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Interstrain Diversity of 2-keto-3-deoxyoctonate Content in Lipopolysaccharides of *Desulfovibrio desulfuricans*

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Abstract

Bacteria of *Desulfovibrio desulfuricans* species are Gram-negative, anaerobic rods selectively reducing sulphates and colonizing oxygen-free ecosystems. They are ubiquitous in the natural environment and have been also found to reside in the human digestive tract. They are suggested to be involved in the pathogenesis of ulcerative colitis and Crohn's disease. The *D. desulfuricans* wild strains were isolated from feces and bioptate of patients suffering from various digestive tract disorders. LPSs were isolated from the wild enteric strains and soil type strain La 2226 of *D. desulfuricans* and analyzed in terms of their 2-keto-3-deoxyoctulosonic acid (Kdo) component content. The obtained spectrophotometric data indicate that Kdo content is characteristic of each of the investigated strains and it ranges from 0.48% to 2.86% (w/w) of the total LPS mass. Statistically significant interstrain differences of Kdo quantity seem to suggest the differences in the O-antigen content. Comparative analysis of Kdo content in LPSs of *D. desulfuricans* strains in relation to that of the reference endotoxin from *Salmonella* spp. allows us to suggest that *D. desulfuricans* bacteria possess O-antigen polysaccharides composed of diverse number of carbohydrate units.

Key words: *Desulfovibrio desulfuricans*, 2-keto-3-deoxyoctulosonic acid, lipopolysaccharide

Introduction

Endotoxin, also called lipopolysaccharide (LPS) is a major factor of pathogenicity responsible for many pathophysiological activities accompanying infections caused by Gram-negative bacteria. This heteropolimer demonstrates significant structural similarity across species of Gram-negative bacteria and is composed of lipid A, core oligosaccharide and O-specific fragment. Lipid A, a biologically active component of LPS, is linked to the polysaccharide constituent of endotoxin through 2-keto-3-deoxyoctulosonic acid (Kdo), which is bound at carbon 6' of nonreducing glucosamine of lipid A. Kdo is believed to be an important structural element of Gram-negative bacteria endotoxin, conditioning essential functions for their life. No bacteria devoid of this sugar in LPS could have been cultured so far. LPS of intracellular pathogens such as *Chlamydia trachomatis* and *Ch. psittaci* has a core region composed of Kdo only (α-Kdo-2,8-Kdo-2,4-α-Kdo) (Brade *et al.*, 1986; Holst *et al.*, 1993; Holst *et al.*, 1994). Capable of growing Re mutants of *E. coli* F515, *S. minnesota* R595 and *P. mirabilis* R45 form LPS consisting of two Kdo residues and lipid A (Brade and Rietschel, 1984, Sidorczyk *et al.*, 1987). LPS of *Haemophilus influenzae* I-69-Rd^{-}/b^{+} mutant contains one phosphorylated molecule of this sugar only (Helander *et al.*, 1988).

Variable numbers of Kdo residues have been identified in various bacterial LPS. Species such as *Campylobacter jejuni* (Aspinall *et al.*, 1993), *Shigella flexneri* (Katzenellenbogen and Romanowska, 1980), *Yersinia enterocolitica* 75R (Radziejewska-Lebrecht *et al.*, 1994), *Vibrio parahaemolyticus* O12 (Kondo *et al.*, 1991), *Citrobacter freundii* O4 and O36 (Romanowska *et al.*, 1988) contain one Kdo, whereas *Klebsiella pneumoniae* (Severn *et al.*, 1996), *Erwinia carotovora* FERM P-7576 (Fukuoka *et al.*, 1997), *Legionella pneumophila* (Knirel *et al.*, 1996) contain two Kdo moieties in their LPS. Three molecules of

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this acid have been found in endotoxin of *Escherichia coli* R3 (Jansson *et al.*, 1981; Haishima *et al.*, 1992), *Coxiella burnetii* (Toman and Skultety, 1996), *Klebsiella pneumoniae* ssp. *pneumoniae* rough strain R20 (O1-K20-) (Süsskind *et al.*, 1998) and even four of it in *Acinetobacter baumannii* strain NCTC 10303 (Vinogradov *et al.*, 1998) oligosaccharide core. Forsberg and Carlson (1998) detected the presence of three Kdo residues in the LPS core region of *Rhizobium etli* CE358 and CE359 strains, and one of them was found in the outer fragment of the LPS component. Similar core structure was identified in the LPS of the wild-type strain CE3 (D'Haeze *et al.*, 2007).

