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A novel type of *meso*-diaminopimelic acid-based peptidoglycan and novel poly(erythritol phosphate) teichoic acids in cell walls of two coryneform isolates from the surface flora of French cooked cheeses

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Abstract. The primary structure of the peptidoglycan and the teichoic acids of two coryneform isolates from the surface flora of French cooked cheeses, CNRZ 925 and CNRZ 926, have been determined. In the peptidoglycan, *meso*-diaminopimelic acid was localized in position three of the peptide subunit. It contained an D-glutamyl-D-aspartyl interpeptide bridge, connecting *meso*-diaminopimelic acid and D-alanine residues of adjacent peptide subunits. The α-carboxyl group of D-glutamic acid in position two of peptide subunits was substituted with glycine amide. The teichoic acid pattern and composition differed between the strains: both contained an erythritol teichoic acid and strain CNRZ 925 also contained an N-acetylglucosaminylphosphate polymer. The erythritol teichoic acids differed in terms of the quality and quantity of substituents, but they both had N,N′-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid in common.

Key words: Cheese flora – Erythritol teichoic acid – N,N′-Diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid – *m*-Dpm based peptidoglycan containing a D-Glu-D-Asp interpeptide bridge

The surface of the French cooked cheeses Gruyère and Beaufort is covered with a thin coat of microorganisms throughout the ripening period, 4–8 months for Gruyère and 6–12 months for Beaufort. The microbial covering is composed of salt tolerant yeasts and bacteria which participate actively in the creation of the particular organoleptic characters of this type of cheese. The bacteria moiety of the flora is composed of 90–95% coryneform bacteria, micrococci and gram-negative bacilli (Accolas et al. 1978). Two coryneform isolates, CNRZ 925 and CNRZ 926, were of special interest in terms of their taxonomy and cell wall biochemistry.

Among the coryneforms, some cell wall components are well known chemotaxonomic markers, which include the primary structure of the peptidoglycan, polysaccharides and teichoic acids (Schleifer and Kandler 1972; Fiedler and Schäffler 1987; Fiedler and Bude 1989).

Both strains contain a novel variant of type A4γ peptidoglycan (Schleifer and Kandler 1972), characterized by a D-glutamyl-D-aspartyl interpeptide bridge. They also contain a novel, erythritol-based teichoic acid, differing between the strains in terms of substitution. These findings are described in the present report.

Materials and methods

Bacterial strains, medium and growth conditions

The strains used were isolated by Accolas et al. (1978) and deposited in the French CNRZ collection at Jouy-en Josas, as CNRZ 925 and CNRZ 926.

The strains are gram-positive, aerobic, coccoid bacteria that exhibit characteristic morphology changes during a growth cycle. In the exponential phase, they are short clubshaped rods, mostly arranged in pairs, and snapping division produces V-shaped arrangements of cells. In the stationary phase, rods become irregular cocci that are arranged predominantly in pairs or tetrads (CNRZ 925) or in irregular clusters (CNRZ 926).

The strains were grown aerobically at 30 °C in a medium containing, per liter, 17 g tryptone, 3 g soy peptone, 5 g yeast extract, 2.5 g glucose, 5 g NaCl, 2.5 g $K_2HPO_4 \cdot 3\ H_2O$ and 1 ml Tween 80, pH 7.3 (Accolas et al. 1978).

Preparation of tryptically digested cell walls, dinitrophenylated cell walls and cell wall fragments

Bacteria were grown in liquid medium until the early stationary phase of growth. Cells were harvested by centrifugation (20,000 × *g*, 20 min, 4 °C) and the pellet was heated at 100 °C for 15 min. Cells were suspended in 10 mM Tris-HCl, pH 7.5, and disintegrated by

Abbreviations: DNP, dinitrophenyl; Ery, erythritol; Gal, galactose; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; $GlcUANAc_2$, N,N′-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid; $HexUANAc_2$, N,N′-diacetyl-2,3-diamino-2,3-dideoxyhexuronic acid; *m*-Dpm, *meso*-diaminopimelic acid; Mur, muramic acid; MurNAc, N-acetylmuramic acid

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shaking with glass beads. Cell disruption was controlled by phase contrast microscopy. Tryptically digested and dinitrophenylated cell walls (CW-TRYP and DNP-CW, respectively) were prepared as described by Schleifer and Kandler (1967).

Dinitrophenylation of peptides (DNP-peptides) was carried out according to Primosigh et al. (1961).

DNP-CW suspended in 1% $NaHCO_3$ were hydrolyzed using egg white lysozyme at 0.2 mg/mg cell wall (Sigma, München, Germany). Incubation was in an atmosphere of toluene with shaking for 16 h at 37 °C. Lysis was recorded photometrically at 578 nm. The lysate was centrifuged (48,000 × g, 20 min, 4 °C) and the supernatant containing cell wall fragments was lyophilized.

