Relationship between Extracellular Polysaccharide and Benzene Tolerance of *Rhodococcus* sp. 33

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Benzene is one of the most toxic and prevailing environmental pollutants. *Rhodococcus* sp. 33 can tolerate and efficiently degrade various concentrations of benzene. Under either resting or growth conditions, rough mutant strains derived from strain 33 were more sensitive to benzene than the mucoidal parent strain. The rough strains did not produce extracellular polysaccharides (EPS), whereas the parental strain 33 did so in large quantity (33 EPS). By the addition of 33 EPS to the rough strains, both the survival and growth of the rough strains in media containing benzene were improved. The 33 EPS was found to be an acidic polysaccharide containing D-galactose, D-glucose, D-mannose, D-glucuronic acid, and pyruvic acid at a molar ratio of 1: 1: 1: 1: 1. These data suggest that the 33 EPS plays an important role in the benzene tolerance in *Rhodococcus* sp. 33, especially helping the cells to survive an initial challenge with benzene.

INTRODUCTION

Rhodococcus sp. 33 was isolated from a contaminated site near a chemical plant at Port Botany in Sydney, Australia, based on its ability to tolerate and efficiently degrade various concentrations of benzene up to its maximal solubility (1.8 g/l) in aqueous media¹. To elucidate the mechanism by which strain 33 tolerates such high concentrations of benzene, Gutierrez *et al.* examined the effects of benzene on cellular fatty acid composition of strain 33², because benzene is widely accepted to cause an increase in fluidity of cell membranes³ and a number of reports have shown that various hydrocarbon-tolerant microorganisms produce qualitative and quantitative changes in their membrane fatty acids in response to toxic organic compounds to decrease the fluidity of their cell membranes^{3–5}. The results showed an increase in the ratio of saturated/unsaturated cellular fatty acids in the presence of benzene from 1.3 (for mannitol-grown cells) to 1.5 (for benzene-grown cells). As expected from the increase in the ratio of saturated/unsaturated cellular fatty acids, the fluidity of the membranes of strain 33 decreased after 18–20 h incubation in the presence of benzene, as found by measuring the generalized polarization of the fluorescent membrane probe laurdan by fluorescence spectroscopy⁶. These data suggested a decrease in the fluidity of the membranes caused by changes in the cellular fatty acid composition as a possible mechanism for benzene tolerance.

Although the fluidity of the membranes was observed to decrease after the cells had been exposed to benzene for approximately 18–20 h, the immediate effect of benzene on the membranes was an increase in fluidity, which was observed for more than 6 h. These data suggest that an adaptive response, set to mitigate benzene toxicity, is induced only after long exposure and that with respect to the organism's tolerance during the first 6 h, *Rhodococcus* sp. 33 may have a well-adapted inherent mechanism to survive an initial challenge with benzene.

Recently, Iwabuchi *et al.* reported that a mucoidal strain of *Rhodococcus rhodochrous* was resistant to an aromatic fraction of the crude oil (AF), whereas its rough derivatives were sensitive; and so they suggested an association between mucoidal morphology and the ability to tolerate the AF⁷. Since *Rhodococcus* sp. 33 forms mucoidal colonies on nutrient agar, we examined a relationship between the mucoidal morphology and the ability to tolerate benzene.

In this study, we isolated morphological mutants from colonies of strain 33 and characterized their tolerance to benzene in order to elucidate mechanisms other than the compositional change in the fatty acids in the cell membranes.

MATERIALS AND METHODS

Bacterial strain and medium

Spontaneous rough mutants, R0, R1, R2, R3, R4, and

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R5, were isolated from *Rhodococcus* sp. 33. These strains were cultivated on PAS liquid media with either mannitol, benzene or both as carbon sources, as described previously¹⁾. *Rhodococcus rhodochrous* S-2 cells⁸⁾ were cultivated on IB agar plates⁹⁾. Nutrient agar (Oxoid, Basingstoke, Hampshire UK) was also used for growth and maintenance of these strains.