The growing interest in bacteria of *Desulfovibrio desulfuricans* species arose from 1970s, when their presence in human feces was demonstrated. It has been suggested that under certain conditions these bacteria can become etiologic factor of various types of enteritis. They have been found to reside in increased population in the digestive tract of patients suffering from ulcerative colitis and Crohn's disease (Florin *et al.*, 1990; Gibson *et al.*, 1991; Tee *et al.*, 1996; Lozniewski *et al.*, 1999).

So far it is little known about the chemical structure of *D. desulfuricans* endotoxin. Gaylarde and Beech (1996) identified heptadecenoic, 8-octadecynoic, 9-octadecenoic, 10-octadecenoic, eicosenoic and tetracosenoic, acids in lipid A, and glucose, galactose, rhamnose, mannose and ribose in polysaccharide chain of these bacteria LPS, although ribose has been suggested to result from contamination of LPS extract by the nucleic acids. However, they did not find Kdo in *D. desulfuricans* endotoxin. Thus, this crucial component of LPS has not been identified in the studied species up to the present.

The aim of the study was to analyze the Kdo occurrence in *D. desulfuricans* lipopolysaccharides and to determine the quantitative relationship between LPS and Kdo in case of Kdo identification.

Experimental

Materials and Methods

Bacterial strains and their cultivation. The *D. desulfuricans* soil strain La2226 (DMS 642, Swiss National Collection of Type Cultures; soil, England) and *D. desulfuricans* wild intestinal strains (DV/A, DV/B, DV/C, DV/H, DV/I, DV/I/1) have been used. Intestinal strains were isolated from feces and caecum specimen derived from the patients diagnosed for diseases specified in Table I. Commercially available lipopolysaccharides from *Salmonella minnesota*, *Salmonella typhimurium*, and *Salmonella typhimurium* (Ra mutant) have been used as references, and were purchased from Sigma.

Bacteria have been cultured in Postgate's medium, pH 7.5, at 30°C for 10 days under anaerobic conditions (80% N_2, 10% H_2 and 10% CO_2) (Postgate, 1984).

LPS isolation and Kdo determination. LPS was isolated from bacterial mass by phenol-water extraction method, according to the procedure described by Johnson and Perry (1976). Kdo content in LPS has been determined spectrophotometrically following their derivatization by the use of thiobarbituric method of Karkhanis *et al.* (1978). The first step of this method involves hydrolysis of LPS with sulphuric (VI) acid at 100°C for 30 minutes which leads to the liberation of Kdo. Then, Kdo reacts successively with periodic acid, sodium arsenite (III) and thiobarbituric acid to form a chromophore whose absorbance is measured at 550 nm and used for determination of Kdo quantity in endotoxins.

The identification of Kdo in investigated bacteria LPS by chromatography. LPS isolated from *D. desulfuricans* bacteria has been methanolyzed for 1 hour at 100°C with 2M methanolic hydrochloric acid (0.5 ml), obtained in the reaction of acetyl chloride with anhydrous methanol. The sample has been evaporated under the stream of argon and obtained methyl glycosides have been acetylated at 100°C by 30 minutes with a mixture of acetic anhydride (100 µl) and pyridine (100 µl). Acetylation reagents have been removed under a stream of argon and sample has been washed by methanol twice and applied on GLC/MS (Rybka and Gamian, 2006). Chromatography has been performed on Hewlett Packard HP5890 apparatus equipped with HP-1MS capillary column (60 m – length, 0.32 mm – internal diameter, 0.25 µm – film) at programmed temperature: 50°C initial temperature for 1 min, raised to 160°C with 20°/min and next raised to 260°C with 4°/min. Separated products have been analyzed by Hewlett Packard HP 5989A mass spectrometer. Ionization has been performed by 70 eV electron impact (ion source temperature – 200°C, quadrupole – 100°C).