Preparation of teichoic acids and teichoic acid fragments

Teichoic acids were extracted from CW-TRYP with 0.025 M glycine/HCl, pH 2.5 (0.2 ml/mg cell wall) for 10 min at 100 °C (Kaya et al. 1985). After centrifugation (48,000 × *g*, 20 min, 4 °C) the sediment was reextracted as above. The supernatants were combined, dialyzed against distilled water with Visking dialysis tubing and lyophilized. Teichoic acids were purified by ion-exchange chromatography on DEAE-Sephacel (Pharmacia, Uppsala, Sweden; 1.6 × 17 cm) applying a linear gradient of 0 to 1 M NaCl in 0.01 M Tris-HCl, pH 7.0, at a flow rate of 20 ml/h. The purified teichoic acids were extensively dialyzed against distilled water. After hydrolysis with 60% (w/v) HF (Riedel-de-Haen, Seelze, Germany) for 16 h at 0 °C (Anderson et al. 1977), nonphosphorylated teichoic acid fragments were separated on a Bio-Gel P-2 (Bio Rad, München, Germany) column (1.5 × 90 cm), which was eluted with distilled water at 8 ml/h in the cold. For the preparation of 2-acetamido-3-amino-2,3-dideoxy-D-glucofuranurono-6,3-lactam (Okuda and Suzuki 1983), teichoic acid was hydrolyzed with 0.5 M HCl for 3 h at 100 °C. The hydrolysate was N-acetylated (Wheat 1966) and components were separated on Bio-Gel P-2 (1.5 × 90 cm, 8 ml/h, distilled water). Diaminoglucuronic acid was determined after hydrolysis (0.5 M HCl, 3 h, 100 °C) by amino acid analysis.

Analytical methods

For total amino acid analysis CW-TRYP were hydrolyzed with 4 M HCl for 16 h at 100 °C. Partial acid hydrolysis of CW-TRYP was carried out with 4 M HCl for 30 min at 100 °C. DNP-peptides were hydrolyzed with 6 M HCl for 6 h at 100 °C. For the analysis of sugars CW-TRYP or HF-extracted material were hydrolyzed with 2 M HCl for 3 h at 100 °C.

Amino acids and amino sugars were quantitatively determined using a Biotronic LC 6001 amino acid analyzer (Biotronic, Maintal, Germany).

The primary structure of the peptidoglycan was determined by examining a partial hydrolysate of CW-TRYP by two-dimensional paper chromatography (Schleifer and Kandler 1972). Peptides were visualized with the ninhydrin reaction or were isolated from chromatograms for further determination. Hydrazinolysis was performed as described by Braun and Schroeder (1967). Photolysis of DNP-peptides was carried out according to Russel (1963a, b). DNP-amino acids were analysed by TLC on silica gel with the following eluents:

I = benzene/acetic acid (80/20, v/v),
II = chloroform/methanol/acetic acid (95/1/1, v/v/v),
III = chloroform/methanol/acetic acid/H_2O (65/25/13/8, v/v/v/v).

Compounds were identified by comparison with authentic DNP-amino acids (Sigma or Serva, Heidelberg, Germany).

The configuration of diaminopimelic acid was determined according to Rhuland et al. (1955), that of alanine and glutamic acid according to methods described in Bergmeyer (1974) and that of aspartic acid according to Bricas et al. (1967). Phosphate was determined with the method of Ames (1966). Erythritol and glucosamine were analyzed and quantified as peracetylated or reduced peracetylated derivatives (Albersheim et al. 1967) with GLC, using a Packard GC 438 gas chromatograph with flame ionization detector. Peracetylates were separated on a 2 × 1,000 mm column of 3% SP 2340 on 100/200 Supelcoport (Supelco, Bellefonte, Pa., USA). Column temperature program was started at 140 °C (2 min) and ended at 270 °C (3 min) with a gradient of 6 °C/min. Injector temperature was 270 °C and detector temperature was 280 °C. Nitrogen was used as carrier gas at 15 ml/min. A mixture of synthetic air (200 ml/min) and purified hydrogen (15 ml/min) was used as combustion gas.

Galactose was measured with galactose dehydrogenase (from *Pseudomonas fluorescens*, Boehringer, Mannheim, Germany), according to Bergmeyer (1974). The anomeric configuration of N-acetyl-glucosaminyl residues was determined with β-N-acetyl-glucosaminidase (from bovine kidney, Boehringer) used under optimal reaction conditions.

Periodate oxidation was performed according to Goldstein et al. (1962). Oxidates were reduced by $NaBH_4$ (10 mg/ml) in 0.1 M NH_4OH. Separation and detection of diaminohexuronic acid was carried out by TLC according to Cummins (1985).

