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# Determination of diamino acids in peptidoglycans from anaerobic bacteria

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**Summary.** Peptidoglycans isolated from two *Fusobacterium* species of anaerobic bacteria were analyzed for constituent amino acids. Hydrolysis conditions were varied to optimize the yield of diamino acids from peptidoglycan. The *o*-phthalaldehyde derivatives of the diamino acid stereoisomers were separated by high-performance liquid chromatography (OPA-HPLC), and variations in the relative areas of the two peaks noticed during analysis of solid samples were attributed to sampling errors. Co-derivatization/injection experiments using standards of the meso and rac forms separated from commercial mixtures demonstrated that *meso*-2,6-diaminopimelic acid and *meso*-lanthionine were peptidoglycan components in *Fusobacterium varium* and *Fusobacterium nucleatum*, respectively. The protonated molecules of 2,6-diaminopimelic acid and lanthionine were detected in peptidoglycan hydrolyzates by off-line, flow-injection electrospray mass spectrometry (ESI-MS). In ESI-MS-MS experiments under identical collision-induced dissociation (CID) conditions, peptidoglycan-derived and standard diamino acids exhibited similar fragmentations. Fragmentation pathways are proposed for each diamino acid. The results confirm that the meso forms of two different diamino acids are utilized in the peptidoglycans of *Fusobacterium* species.

**Keywords:** Amino acids – 2,6-Diaminopimelic acid – *Fusobacterium nucleatum* – *Fusobacterium varium* – HPLC – Lanthionine – Electrospray mass spectrometry – Peptidoglycan

## Introduction

The peptidoglycan layer in bacterial cell walls is an important structural component, providing shape, rigidity, and resistance to osmotic stress (Bugg and Walsh, 1992; Labischinski and Maidhof, 1994). The glycan chains within the peptidoglycan network are composed of alternating *N*-acetylglucosamine

and *N*-acetylmuramic acid moieties; each chain is linked to others by peptide bridges attached to the lactyl groups of the *N*-acetylmuramic acid subunits (Bugg and Walsh, 1992; Labischinski and Maidhof, 1994). While D- and L-alanine, D-glutamic acid, and a diamino acid are common residues in the peptide crosslink, there is considerable variation in the amino acid composition among bacteria, particularly the nature and the stereochemistry of the diamino acid (Cummins, 1974; Labischinski and Maidhof, 1994; Schleifer and Kandler, 1972). In the peptidoglycan of Gram-positive bacteria, lysine and 2,6-diaminopimelic acid ($A_2$pm, **1**) occur more frequently than other diamino acids, such as ornithine and 2,4-diaminobutyric acid, whereas 2,6-diaminopimelic acid is the predominant diamino acid utilized in peptidoglycan crosslinks by Gram-negative bacteria (Schleifer and Kandler, 1972). In several *Fusobacterium* species of anaerobic bacteria, however, 2,6-diaminopimelic acid (**1**) is replaced by lanthionine (Lan, **2**), a sulfur analogue (Kato et al., 1981; Miyagawa et al., 1981; Vasstrand et al., 1982).

The amino acid composition of peptidoglycan provides valuable information for structure elucidation and bacterial taxonomy (Schleifer and Kandler, 1972; Work, 1970). High-performance liquid chromatography (HPLC) has become the most popular method for the analysis of diverse biological samples, particularly for the detection of diamino acids. HPLC determinations of 2,6-diaminopimelic acid have been useful for chemotaxonomy studies (McKerrow et al., 2000; Takahashi et al., 1989; Zanol and Gastaldo, 1991), the characterization of bacterial mutants (Richaud et al., 1987), the investigation of bacterial metabolism (El-Waziry et al., 1996; Mengin-Lecreulx et al., 1988), and as an indicator of the bacterial content of feeds (Puchała et al., 1992; Webster et al., 1990) and rumen digesta (Czauderna and Kowalczyk, 1999; Dugan et al., 1992; Webster et al., 1990). Enzyme-catalyzed reactions involving 2,6-diaminopimelic acid (Weir et al., 1989) or lanthionine (Costa et al., 1989) have been monitored by HPLC, and HPLC methods have been used in conjunction with metabolic studies of sulfur-containing amino acids (Richaud et al., 1993; Yu et al., 1997) and the determination of amino acids in plasma and protein hydrolyzates (Krause et al., 1995).

The HPLC determination of 2,6-diaminopimelic acid (**1**) and lanthionine (**2**) is complicated by the fact that these diamino acids occur as three stereoisomers (Fig. 1): a meso form (2*R*,6*S*), and a pair of enantiomers (2*R*,6*R*) and (2*S*,6*S*). Achiral chromatographic methods achieve a separation of the diastereomeric meso and rac forms (Czauderna et al., 1999; Czauderna and Kowalczyk, 1999; Dugan et al., 1992; Mengin-Lecreulx et al., 1988; Puchała et al., 1992; Richaud et al., 1987; Webster et al., 1990; Weir et al., 1989), whereas derivatization with a chiral reagent or use of a chiral solid support is needed to achieve separation of all three stereoisomers (El-Waziry et al., 1996; McKerrow et al., 2000; Nagasawa et al., 1993; Takahashi et al., 1989; Zanol and Gastaldo, 1991). Standard samples of the pure stereoisomeric forms of **1** and **2** are not readily available, making the assignment of the elution order of the diamino acid isomers difficult and limiting the interpretation of results obtained by chromatographic analysis. In the present investigation, an achiral HPLC method, calibrated using *meso*- and *rac*-diamino acid