Extraction and purification of rhodococcal EPS

EPS produced by *Rhodococcus* sp. 33 (33 EPS) and by *Rhodococcus rhodochrous* S-2 (S-2 EPS) were removed from the cells by shaking and partially purified by enzymatic treatments, phenol/chloroform treatment and ethanol precipitation as described by Urai *et al.*¹⁰⁾ These partially purified EPS were further purified by DEAE-Toyopearl 650M column chromatography as described by Urai *et al.*¹¹⁾

Benzene tolerance test

Growth conditions: A loopful of bacterial cells grown on nutrient agar plates was inoculated into 50 ml of PAS medium containing 1% mannitol in a 250-ml shake flask and incubated with shaking for 3 days. After incubation, 2.5 ml of this culture was inoculated into 50 ml of fresh PAS medium containing 1% mannitol and incubated for 24 h. Then, 2.5 ml of this culture was transferred into 50 ml of PAS medium equilibrated with benzene in a 250-ml shake flask, and the purified 33 EPS was added to the media at a final concentration of 0.1 mg/ml. The media were incubated with shaking. PAS medium equilibrated with benzene was prepared as follows: Fifty milliliters of PAS medium was dispensed into a 250-ml shake flask, and an empty test tube (100 mm × 10 mmφ) was placed inside the flask. Benzene was added to the test tube to allow its vapor to diffuse into the medium and the headspace. The flask was plugged with cotton to allow diffusion of air into the system. The flask was preincubated with shaking (100 rpm) for 1 h before inoculation. All procedures were performed at 28°C.

Resting conditions: Bacterial cells grown on nutrient agar plates were harvested and resuspended in 37.5 mM potassium-phosphate buffer (pH 6.9) at approximately 10⁹ cfu / ml. Two milliliters of the cell suspension were transferred into 50 ml of 37.5 mM potassium-phosphate buffer (pH 6.9) equilibrated with benzene as described above, and the purified 33 EPS was added to the buffer at a final concentration of 0.1 mg/ml. The cell suspensions were incubated with shaking for 1 h. All procedures were performed at 28°C.

Analysis of neutral sugars and uronic acid

The purified 33 EPS was hydrolyzed as follows: Ten microliters of 33 EPS aqueous solution (5 mg/ml) was mixed with 20 µl of 99 % trifluoroacetic acid, incubated at 100°C for 18 h, and then dried *in vacuo*. The hydrolyzate was dissolved in 10 µl of MilliQ water, mixed with

40 µl of 4-aminobenzoic acid ethyl ester (ABEE) labeling reagent (Seikagaku Corporation, Tokyo, Japan), and heated at 80°C for 1 h. After cooling to room temperature, 200 µl of MilliQ water and 200 µl of chloroform were added; and the 33 EPS was then centrifuged at 10,000 × *g* for 10 min. The aqueous phase was recovered and filtered through a 0.2-µm-pore-size membrane filter to remove particulate materials. ABEE-labeled saccharides were analyzed by high-performance liquid chromatography (HPLC; Shimadzu LC-6A; Shimadzu, Kyoto, Japan) with a Honenpak C18 column (75 mm × 4.6 mmφ; Honen Co., Tokyo, Japan) and a mixture of 200 mM borate buffer and acetonitrile (93:7, V/V). A mixture of L-arabinose, L-fucose, D-galactose, D-glucose, D-mannose, L-rhamnose, D-xylose, D-galacturonic acid, and D-glucuronic acid (1 mg/ml each) was treated as described above and then employed as authentic substances for reference.

Chemical modification of 33 EPS

Carboxyl reduction of 33 EPS: Ten milligrams of 33 EPS was reduced as described by Richards *et al.*¹²⁾ The HPLC analysis of the reduced 33 EPS hydrolyzate revealed that most of the glucuronic acid residues had been reduced to glucose (data not shown).