NMR spectra were recorded on a Bruker AM 360 spectrometer at 20 °C. For ^{1}H-NMR spectra samples were dissolved in D_2O, and in d_6-DMSO for proton decoupled ^{13}C-NMR spectra.

Results

Components of peptidoglycans

The amino acids and amino sugars in CW-TRYP of the strains investigated are listed in Table 1. The identical composition and the same molar ratio of amino acids in both organisms indicate that the peptidoglycans are identical.

Diaminopimelic acid exhibited the *meso*-configuration, glutamic and aspartic acids were present in the D-configuration, and alanine was present in an equimolar ratio of D- and L-forms.

Primary structure of peptidoglycans

Determined by hydrazinolysis in the peptidoglycan of both strains, the C-terminal amino acids were found to be D-alanine and glycine (8% of total alanine and 6% of total glycine). Two mol ammonia/mol *meso*-diaminopimelic acid were released by acid hydrolysis (2 M HCl, 1 h, 100 °C) indicating two carboxyl amide residues per peptidoglycan repeating unit. DNP-glutamic acid and, to a lesser extent, DNP-aspartic acid and mono-DNP-*meso*-diaminopimelic acid were released by acid hydrolysis from DNP-CW.

Lysozyme treatment of DNP-CW was used to prepare DNP-muropeptides in which glutamic and aspartic acid were the N-terminal amino acids. After UV photolysis, DNP-Glu disappeared totally, whereas DNP-Asp was not destroyed. This indicates that the amino group of glutamic acid prone to dinitrophenylation was in α-position to a free carboxyl group. In the case of DNP-Asp, the α-carboxyl group was involved in the peptide linkage.

The amino acid sequence of the peptide moiety was further investigated by analyzing peptides obtained by the partial hydrolysis of DNP-CW (4 M HCl, 30 min,

Table 1. Quantitative composition of peptidoglycan components (nmol/mg cell wall) in acid hydrolysates (4 M HCl, 16 h, 100 °C) of CW-TRYP (a) and molar ratios of amino acids, amino sugars and ammonia, where *m*-Dpm is set as 1 (b)

		m-Dpm	Glu	Asp	Ala	Gly	GlcN	NH_3	Mur
CNRZ 925	a	281	576	298	561	322	312	633	281
	b	1	2.05	1.06	2.00	1.15	1.23	2.25	1.00
CNRZ 926	a	287	595	315	619	348	358	603	294
	b	1	2.08	1.10	2.16	1.21	1.24	2.10	1.02

100 °C). Hydrolysates were separated by two-dimensional paper chromatography (data not shown). In addition to free amino acids, muramic acid, and glucosamine, the following dipeptides were determined by sequence analysis: Mur-Ala, Ala-D-Glu, α-Gly-D-Glu, γ-D-Glu-*m*-Dpm, *m*-Dpm-D-Ala, α-D-Asp-*m*-Dpm (from photolysis data), γ-D-Glu-D-Asp (from photolysis data), D-Ala-D-Glu, D-Ala-D-Ala. Based on these peptides, the peptidoglycan peptide subunit linked to an N-acetylmuramyl residue of a glycan strand was Ala-γ-D-Glu-*m*-Dpm-D-Ala-(D-Ala). The occurrence of α-Gly-D-Glu strongly suggested a substitution of Gly for the α-carboxyl group of D-Glu. Since only 6% of total glycine were found in hydrazinolysates of CW-TRYP, its carboxyl group was assumed to be amidated in the peptidoglycan.

Among the peptides obtained from DNP-CW, DNP-D-Glu-D-Asp was also present. Photolysis destroyed DNP-Glu, in agreement with the above mentioned findings. γ-D-Glu-D-Asp is the interpeptide bridge of the peptidoglycans, made obvious by the peptides γ-D-Glu-D-Asp, α-D-Asp-*m*-Dpm, and D-Ala-D-Glu. This result corresponds with the dinitrophenylation of D-Glu, D-Asp and *m*-Dpm in CW-TRYP. Since only small amounts of D-Asp and negligible amounts of *m*-Dpm were dinitrophenylated, the interpeptide bridge is seldomly reduced to D-Asp and is only rarely missing. Peptide D-Ala-D-Ala was found only in small quantities, in line with the fact that only 18% (CNRZ 926) and 24% (CNRZ 925) of the interpeptide bridges were not crosslinked. These data were corroborated by hydrazinolysis of CW-TRYP, after which 8–10% of alanine were released. Consequently, these peptidoglycans were found to exhibit a high degree of crosslinkage.

Occurrence of teichoic acids in cell walls

A high phosphate content of 0.73 and 0.64 μmol/mg CW-TRYP of CNRZ 925 and CNRZ 926, respectively, indicates the presence of teichoic acids. By comparing this phosphate content with that of glycine-HCl-extracted CW-TRYP or the extracted material, 56% and 63% of phosphorus, respectively, could be released from CW-TRYP.