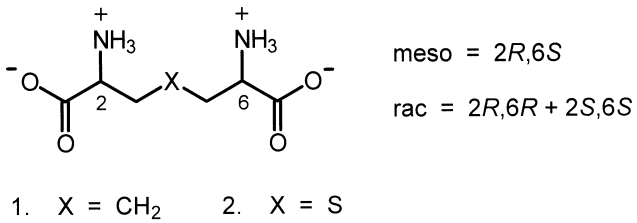


**Fig. 1.** Stereoisomers of 2,6-diaminopimelic acid (**1**) and lanthionine (**2**)

standards and off-line, electrospray mass spectrometry (ESI-MS-MS) have been used to identify amino acids in hydrolyzates of partially purified peptidoglycan samples. These studies further characterize differences of amino acid metabolism in two Gram-negative *Fusobacterium* species (Ramezani et al., 1999; Gharbia and Shah, 1991) that have been implicated in periodontal disease and other human infections (Satyanarayana and White, 1999).

## Materials and methods

### *OPA-HPLC determination of amino acids*

Fluorescent isoindole derivatives of amino acids were formed using *o*-phthalaldehyde (OPA) – 2-mercaptoethanol derivatization reagent and injected onto a Beckman ODS Ultrasphere column (5 $\mu$m, 45 $\times$ 4.6 mm). Separations were achieved at a total flow rate of 2.5 mL/min using a gradient formed between 0.1 M sodium acetate (adjusted to pH 6.2 with 6 M HCl; water-aspirator degassed)-methanol-THF (900 : 95 : 5 v/v) and methanol (Ramezani et al., 1999; White et al., 1989) with the following composition (min, % methanol): 0.0, 0; 0.5, 15; 3.0, 15; 3.25, 40; 5.75, 40; 6.0, 100; 6.5, 100; 7.0, 0. Solid samples of 2,6-diaminopimelic acid and lanthionine were dissolved in water or sodium borate buffer (100 mM, pH 9.5), respectively, at 1–2 mg/10 mL for OPA-HPLC analysis. Filtrates from crystallizations and 2,6-diaminopimelic acid solutions obtained by eluting paper chromatograms were analyzed directly by OPA-HPLC. The relative peak areas are expressed as the mean $\pm$ standard deviation calculated from three or more replicate injections. Fluorescence spectra of lanthionine OPA derivatives in 0.1 M sodium acetate-methanol (60 : 40 v/v) were obtained on a Shimadzu spectrofluorophotometer, model RF-5301PC.

### *Separation of diamino acid stereoisomers*

*2,6-Diaminopimelic acid:* Two consecutive recrystallization of 2,6-diaminopimelic acid (790 mg, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) from aqueous ethanol (Work, 1963) yielded crystals of the meso isomer (7 mg, 92% meso). Chromatography of 2,6-diaminopimelic acid (16.4 mg) on Whatman #1 chromatographic paper (Rhuland et al., 1955) yielded (2*S*,6*S*)-diaminopimelic acid (90% 2*S*,6*S*) upon elution of the $R_f$ 0.15 band after development by descending paper chromatography using methanol/water/pyridine (80 : 10 : 10 v/v).

*Lanthionine:* Recrystallization of lanthionine (103 mg, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) from aqueous ethanol (Horn et al., 1942) yielded lanthionine crystals and a filtrate highly enriched with the more soluble isomer (22 mg, 83% rac). A sample of *meso*-lanthionine (10 mg, 86% meso) was obtained by extracting solid lanthionine (99 mg) with two successive portions of water at ambient temperature.

### *Growth of anaerobic bacteria*

*Fusobacterium nucleatum* (ATCC 25586) and *Fusobacterium varium* (NCTC 10560) were maintained on 5% sheep-blood agar plates (Queen Elizabeth II Health Sciences Centre, Halifax, NS, Canada) with weekly transfers to fresh plates. Liquid cultures were initiated by inoculating peptone medium (50 mL) (Ramezani et al., 1999) with one or two colonies of *F. nucleatum* or *F. varium* from a sheep-blood agar plate. After 24 h of incubation, the culture was used to inoculate a larger volume of fresh peptone medium (5 L) which was incubated for a further 25 h. All incubations were carried out at 37°C under an atmosphere of $H_2/CO_2/N_2$ (10:10:80 v/v).

### *Isolation of peptidoglycan*

Cells from a 24-h peptone culture (5 L) were collected by centrifugation (8,000 × g, 5 min, 10°C) and resuspended in water (55 mL). A 10% solution of trichloroacetic acid (55 mL) was added immediately and the mixture was shaken. After storage at 4°C for at least 30 min, the suspension was centrifuged (13,200 × g, 5 min, 10°C); the pellets were washed three times in water (15,850 × g, 10 min) and resuspended in 50 mM sodium acetate (90 mL, pH 5.3). An 8% solution of sodium dodecyl sulfate (SDS) (90 mL) was added; the mixture was heated to 95°C with stirring for 1 h and stored at ambient temperature overnight. The suspension was centrifuged at 25–30°C (38,700 × g, 60 min); the pellets were washed three times in water (15,850 × g, 10 min) and freeze-dried. *F. nucleatum* and *F. varium* cultures yielded peptidoglycan at 19 mg/L and 93 mg/L, respectively.