Statistical analysis. To validate the results regression line model $y=ax+b$ estimated by the least squares method has been used. The significance of the regression coefficient (a) and y intercept (b) has been verified by the *t*-test. The strength of linear relationship has been assessed by Pearson's correlation coefficient (r), whose significance has also been verified by the *t*-test.

One-factor analysis of variance (ANOVA) has been used to analyze the statistical significance of differences between Kdo mean quantities in LPS from investigated enteric strains of *D. desulfuricans*. The multiple comparison of each mean quantity of Kdo in 1 mg of LPS derived from enteric strains has been performed using the Tukey's post-hoc test.

Differences between mean quantities of Kdo in LPS from the La2226 standard soil strain and each of

Table I
The sources of intestinal strains (Dzierżewicz *et al*., 1994)

Wild intestinal strains of *D. desulfuricans*	Diagnosis	Material
DV/A	asiderotic anaemia, cholestasis	feces
DV/B	hepatitis	
DV/C	Crohn's disease, asiderotic anaemia, megaloblastic anaemia	
DV/H	colon polypus, cholelithiasis, erosive gastritis, urinary tract infection, appendectomy hysterectomy in the past, and adnexectomy in the past	
DV/I	colonic diverticulosis, cholecystectomy in the past, viral hepatitis B in the past	feces
DV/I/1		biopsy specimen from caecum

Table II
Quantitative characteristics of Kdo of lipopolysaccharides from the studied bacteria

Bacteria		$A_{n=6}$ ± SD	m_{Kdo} ± SD [µg/mg LPS]	n_{Kdo} ± SD [µmol/mg LPS]	*p (test t)	ANOVA
D. desulfuricans	*La2226*	1.2074 ± 0.048	28.60 ± 1.13	0.120 ± 0.005		
	DV/A	1.0882 ± 0.022	25.77 ± 0.52	0.108 ± 0.002	0.0001	F (5.30) = 2381.1; p<0.0001
	DV/B	0.2472 ± 0.011	5.86 ± 0.26	0.025 ± 0.001	<0.000001	
	DV/C	0.5932 ± 0.014	14.05 ± 0.32	0.059 ± 0.001	<0.000001	
	DV/H	0.7001 ± 0.022	16.58 ± 0.53	0.070 ± 0.002	<0.000001	
	DV/I	0.5056 ± 0.009	11.98 ± 0.21	0.050 ± 0.001	<0.000001	
	DV/I/1	0.2021 ± 0.015	4.79 ± 0.36	0.020 ± 0.002	<0.000001	
S. minnesota		0.5877 ± 0.017	13.92 ± 0.40	0.058 ± 0.002		
S. typhimurium		0.6708 ± 0.011	39.72 ± 0.64	0.167 ± 0.003		
S. typhimurium mutant Ra		1.0239 ± 0.043	60.63 ± 2.57	0.255 ± 0.011		

$A_{n=6}$ – absorbance of Kdo derivative measured at 550 nm and expressed per 1 mg of LPS
m_{Kdo} – Kdo quantity [µg] per 1 mg of LPS
n_{Kdo} – Kdo quantity [µmol] per 1 mg of LPS
SD – standard deviation
* – comparison of Kdo mean quantities in LPS between *D. desulfuricans* soil strain La2226 and enteric strains

the enteric strains have been verified by the *t*-test for independent samples. If variances of the two compared samples appeared to be significantly different, *t*-test with independent variance estimation (Cochran-Cox test) has been applied. Normality of distributions has been verified by the Shapiro-Wilk test, while the homogeneity of variances has been ascertained by the *F*-test or the Levene test. The results have been considered significant when $p<0.05$. Statistical analysis have been carried out using the Statistica v 6.0 software.