Composition of teichoic acids

To determine the composition of teichoic acids, CW-TRYP were exposed to 60% HF. The HF-extract was subsequently hydrolyzed and subjected to GLC after appropriate derivatization. Components recognized are shown in Fig. 1. Similar profiles were obtained from extracted teichoic acids. By comparison with a reference mixture containing erythritol and threitol, the polyol component of teichoic acids was clearly shown to be erythritol. The occurrence of erythritol could be further affirmed by NMR-spectroscopy (see below). Other constituents of teichoic acids were galactose and glucosamine, which is N-acetylated in native teichoic acids (see below).

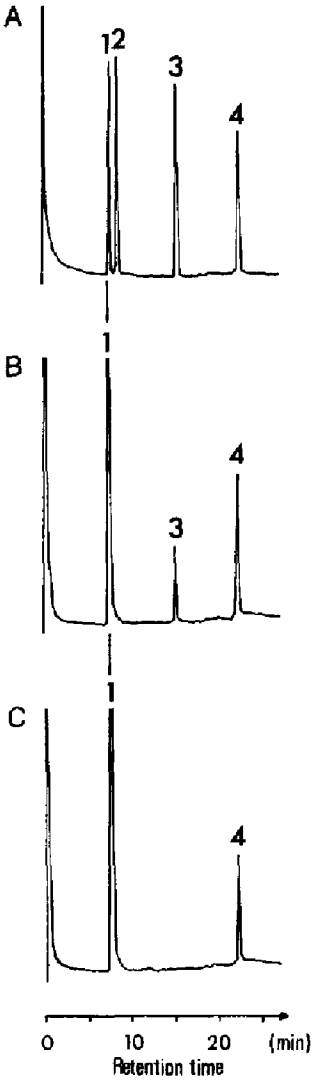

Fig. 1. GLC of HF-hydrolysates of CW-TRYP. Reference mixture (**A**), CNRZ 925 (**B**) and CNRZ 926 (**C**). CW-TRYP were hydrolyzed with 60% HF. Supernatants containing teichoic acid constituents were subjected to acid hydrolysis (2 M HCl, 100 °C, 3 h) and derivatized for GLC according to Albersheim et al. (1967). Peracetylates were separated by GLC. *1* Tetra-O-acetyl-erythritol, *2* tetra-O-acetyl-threitol, *3* hexa-O-acetyl-galactitol, *4* N-acetyl-penta-O-acetyl-glucosaminitol

Amino acid analysis of acid hydrolysates of CW-TRYP and extracted teichoic acids revealed an unknown ninhydrin-positive compound with a relative elution index of 1.24 compared to glucosamine. The elution indices of fucosamine and ornithine were 1.22 and 1.27. To clarify the nature of this compound, we considered the occurrence of a diaminodideoxyhexuronic acid, since such a sugar has been described as a constituent of cell wall polysaccharides (Cummins and White 1983) and lipopolysaccharides (Dmitriev et al. 1982; Okuda and Suzuki 1983). Using the procedure described (Cummins 1985), the presence of a diaminodideoxyhexuronic acid in hydrolysates of CW-TRYP and in extracted teichoic acids was confirmed by the appearance of a characteristic bright blue spot on TL chromatograms. Further confirmation that this substance was a diaminodideoxyhexuronic acid was furnished by NMR-spectroscopy of isolated teichoic acid fragments (see below).

Purification and structural characterization of teichoic acids

Phosphorus containing material was separated by subjecting extracted teichoic acids to ion-exchange chromatography on DEAE-Sephacel (Fig. 2). The existence of two teichoic acids was indicated in strain CNRZ 925 and one was indicated in strain CNRZ 926.

In order to identify the constituents of teichoic acids, fractionated aliquots of pooled fractions I to III were subjected to GLC following derivatization. The constituents found are listed in Table 2.

The material in pooled fractions I and II of strain CNRZ 925 consisted of poly-N-acetylglucosaminyl phosphate which was 50% substituted with galactose in fraction II. Since the glycoside bond of galactose was very labile to 60% HF, a furanoide configuration of galactose was considered (Fiedler, unpublished data).