### *Hydrolysis of peptidoglycan*

Peptidoglycan and 4 M HCl (typically 1 mg/mL HCl) were heated under $N_2$ in a sealed tube at 100°C for 6 h. The mixture was concentrated at 50°C *in vacuo* Four portions of water (typically 2 mL/mg peptidoglycan) were successively added and removed *in vacuo* at 50°C. The residue was dried *in vacuo* over NaOH. Sodium borate buffer (0.5 mL, 100 mM, pH 9.5) and water (0.5 mL) were added to the *F. nucleatum* and *F. varium* samples, respectively. The samples were filtered (0.45 $\mu$m), and the filtrate was titrated to pH 6–8 with 5% NaOH prior to OPA-HPLC analysis. Samples for MS studies were applied to a Dowex 50W-X8 cation-exchange column (50–100 mesh, 5 mL). The column was washed with water and eluted with 0.5 M $NH_3$. Fractions testing positive to ninhydrin were pooled, concentrated to dryness, and stored *in vacuo*.

### *Mass spectrometry*

Positive ion electrospray ionization (ESI) mass spectra were obtained on a Micromass Quattro Triple Quadrupole Mass Spectrometer. The background solution of 2% aqueous formic acid was introduced at a rate of 30 $\mu$L/min from a syringe pump through a Rheodyne valve equipped with a 10-$\mu$L sampling loop. All samples were dissolved in 2% aqueous formic acid. The cone voltage was maintained between 3–10 V, and the collision energy for collision-induced dissociation experiments (CID) was between 20–30 eV using argon gas at a nominal pressure of $1 \times 10^{-4}$ Torr.

## Results and discussion

### *OPA-HPLC: method development*

The mixture of stereoisomeric, fluorescent isoindoles, obtained by precolumn derivatization of each commercial diamino acid, separated into two peaks

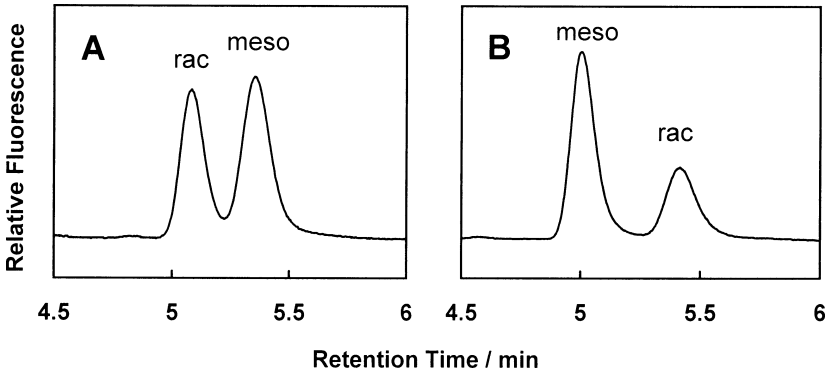


**Fig. 2.** Chromatographic separation of diamino acid stereoisomers: **A** 2,6-diaminopimelic acid (**1**, 0.74 mM); **B** lanthionine (**2**, 0.77 mM)

under achiral chromatographic conditions (Fig. 2). Baseline separation within six minutes was achieved for the lanthionine derivatives and was nearly attained for the 2,6-diaminopimelic acid isoindoles. Significantly longer times are reported in published OPA methods for the chromatographic determination of 2,6-diaminopimelic acid (Czauderna and Kowalczyk, 1999; Czauderna et al., 1999; Dugan et al., 1992; McKerrow et al., 2000; Pittenauer et al., 1993; Puchała et al., 1992; Webster et al., 1990; Weir et al., 1989) and lanthionine (Costa et al., 1989). For lanthionine, a reduction in the amount of the OPA reagent used and/or the derivatization time (to 5–30 sec) led to the appearance of a third peak at a retention time of 1.6 min, suggesting the formation of a mono-OPA derivative.

For the assignment of the two OPA-HPLC peaks, the commercial diamino acid mixtures were fractionated to provide standards enriched in only one or two stereosiomers. Crystallizations from aqueous ethanol yielded *meso*-2,6-diaminopimelic acid crystals (Work, 1963), which eluted as the second OPA-HPLC peak (Fig. 2A). (2*S*,6*S*)-Diaminopimelic acid, obtained by paper chromatography of the commercial sample (higher $R_f$ band, Rhuland et al., (1955)), eluted as the first HPLC peak (Fig. 2A), confirming the elution order established for similar chromatographic conditions by Weir et al. (1989). Analogous crystallization and solubility experiments demonstrated that the OPA derivative of the less soluble meso isomer of lanthionine (Horn et al., 1942) had a shorter retention time than that of the more soluble racemic mixture (Fig. 2B). The relative order of elution of *meso*- and *rac*-lanthionine in the OPA-HPLC method has apparently not been assigned: the only previous report (Costa et al., 1989) employed (2*S*,6*S*)-lanthionine as a standard. For subsequent OPA-HPLC analyses, difficulties associated with dissolving lanthionine were avoided by using borate buffer, the buffer commonly employed for the OPA derivatization reaction.