Results and Discussion

Numerous functional groups (carboxylic, ketonic and hydroxylic) present in the Kdo structure make this endotoxin component troublesome in respect of chemical analysis. This unique sugar is readily decomposed during derivatization (Kiang *et al.*, 1997; Tacken and Brade, 1986; Tacken *et al.*, 1986), and therefore its determination by chromatography is very difficult.

Gaylarde and Beech (1996) and Bradley and Gaylarde (1988) suggested the lack of Kdo in LPS from *D. desulfuricans* bacteria. In the present study we made an attempt at identifying Kdo presence in these bacteria by chromatography (GLC/MS) of acetylated methyl glycosides (Rybka and Gamian, 2006) and spectrophotometry following the use of thiobarbituric method (Karkhanis *et al.*, 1978; Lee and Tsai, 1999).

The GLC/MS analysis of acetylated methyl glycosides confirmed the presence of Kdo in investigated structures. The chromatogram and electron impact mass spectra of Kdo derivatives are shown on Fig. 1.

Comparative spectral characteristics of Kdo standard derivative and Kdo derived from *D. desulfuricans* LPS let us also suggest the presence of Kdo in endotoxins of the all studied *D. desulfuricans* strains. Spectrum of Kdo of *D. desulfuricans* soil strain and that of the standard Kdo are shown in Fig. 2. The Kdo contents in endotoxins isolated from the all studied strains are presented in Table II. They are expressed