Material in pooled fractions III and pooled fractions I and II of strain CNRZ 926 consisted of erythritol-containing teichoic acids, accompanied by diaminodideoxyhexuronic acid. Comparing erythritol released in HF-

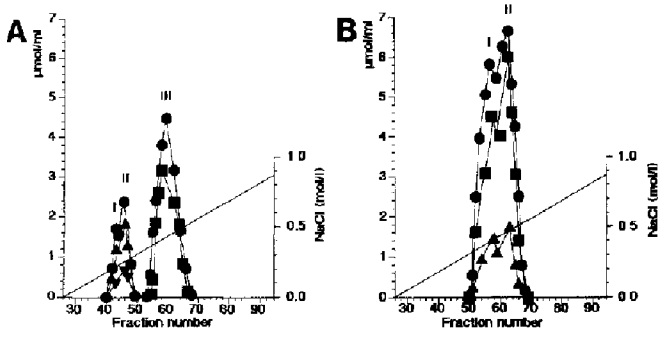

Fig. 2. DEAE-Sephacel chromatography of teichoic acids extracted from CW-TRYP of strains CNRZ 925 (**A**) and CNRZ 926 (**B**). Fractions (1.6 ml) were collected and analyzed for phosphorus (●—●), erythritol (■—■), glucosamine (▲—▲) and galactose (▼—▼). Sugars were determined after HF-treatment of samples followed by hydrolysis (2 M HCl, 3 h, 100 °C) and derivatization for GLC

Table 2. Quantitative composition of pooled fractions of Fig. 2

Strain	Pooled fraction	µmol Total			
		P_i	Ery	GlcN	Gal
CNRZ 925	I	3.33	–	3.86	–
	II	24.21	–	24.52	12.40
	III	75.73	67.96	–	–
CNRZ 926	I	38.85	38.04	12.36	
	II	25.39	22.66	13.53	

hydrolysates and acid treated HF-hydrolysates (2 M HCl, 3 h, 100 °C), there was more after acid treatment, indicating that erythritol was substituted. This observation was supported by periodate oxidation of fraction III material, after which only 40% of the erythritol disappeared. The destruction of erythritol by periodate is an additional and important indication of the structure of erythritol teichoic acids present: adjacent erythritol residues are phosphodiester-linked between hydroxyl groups of carbons 1 and 4. This linkage of erythritol residues was also deduced from erythritol phosphates present in an acid hydrolysate (0.5 M HCl, 3 h, 100 °C) of erythritol teichoic acid (data not shown).

In order to further determine the structural features of erythritol teichoic acids and to define the structure and the structural role of diaminodideoxyhexuronic acid, nonphosphorylated repeating units were investigated on Bio-Gel P-2. As shown (Fig. 3) teichoic acid from strain CNRZ 925 (Fig. 2A, pooled fraction III) yielded four fragments whereas in strain CNRZ 926 (Fig. 2B, pooled fractions I and II), three fragments were separated. Their composition is given in Table 3.

The prominent fragments I, III and IV of strain CNRZ 925 were analyzed by mass spectrometry and by ^{1}H-NMR spectroscopy. It was confirmed that fragment IV was erythritol, while fragment III was found to be 2-O-

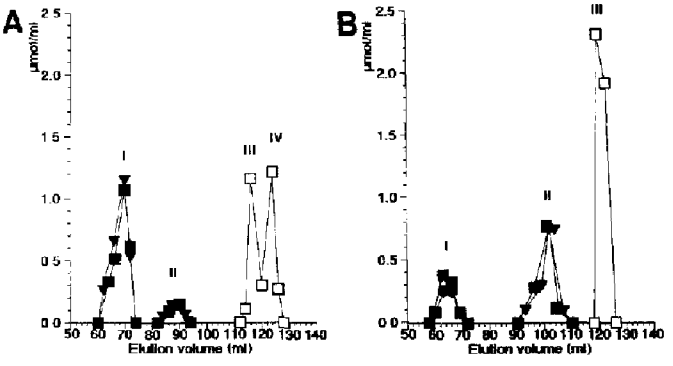

Fig. 3. Bio-Gel P-2 chromatography of HF degradation products obtained from DEAE-Sephacel-purified erythritol teichoic acids of strains CNRZ 925 (**A**) and CNRZ 926 (**B**). Fractions (1 ml) were collected and analyzed by GLC or amino acid analysis after acid hydrolysis (2 M HCl, 3 h, 100 °C: *filled symbols*) or directly derivatized for GLC (*open symbols*): erythritol (■—■) and (□—□), respectively, glucosamine (▼—▼), glucosamine/diaminohexuronic acid (●—●). Diaminohexuronic acid was quantified by assumption that fragment I in strain CNRZ 926 is equally substituted by glucosamine and diaminohexuronic acid

Table 3. Quantitative composition of pooled fractions of Fig. 3. Hydrolysates of fractions (2 M HCl, 3 h, 100 °C) were determined by means of amino acid analysis and GLC

Strain	Pooled fraction	µmol total		
		Ery	GlcN[a]	$HexUANAc_2$[b]
CNRZ 925	I	6.06	—	9.84
	II	1.09	—	0.95
	III	4.38	—	—
	IV	4.97	—	—
CNRZ 926	I	2.66	2.51	2.51
	II	4.58	4.56	—
	III	12.32	—	—

[a] Determined as GlcNAc, β-glycosidically bound, since GlcN was liberated quantitatively by β-N-acetylglucosaminidase