The two diamino acid peaks observed in each standard chromatogram had unequal areas. The relative areas (56.1 : 43.9 ($\pm$0.4)) of the two 2,6-diaminopimelic acid peaks (Fig. 2A) are consistent with the approximately equal areas noted by Czauderna et al. (1999), Puchała et al. (1992) and Weir

**Table 1.** Variation of the relative areas of the lanthionine peaks attributed to sampling error

| Solution | Lanthionine mass (mg) | Number of injections | Relative area (%)[a] | |
|---|---|---|---|---|
| | | | *meso*-lanthionine | *rac*-lanthionine |
| 1 | 1.2 | 6 | 62.7 ± 0.3 | 37.3 ± 0.3 |
| 2 | 1.4 | 3 | 64.9 ± 0.4 | 35.1 ± 0.4 |
| 3 | 1.6 | 6 | 69.1 ± 0.3 | 30.9 ± 0.3 |
| 4 | 8.1 | 17 | 71.0 ± 0.6 | 29.0 ± 0.6 |

[a] mean ± standard deviation. The significance of the differences between solutions is supported by a greater than 99% confidence interval calculated by the t test.

et al. (1989). The very different relative peak areas (67:33 (±3)) observed for the lanthionine peaks appear to be inconsistent with equal amounts of the meso and rac forms in the commercial standard. Since identical excitation (336 nm) and emission maxima (446 nm) were measured for the *meso*- and *rac*-lanthionine derivatives, the lower fluorescence response of the *rac*-lanthionine derivative was attributed to intramolecular quenching, by analogy with the known lower fluorescence of lysine derivatives (Alvarez-Coque et al., 1989), and the unequal fluorescence responses obtained for different amino acids (Aberhart, 1988; White et al., 1989).

During the analysis of standard lanthionine solutions in borate buffer, a significant variation in the relative areas of the two HPLC peaks was noted each time a new solution was prepared (Table 1). No change in the relative peak areas measured for an individual solution was observed upon storage or dilution. Similarly, the relative peak areas were not influenced by the amount of OPA used in the derivatization reaction or by the derivatization time, Since very small samples of solid were used to prepare each lanthionine solution, the variation in the relative peak areas (Table 1) was attributed to an uneven distribution of diastereomeric forms in the solid, commercial sample, leading to a sampling error (Kratochvil and Taylor, 1981). Sampling errors were minimized by averaging the relative peak areas measured for several standard lanthionine solutions, and these values were used for the calculations related to the separation of diastereomers. Sampling errors were less noticeable for 2,6-diaminopimelic acid.

The retention times of the 2,6-diaminopimelic acid and lanthionine derivatives are quite similar (Fig. 2). Co-derivatization/injection of the two diamino acids gave partial resolution of the peaks at shorter retention times (i.e., *rac*-$A_2$pm and *meso*-Lan), but failed to achieve separation of *meso*-$A_2$pm and *rac*-Lan. It is noteworthy that the elution order of the stereoisomers is reversed, allowing the meso isomers of the diamino acids (or the racemic mixtures) to be easily distinguished from each other. Further information to distinguish the two amino acids could be provided by oxidation of lanthionine to a sulfone or a sulfoxide. OPA-HPLC analysis revealed that negligible amounts of residual lanthionine remained upon oxidation with performic acid (2 h at 0°C). However, several products were present, confirming a previous report (Vasstrand

et al., 1982), which suggests that lanthionine cannot be cleanly oxidized to a single product for chromatographic analysis. Moreover, lanthionine sulfoxide and lanthionine sulfone decompose readily in acidic solution (Zahn and Baschang, 1959), excluding the possibility of oxidizing lanthionine prior to peptidoglycan hydrolysis (*vide infra*).

### *Isolation and analysis of peptidoglycan*

In initial studies, peptidoglycan was isolated from *F. nucleatum* by a hybrid of two published methods (Kato et al., 1979; Vasstrand et al., 1979), but improved yields of lanthionine (determined by OPA-HPLC) were achieved when an isolation procedure was developed from that described for the isolation of peptidoglycan from *Neisseria gonorrhoeae*, another Gram-negative bacterium (Rosenthal and Dziarski, 1994).

Peptidoglycan hydrolyzates were prepared originally using 6 M HCl and 110°C for 18 h. Relatively small, diamino acid peaks were detected by OPA-HPLC, however, and partial epimerization of lanthionine has been observed under these conditions (Jacobson et al., 1974; Vasstrand et al., 1982). The stability of lanthionine in aqueous acid was tested by treating a standard sample (2.5 mg/mL HCl) under the initial hydrolysis conditions. OPA-HPLC analysis revealed that about 50% of the lanthionine decomposed; only small amounts of cysteic acid and serine were detected, suggesting that other decomposition products were not primary amines.

