoprotein in the cell wall and by a lower GC content of the deoxyribonucleic acid (2).

A murein type somewhat similar to that of the planococci was found in *M. varians* NTCC 7281 and a strain isolated from an airborne contaminant in our laboratory (*Micrococcus* sp. 1). It contains 3 moles of D-glutamic acid (8). Two moles are involved in the interpeptide bridge (Fig. 7). As in *M. lysodeikticus*, the α-carboxyl group of the D-glutamic acid of the peptide subunit is substituted by glycine (*unpublished data*). In contrast to all the other micrococci, the dibasis amino acid is meso-diaminopimelic acid. Other strains also named *M. varians* (ATCC 399, 13099, 13100) were found to contain the usual L-Lys-L-Ala$_3$-type murein. This indicates that the strain NTCC 7281 is different from the other strains of *M. varians*. Baird-Parker (2) mentioned that this strain should be separated because of its morphological and physiological properties. The strain *Micrococcus* sp. 1 isolated in our laboratory shows a life cycle similar to the

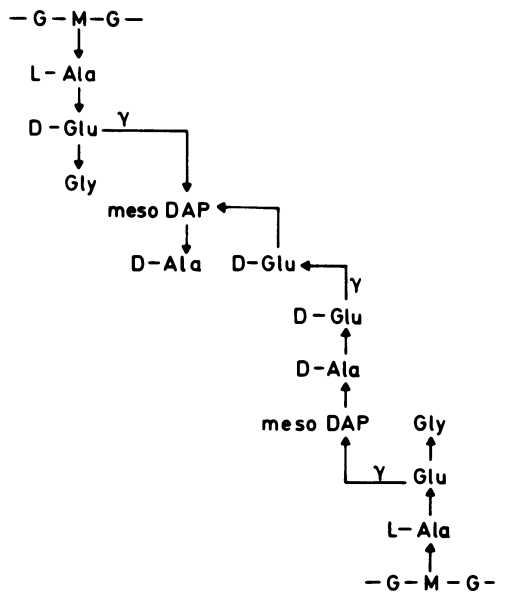

FIG. 7. *The murein of M. varians NCTC 7281 and a micrococcus strain (own isolate).*

coryneform bacteria and is probably an *Arthrobacter.*

In general, it is necessary to reconsider the taxonomic position of all strains containing murein types different from the majority of the micrococci and staphylococci.

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