[b] Since no standards are available for diaminohexuronic acids, $HexUANAc_2$ was quantified by assuming a molar ratio of 1:1:1 for GlcN:$HexUANAc_2$:Ery in pool I of strain CNRZ 926

acetylerythritol. The molecular mass of fragment I was 380 Da and it could be defined as erythritol substituted by N,N′-diacetyl-2,3-diamino-2,3-dideoxyhexuronic acid. The ^{3}H-NMR spectrum revealed a β-glycoside linkage but it could not be ascertained whether a glucuronic acid or a galacturonic acid was present. In order to resolve this question, 2-acetamido-3-amino-2,3-dideoxy-D-glucofuranurono-6,3-lactam (Okuda and Suzuki 1983) was prepared from an acid hydrolysate of erythritol teichoic acid (Fig. 2A, pooled fraction III). The elution profile is shown in Fig. 4. Analysis of this compound by ^{13}C- and ^{3}H-NMR (Figs. 5 and 6) clearly showed that N,N′-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid is the substituent on erythritol. The other fragments in Fig. 3 were not further investigated and structural features were tentatively deduced from the quantitative composition and chromatographic behaviour. The material in pooled fraction II of Fig. 3 B consisted of β-N-acetylglucosaminyl-erythritol and pooled fraction I of Fig. 3B contained erythritol simultaneously substituted with N-acetylglucosamine and N,N′-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid. In comparison with the unambiguous data for N,N′-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid-substituted erythritol in pooled fraction I of Fig. 3A, the structure of N,N′-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid-substitued erythritol in pooled fraction II of Fig. 3A is unclear.

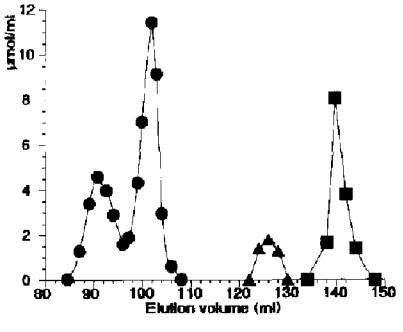

Fig. 4. Bio-Gel P-2 chromatography of erythritol teichoic acid of strain CNRZ 925 after hydrolysis (0.5 M HCl, 100 °C, 3 h) and N-acetylation. Fractions (1 ml) were analysed for phosphate, erythritol by GLC and diaminohexuronic acid after hydrolysis (0.5 M HCl, 100 °C, 3 h) by amino acid analysis erythritol phosphate (●——●), erythritol (▲——▲), N-acetyldiaminoglucuronic acid lactam (■——■)

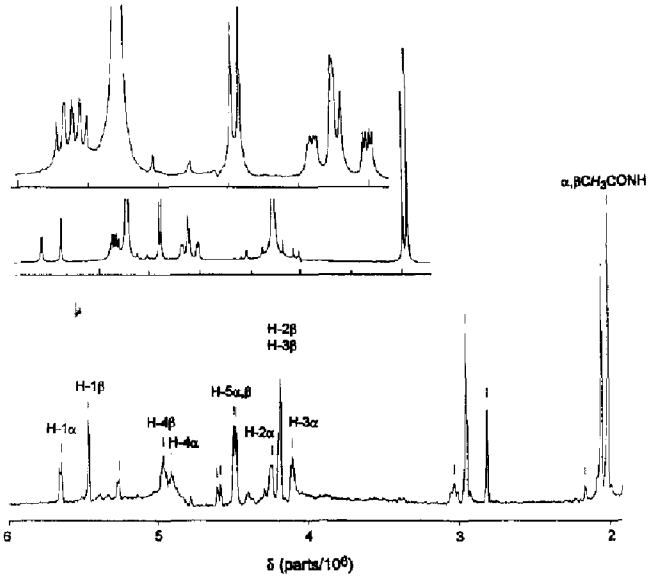

Fig. 5. ^{3}H-NMR-spectrum (360 MHz) of 2-acetamido-3-amino-2,3-dideoxy-D-glucofuranurono-6,3-lactam. The spectrum was recorded in ^2H_2O at 20 °C. *Top left insert*: 270 MHz ^{3}H-NMR-spectrum of 2-acetamido-3-amino-2,3-dideoxy-D-glucofuranurono-6,3-lactam (without any indications) as determined by Okuda and Suzuki (1983). δ_H (parts/10^6): 5.65 (1 H, d, *H*-1α), 5.47 (1 H, d, *H*-1β), 4.97 (1 H, dd, *H*-4β), 4.91 (1 H, dd, *H*-4α), 4.50 (2 H, d, *H*-5α and 5β), 4.24 (1 H, dd, *H*-2α), 4.21 (1 H, dd, *H*-2β), 4.19 (1 H, dd, *H*-3β), 4.11 (1 H, dd, *H*-3α), 2.02 (3 H, s, α-NHCOC*H*$_3$), and 2.07 (3 H, s, β-NHCOC*H*$_3$). Peaks were attributed according to Okuda and Suzuki (1983)