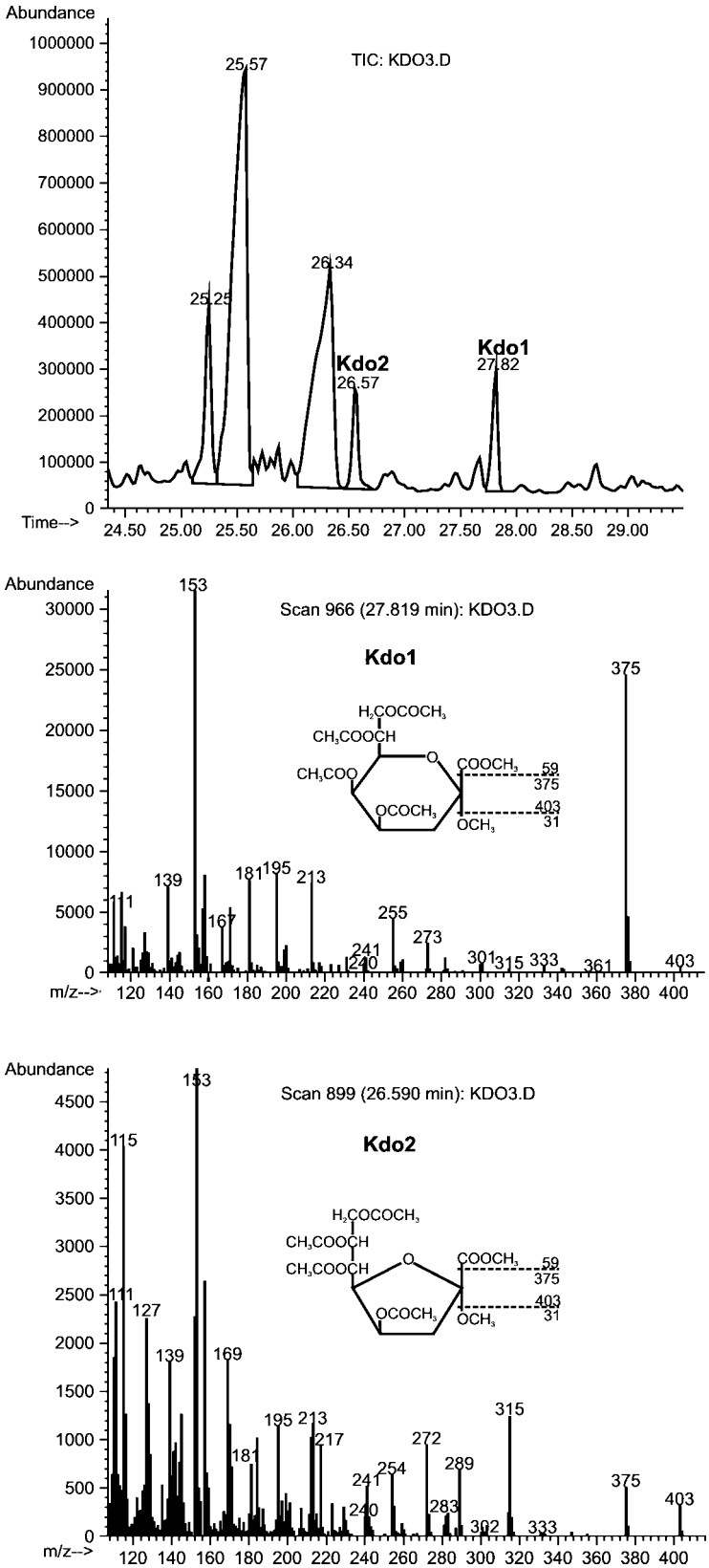

Fig. 1. The chromatogram and electron impact mass spectra of Kdo acetylated carboxymethyl ester methyl glycosides obtained from LPS of *D. desulfuricans* strain DV/A bacteria

in terms of the mean absorbance values (at $\lambda = 550$ nm) of Kdo derivative per 1 mg of LPS, the amount of Kdo (μg) calculated from the regression equation plot ($y = 0.0844x$, $r = 0.9986$) per 1 mg of LPS, μmoles of Kdo per 1 mg of LPS. The quantities of Kdo in endotoxins from *D. desulfuricans* strains ranged from 4.8 μg/mg as found for the DV/I/1 strain to 28.6 μg/mg for La2226 strain (Table II).

Earlier extensive studies performed by Dzierżewicz *et al.* (1994) on the phenotype characteristics of