To optimize the recovery of diamino acid, particularly lanthionine, compromise conditions were sought to maximize the yield of amino acid from peptidoglycan hydrolysis and to minimize decomposition and epimerization of the diamino acid. The least strenuous conditions for peptidoglycan hydrolysis were determined by subjecting *F. varium* peptidoglycan to various concentrations of HCl (0.1–12 M) for 14 h at 100°C. Incomplete hydrolysis occurred at concentrations less than 3 M and similar chromatographic profiles of the products were obtained at 3–6 M HCl. The minimum time needed for peptidogycan hydrolysis in 4 M HCl at 100°C was determined for peptidoglycan from both *Fusobacterium* species; maximum yields of hydrolysis products were attained by 6 h, a significantly shorter time than that employed by Vasstrand et al. (1982) for the same acid concentration.

A series of co-derivatization/injection experiments using the meso and rac standards were carried out to assign peaks in the chromatograms of the peptidoglycan hydrolyzates to a stereoisomer of **1** or **2**. The peak at 5.49 min in the chromatogram of the *F. varium* hydrolyzate (Fig. 3A) co-eluted with *meso*-2,6-diaminopimelic acid; no peaks were detected at the retention times of *rac*-2,6-diaminopimelic acid and *meso*-lanthionine. The peak at 4.95 min in the chromatogram of the *F. nucleatum* hydrolyzate (Fig. 3B) co-eluted with *meso*-lanthionine and was resolved partially from *rac*-2,6-diaminopimelic acid, whereas a peak at 5.45 min had a slightly different retention time than that of *rac*-lanthionine and broadened significantly when co-injected with *rac*-lanthionine. The chromatographic results are most consistent with *meso*-2,6-

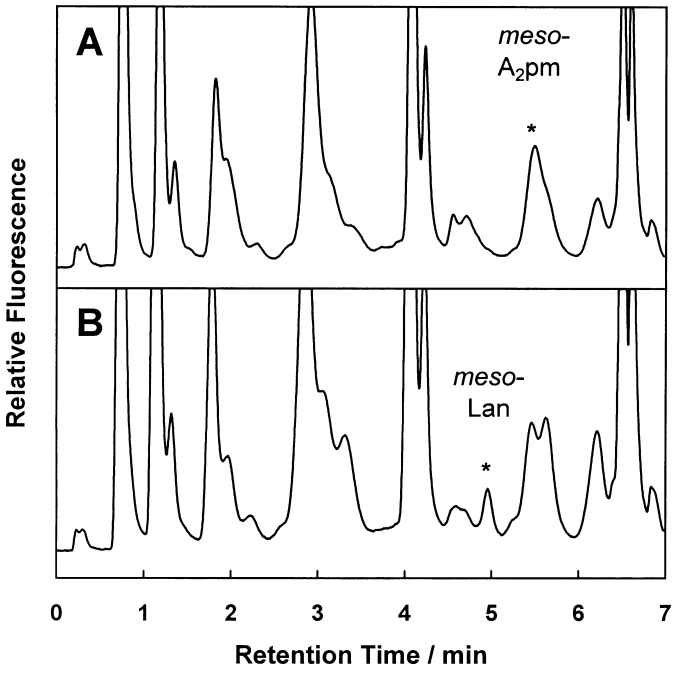


**Fig. 3.** Chromatograms of peptidoglycan hydrolyzates from (**A**) *F. varium* and (**B**) *F. nucleatum*. Major peaks assigned to Asp (0.7 min), Glu (1.1 min), Ser (1.7 min), Gly (2.8 min), Thr (3.0 min), Arg (3.2 min), Ala (4.0 min), Tyr (4.2 min), *meso*-Lan (4.9 min), *meso*-$A_2$pm (5.5 min), Leu/Ile (6.5 min) and Lys (6.6 min)

diaminopimelic acid and *meso*-lanthionine as peptidoglycan constuents in *F. varium* and *F. nucleatum*, respectively.

OPA-HPLC analysis revealed that several other amino acids were also present in the hydrolyzates of the partially purified peptidoglycan (Fig. 3). Co-injection experiments using amino acid standard solutions assigned the major chromatographic peaks to aspartic acid, glutamic acid, serine, glycine, threonine, arginine, alanine, tyrosine, leucine/isoleucine, and lysine. Ions corresponding to the $M+H]^+$ ions of these amino acids, as well as those of proline, valine, methionine, phenylalanine, histidine, and the aminosugars glucosamine and muramic acid, were detected by electrospray mass spectrometry (ESI-MS) (Figs. 4A and 5A) using a low cone voltage to minimize in-source fragmentations. The ESI mass spectra of the respective peptidoglycan hydrolyzates also exhibited ions at the *m/z* values expected for protonated 2,6-diaminopimelic acid (*m/z* 191, Fig. 4A) and lanthionine (*m/z* 209, Fig. 5A). Only one of the diamino acid ions was present in each mass spectrum, suggesting that 2,6-diaminopimelic acid and lanthionine are specifically associated with *F. varium* and *F. nucleatum*, respectively.