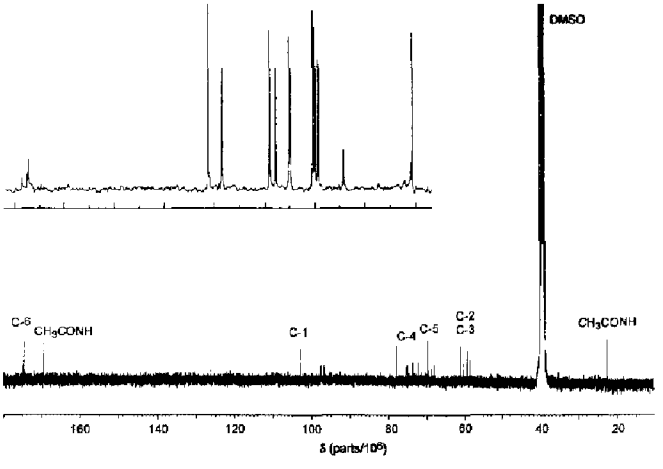

Fig. 6. Proton-decoupled ^{13}C-NMR-spectrum (360 MHz) of 2-acetamido-3-amino-2,3-dideoxy-D-glucofuranurono-6,3-lactam. The spectrum was recorded in d_6-DMSO at 20 °C. *Top left insert*: 270 MHz ^{13}C-NMR-spectrum of N-acetylglucuronic acid lactam (without any indications) as determined by Okuda and Suzuki (1983). Major peaks occurred at 174.86 and 174.58 (*C*-6α and -6β), 169.52 and 169.40 (α- and β-NH_3COCH_3), 102.75 and 97.47 (*C*-1α and -1β), 77.80 and 75.44 (C-4α and -4β), 69.82 and 69.66 (*C*-5α and -5β), 61.09, 60.30, 59.23 and 58.52 (*C*-2α and -2β; C-3α and -3β) and 22.64 and 22.52 (α- and β-NH_3COCH_3). Peaks were assigned according to Okuda and Suzuki (1983)

Discussion

Analytical data obtained for the peptidoglycans present in strains CNRZ 925 and CNRZ 926 are consistent with a common primary structure (Fig. 7). This peptidoglycan is *m*-Dpm-based and contains an interpeptide bridge consisting of dicarboxyamino acids. It is thus a type of the A4γ-variation of peptidoglycan, according to Schleifer and Kandler (1972). The interpeptide bridge is composed of the novel dipeptide D-Glu-D-Asp. The occurrence of D-Asp in this peptidoglycan-type corresponds to an unusual mode of attachment to the peptide subunit. The finding that DNP-D-Asp in dinitrophenylated muropeptides was stable to photolysis implies that D-Asp is bound by its α-carboxyl group to the ω-amino group of *m*-Dpm. In all other peptidoglycans of this variation described so far, the linkage was formed via the ω-carboxyl group of the dicarboxyamino acid (Staudenbauer 1968; Schleifer and Kandler 1972). Another characteristic of this peptidoglycan-type is the glycine amide substitution of the α-carboxyl group of D-Glu in position 2 of the peptide subunit. Whereas substitution with amide in this position is very common, larger substituents are more rarely found (Schleifer and Kandler 1972). The amidation site of the second amide group was not known and has not been determined, but possible amidation sites are shown in Fig. 7.

Besides peptidoglycans containing γ-D-Glu-D-Glu as an interpeptide bridge (Bogdanovsky et al. 1971), the peptidoglycan-type described here is another example that an interpeptide bridge may develop in *m*-Dpm-containing peptidoglycans. Thus, the determination of *m*-Dpm alone does not indicate a directly crosslinked peptidoglycan.

Another important characteristic of the cell walls of the organisms studied is the presence of unusual teichoic acids. We did not detect glycerol, ribitol or mannitol, the polyols generally composing teichoic acids (Ward 1981; Anderton and Wilkinson 1980; Fiedler et al. 1981). Rather, an unusual polyol, erythritol, was present in the cell wall teichoic acids of strains CNRZ 925 and CNRZ 926. Other components identified as teichoic acid constituents were N-acetylglucosamine, galactose, and the unusual components acetate and N,N'-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid. Detailed analysis of purified teichoic acids showed differences between the two strains. Thus, CNRZ 926 possessed erythritol teichoic acid as the sole teichoic acid, whereas in CNRZ 925 erythritol teichoic acid was combined with poly-N-acetylglucosaminitol. Erythritol teichoic acids belong to the "classical" teichoic acid type with erythritol phosphate as repeating unit of the backbone. From the fragments obtained by HF-hydrolysis of teichoic acids, it was concluded that erythritol teichoic acid in strain CNRZ 925 is one-third substituted with N,N'-diacetyl-2,3-diamino-2,3-dideoxyglucuronic acid and another third with acetate. Part of the diaminoglucuronic acid may have been deacetylated during preparation (Fig. 3A, fragment II). Erythritol teichoic acid of strain CNRZ 926 is 20% substituted with N-acetylglucosamine and 10% each with N-acetylglucosamine and diaminoglucuronic acid. We did not determine if these substituents were bound to different carbon atoms or as a disaccharide. Fragments common to both teichoic acids are shown in Fig. 8. In addition to erythritol teichoic acid, strain CNRZ 925 contains a poly-N-acetylglucosaminyl phosphate polymer. This teichoic acid is 50% substituted with furanogalactose.