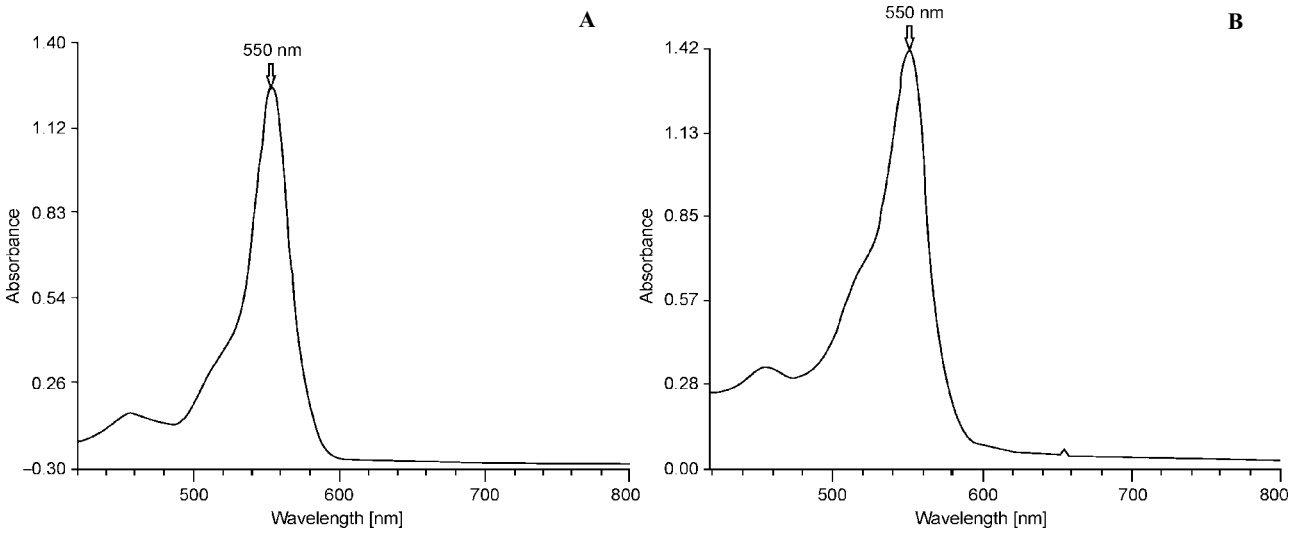

Fig. 2. Spectra of Kdo chromophore in reaction with thiobarbituric acid (A – Kdo standard, B – LPS from *D. desulfuricans* La2226 strain)

D. desulfuricans strains isolated from human digestive tract and derived from the soil revealed essential interstrain differences related to the different types of environment. Therefore, in this work the Kdo content calculated per 1 mg of LPS isolated from each of the investigated enteric strains has been compared with that of the soil strain (La2226), and have been observed statistically significant differences.

A comparison of the Kdo quantities also showed significant differences among all investigated *D. desulfuricans* strains (one-way ANOVA, Table II). The Tukey's test confirmed high statistical significance ($p<0.001$) of Kdo mean quantities for each of the analysed pairs.

The differences in Kdo contents in LPS of *D. desulfuricans* strains may result from macromolecular heterogeneity of their LPS. On the basis of electrophoretic analysis, Dzierżewicz *et al.* (2005) suggested higher content of molecules with long carbohydrate chains in endotoxin isolated from the DV/A enteric strain than that in the soil strain LPS. Smooth-type lipopolysaccharides, composed of lipid A, core oligosaccharide and O-antigen are known to have molecular weight greater than rough-type LPS devoid of O-antigen portion (Lee and Tsai, 1999). As a consequence, a percentage of Kdo in smooth forms LPS is relatively lower when compared to that in LPS with shorter carbohydrate fragment. Small percentage content of Kdo in endotoxins from *D. desulfuricans* in contrast to that in LPS from *Salmonella* Ra LPS suggests that *D. desulfuricans* belongs to microorganisms containing O-antigen in its lipopolysaccharide. Based on the foregoing assumption it can be expected that the most smooth LPS are those of DV/I/1 and DV/B strains whereas the most rough are represented by La2226 and DV/A strains. The reliability of this suggestion may be supported by the findings of Dzierżewicz *et al.* (2001) whose study revealed 100% compatibility of the DV/A enteric strain with the soil strain La2226 in their susceptibility to various antibiotics, and the known relationship between the length of LPS polysaccharide chain and the antibiotic resistance of microorganisms.

Furthermore, a significant statistical difference ($p<0.001$) in the Kdo content between DV/I and DV/I/1 strains isolated from the same host has been found. It is worth to note that the findings of Dzierżewicz *et al.* (2003) indicated relatively small similarity between genetic profiles of those two strains, their different susceptibility to antibiotics (Dzierżewicz *et al.*, 2001) and relatively low similarity (98.2%) of cellular fatty acid profiles (Dzierżewicz *et al.*, 1996).

The thiobarbituric assay cannot be applied for determination of Kdo substituted at C4 or C5, due to impossibility of its oxidation by periodic acid acid (HIO_4) (Lee and Tsai, 1999). Caroff *et al.* (1987) also suggested the lack of Kdo reactivity in case of its substitution at C4 by phosphate or phosphoethanolamine. This has also been observed for LPS from *Bordetella pertussis*. These bacteria produce two kinds of lipopolysaccharides, one of which reacts with TBA to give chromophore product and the other is devoid of this ability due to the phosphorylation of Kdo. Based on our findings, it can be concluded that Kdo in the endotoxins of *D. desulfuricans* is not substituted at C4 or C5.

It should be also mentioned, that 3,6-dideoxyhexoses – abequose and colitose have the ability to react with TBA (Kiang *et al.*, 1997), but their presence in endotoxins of *D. desulfuricans* has been excluded by our results of chromatographic analysis (Lodowska *et al.*, 2003).

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