With tandem MS instrumentation, it is possible to select one ion from a complex mixture and generate an MS-MS spectrum by subjecting the ion to collision-induced fragmentation. If the MS-MS spectrum of the selected ion is identical to the spectrum of the corresponding ion derived from a standard sample, there is a high probability that the standard analyte is a component in

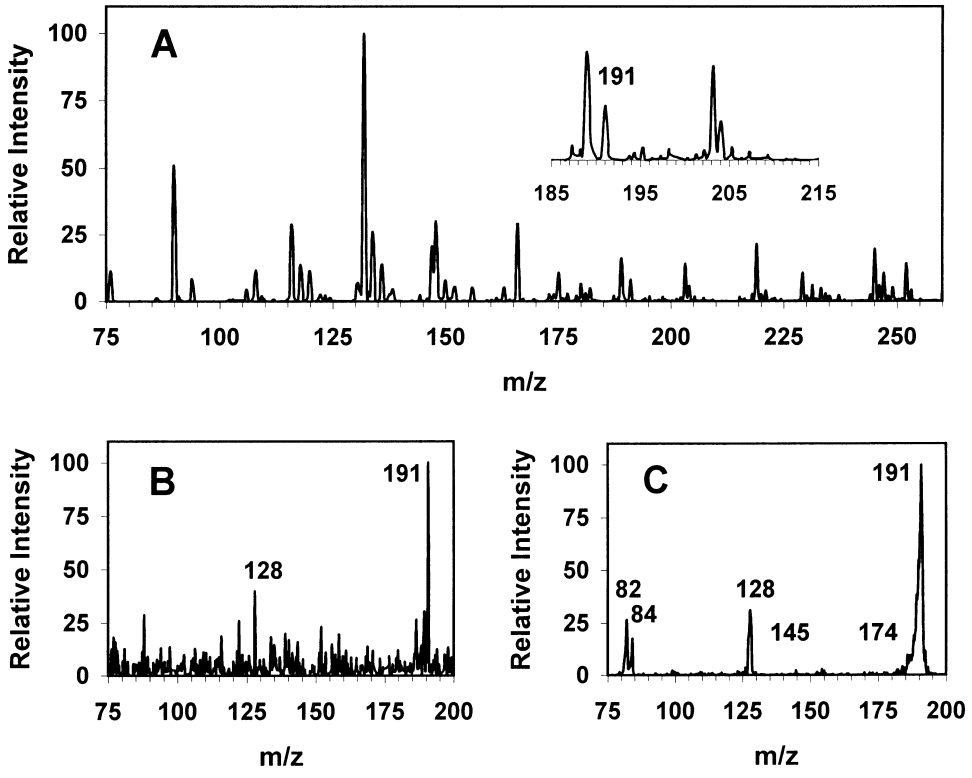


**Fig. 4.** MS Analysis: **A** ESI mass spectrum of a peptidoglycan hydrolyzate from *F. varium*. Ions correspond to $M+H]^+$ of Gly (*m/z* 76), Ala (*m/z* 90), Ser (*m/z* 106), Pro (*m/z* 116), Val (*m/z* 118), Thr (*m/z* 120), Leu/Ile (*m/z* 132), Asp (*m/z* 134), Lys (*m/z* 147), Glu (*m/z* 148), His (*m/z* 156), Phe (*m/z* 166), Arg (*m/z* 175), glucosamine (*m/z* 180), $A_2$pm (*m/z* 191), and muramic acid (*m/z* 252); **B** CID mass spectrum of the ion at *m/z* 191 in **A**; **C** CID mass spectrum of standard 2,6-diaminopimelic acid (**1**)

the sample mixture. This approach was used to confirm the assignment of ions to the diamino acids. However, the signal strength of the ion at *m/z* 191 as shown in Fig. 4A is low, which made it difficult to obtain a good MS-MS spectrum from this ion. Nevertheless, the spectrum shown in Fig. 4B was reproducible in several different runs, consistently generating a fragment ion at *m/z* 128. On the other hand, a prominent ion at *m/z* 120 (Fig. 5B) was obtained by fragmentation of the *m/z* 209 ion. Under identical CID conditions, standard samples of 2,6-diaminopimelic acid and lanthionine yielded major fragment ions at *m/z* 128 (Fig. 4C) and *m/z* 120 (Fig. 5C), respectively. The formation of identical major fragment ions from the sample and standard provides strong supporting evidence for the presence of 2,6-diaminopimelic acid and lanthionine in the peptidoglycan hydrolyzates.

Many mass spectral fragmentations of protonated α-amino acids induced by collisional activation show characteristic patterns, which can be observed by MS-MS and used to assist with their identification. It is generally agreed that loss of $H_2O$ and CO is an important fragmentation pathway of protonated amino acids (Dookeran et al., 1996; O'Hair et al., 2000; Rogalewicz et al.,