In most cases, classical teichoic acids contain a poly(glycerophosphate) or a poly(ribitol phosphate) backbone (Ward 1981; Baddiley 1988). In a few bacteria, a poly(mannitol phosphate) backbone has been described (Anderton and Wilkinson 1980; Fiedler et al. 1981). The present work is the first report of teichoic acids with a poly(erythritol phosphate) backbone. Thus, erythritol as a component of the teichoic acid backbone completes the homologous series of polyols, from glycerol via ribitol to mannitol.

The presence of an uronic acid as substituent is to our knowledge a novel feature of teichoic acids. Thus far,

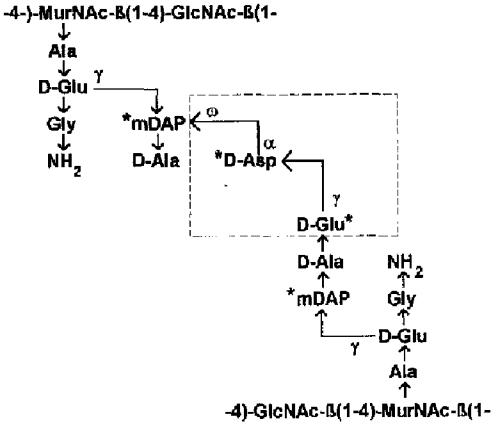

Fig. 7. Sequence of the primary structure of the peptidoglycan of strains CNRZ 925 and CNRZ 926. Interpeptide bridge marked by a dashed frame; asterisks indicate the positions of possible amidation

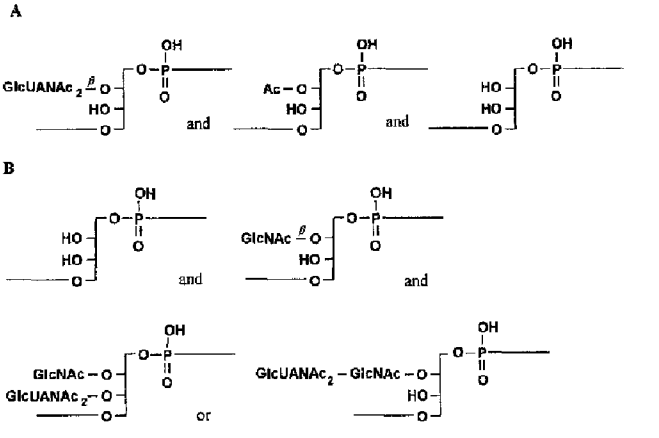

Fig. 8. Fragments of the erythritol teichoic acids of strain CNRZ 925 (A) and CNRZ 926 (B). Fragments of the teichoic acids are shown separately, since percentage of substitution is different. All substituents are assumed to be part of one teichoic acid chain since erythritol teichoic acids of both strains eluted in a single peak on DEAE-Sephacel

only D-alanine, neutral sugars or N-acetylaminosugars are known as teichoic acid substituents (Ward 1981; Baddiley 1988). In addition to neutral sugars, uronic acids are constituents of acidic polysaccharides, teichuronic acids (Janczura et al. 1961). Teichuronic acids are believed to play the role of teichoic acids in one aspect: that of cation scavengers. Teichuronic acids can coexist with teichoic acids in cell walls (Hughes et al. 1968) or replace teichoic acids under conditions of phosphate limitation (Tempest et al. 1968). This means that until the present uronic acid – as part of a cell wall polymer – has been found concomitantly with teichoic acid but never in the same molecule. It is not clear if and how this influences the properties of the teichoic acid, e.g. binding of cations since there are additional negative charges introduced in the molecule.

These two unique features – *m*-Dpm containing peptidoglycan with a D-Glu-D-Asp-interpeptide bridge and the poly(erythritol phosphate) teichoic acid – are believed to be taxonomically relevant, strongly indicating that the two dairy isolates CNRZ 925 and CNRZ 926 constitute coryneform organisms that merit classification.

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