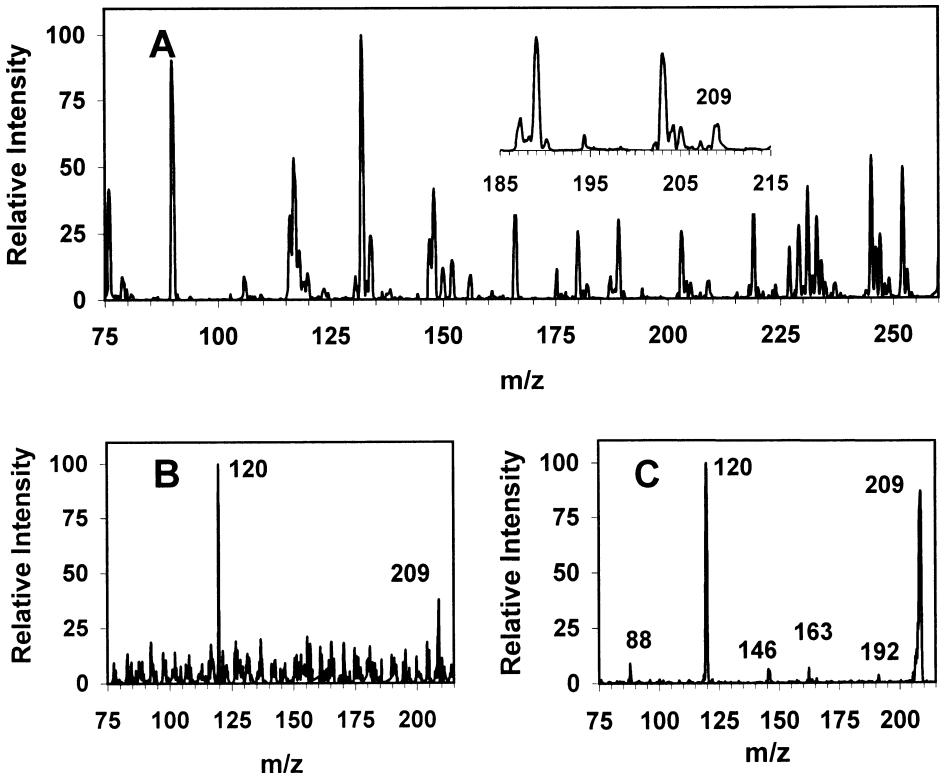


**Fig. 5.** MS Analysis: **A** ESI mass spectrum of a peptidoglycan hydrolyzate from *F. nucleatum*; $M+H]^+$ ion of Lan (*m/z* 209) and other ions as described in Fig. 4A, except $A_2$pm; **B** CID mass spectrum of the ion at *m/z* 209 in A; **C** CID mass spectrum of standard lanthionine (**2**)

2000). The ions at *m/z* 132 ($M+H]^+$ ion of leucine/isoleucine) in the *F. varium* hydrolyzate and *m/z* 166 ($M+H]^+$ ion of phenylalanine) in the *F. nucleatum* hydrolyzate showed major fragment ions due to the loss of 46 mass units, but the $M+H]^+ - 46$ ion (**1f**, Fig. 6) is a minor species in the mass spectra of the standard diamino acids (Figs. 4C and 5C).

The structure of protonated diaminopimelic acid, **1a**, is closely related to that of protonated lysine; each contain an amino group at the α and ε position. The ESI mass spectrum of protonated lysine shows another important fragmentation, namely the loss of $NH_3$, in addition to the loss of $H_2O$ and CO (Rogalewicz et al., 2000; Yalcin and Harrison, 1996). In the gas phase, the second amino group is unlikely to be protonated as part of a zwitterion, and is able to assist the loss of $NH_3$ (Rogalewicz et al., 2000). The major fragment ion at *m/z* 128 arising from protonated diaminopimelic acid is consistent with the loss of $H_2O$, CO and $NH_3$ from **1a** (Fig. 6). A feature of the proposed structure for **1a** is that it contains three reasonably strong hydrogen bonds (not shown), which should stabilize this conformation of the ion in the gas phase and promote the proposed attack of the free amino group at the α position on the proton at the δ carbon. Ion **1c** is the result of two favorable fragmentations, and is analogous to the lysine fragmentation pathway. In the

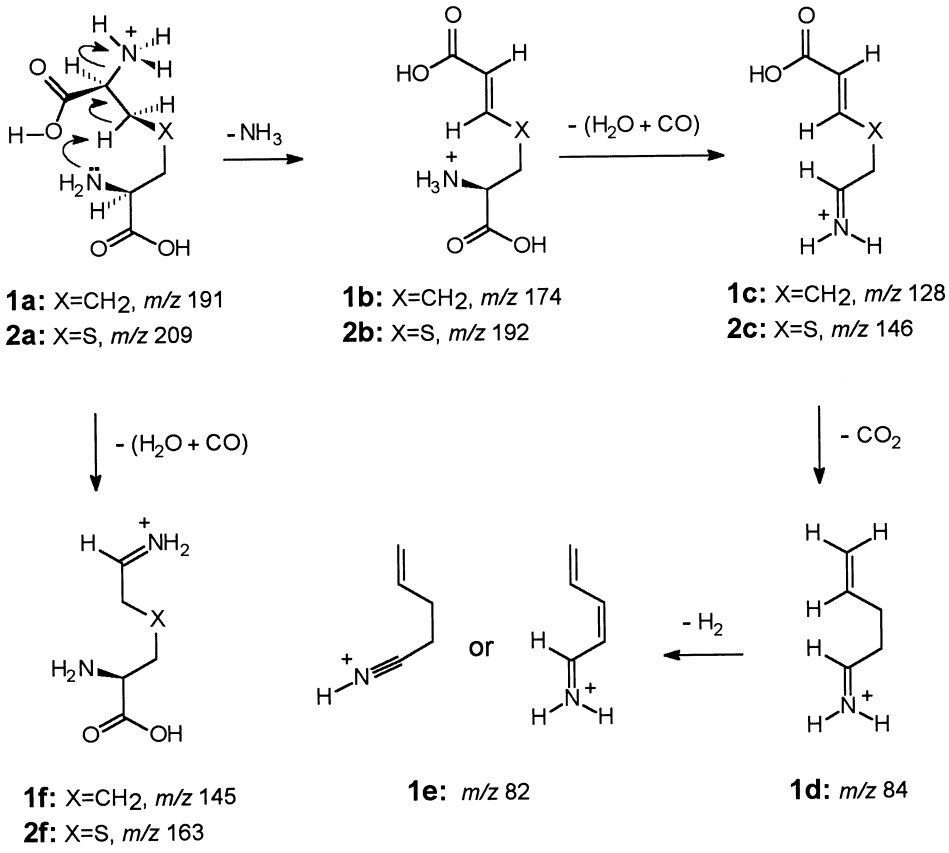


**Fig. 6.** Collision-induced fragmentation pathway of protonated 2,6-diaminopimelic acid (**1a**) and the corresponding minor fragmentations of protonated lanthionine (**2a**)

case of 2,6-diaminopimelic acid, however, the fragmentation pathway is extended by loss of the second carboxylic acid group, generating **1d**, which subsequently loses $H_2$, forming **1e**, shown as two feasible structures in Fig. 6.

Protonated lanthionine, **2a**, should exist in a very similar conformation to that of **1a**, and a similar fragmentation pathway would be expected. Indeed the MS-MS spectrum (Fig. 5C) of **2a** displays low intensity peaks for the analogous fragment ions **2b**, **2c**, and **2f** (Fig. 6), but the intense ion at *m/z* 120 indicates that another fragmentation pathway becomes accessible when sulfur is present. Generation of an ion at *m/z* 120 most likely involves carbon–sulfur bond cleavage (Fig. 7). That ion **2g** appears to be formed more readily than any of the ions in the MS-MS spectrum of **2a** is possibly the result of at least two strong intramolecular hydrogen bonds in **2g**. It may be that **2g** could exist in other tautomeric forms, but again, these would also possess hydrogen bonds. Thus, **2g** provides a useful diagnostic tool for the detection of lanthionine by ESI-MS-MS in complex mixtures (Fig. 5A).

In conclusion, a reversed phase HPLC method with pre-column derivatization was developed and used to detect *meso*-2,6-diaminopimelic acid and *meso*-lanthionine in peptidoglycan hydrolyzates of *F. varium* and

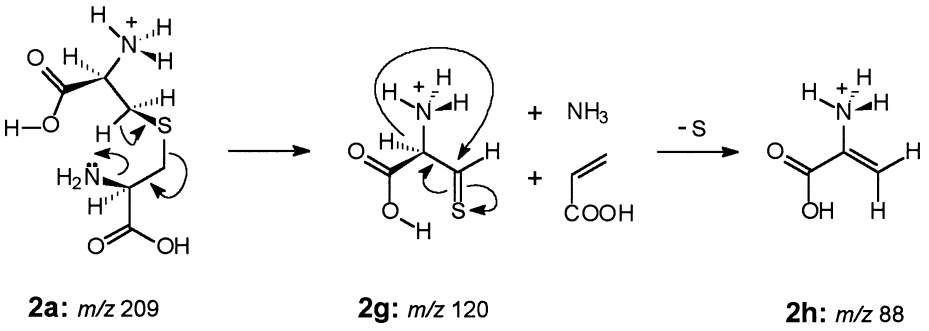


**Fig. 7.** Major collision-induced fragmentation pathway of protonated lanthionine (**2a**)

*F. nucleatum*, respectively. The chromatographic identification of 2,6-diaminopimelic acid and lanthionine was confirmed by ESI-MS and ESI-MS-MS analysis of peptidoglycan hydrolyzates, demonstrating that extensive sample purification is not required to identify diamino acids in these complex mixtures. The chromatographic and MS results strongly suggest that neither organism utilizes both 2,6-diaminopimelic acid and lanthionine in the peptidoglycan crosslinks. The present results are consistent with previous reports of *meso*-2,6-diaminopimelic acid in *F. varium* and *meso*-lanthionine in *F. nucleatum* (Kato et al., 1981; Miyagawa et al., 1981; Vasstrand et al., 1982).

The importance of the peptidoglycan layer for maintaining the integrity of bacterial cells and the success of the β-lactam and glycopeptide antibiotics, which inhibit cross-linking of the glycan chains (Bugg and Walsh, 1992), have stimulated searches for new antibiotics that disrupt other steps of peptidoglycan biosynthesis (Bugg and Walsh, 1992), particularly inhibitors of 2,6-diaminopimelic acid biosynthesis (Cox et al., 2000). In addition to differences in peptidoglycan composition, these two *Fusobacterium* species exhibit different preferences for the utilization of amino acids (Ramezani et al., 1999) and are reported to utilize different pathways for the catabolism of glutamate (Gharbia and Shah, 1991). Differences in the production of energy by anaerobic bacteria may reveal essential steps for the catabolism of amino acids that could serve as metabolic targets for novel antibiotics. Ongoing studies in our laboratory are being carried out to further define the metabolic differences between *F. varium* and *F. nucleatum*.

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