Partially-hydrolyzed 33 EPS: Ten milligrams of 33 EPS was dissolved in 10 ml of 2% trifluoroacetic acid, heated at 100°C for 3 h, and then lyophilized. The lyophilized sample was dissolved in 1 ml of 0.2 M acetic acid, applied onto a Bio-Gel P-2 gel filtration column (900 mm × 15 mmφ), and eluted with 0.2 M acetic acid. Fractions containing oligosaccharides ranging from pentamers to dodecamers were combined and lyophilized (data not shown). Almost all of the acetal bonds between pyruvic acid and saccharide chain should be cleaved under the conditions of hydrolyzation described above.

Analytical methods

Reducing sugar content was determined by the phenol-sulfuric acid method¹³⁾. Pyruvic acid content was enzymatically determined with an F-kit Pyruvic acid (J. K. International Co., Tokyo, Japan), as described by the supplier, after hydrolysis with 2% TFA at 100°C for 120 min. Fatty acid analyses were performed as described previously¹⁰⁾.

Gel filtration column chromatography for native 33 EPS was performed by using Sephacryl S1000 (850 mm × 15 mmφ; Amersham Biosciences UK Limited, Buckinghamshire, UK) as resin, 1 mM Tris-HCl (pH8.0) containing 0.1 M NaCl as eluent, and Dextran T2000 (Amersham Biosciences UK Limited, Buckinghamshire, UK) as size marker.

The purified 33 EPS was dissolved in 0.15 M Tris-HCl (pH 7.4) at 1 mg/ml, and its reduced viscosity was determined by using an Ostwald viscosity meter No. 1 operated at 27°C.

RESULTS

Isolation of rough mutant strains

Six spontaneous rough mutants, R0, R1, R2, R3, R4, and R5, were isolated from *Rhodococcus* sp. 33 on nutrient agar plates or PAS agar plates containing 1% mannitol as the carbon source. In the presence of benzene these spontaneous rough mutants showed no or little growth ($OD_{600} < 0.1$), whereas their parental strain, *Rhodococcus* sp. 33, showed good growth ($OD_{600} = 0.65 \pm 0.04$)¹⁾, which was as good as the growth on mannitol as carbon source (Table 1). These rough strains did not produce the extracellular polysaccharides (EPS), whereas the parental strain did.

Purification and physicochemical characterization of 33 EPS

EPS produced by the *Rhodococcus* sp. 33 was extracted and purified by ion-exchange chromatography (Fig. 1). The purified 33 EPS was eluted from the Sephacryl S1000 gel-filtration column as a broad single peak earlier than Dextran T2000 (Fig. 2), suggesting its apparent molecular weight to be greater than 2,000,000.

The purified 33 EPS was a white fibrous material soluble in water and alkalis but not in acids, methanol, ethanol, acetone, or dimethyl sulfoxide. Spectrophotometrically, absorption was detected at neither 280 nm nor 255 nm, suggesting that the 33 EPS preparation did not contain proteins or nucleic acids. Its aqueous solution was viscous, and its reduced viscosity was determined to be 15.6 dl g^{-1} ($= 1.56 \text{ m}^3 \text{ kg}^{-1}$) by the use of an Ostwald viscosity meter.

The lyophilized 33 EPS was hydrolyzed by TFA, labeled with ABEE, and analyzed by HPLC. Consequently, D-galactose, D-glucose, D-mannose, and D-glucuronic acid were found (Fig. 3). Based on these data, the proportions of the constituent sugars (% W/W) were calculated to be the following: D-galactose, 22.1 %; D-glucose, 22.4%; D-mannose, 21.5%; and D-glucuronic acid, 18.4%.

Pyruvic acid was detected in the hydrolysate of 33 EPS obtained with 2% TFA, and its content was calculated to be 15.6% (W/W). Fatty acids other than pyruvic acid were not detected in either acid- or alkali-hydrolyzed 33 EPS.

Benzene tolerance test

Rough mutant strains showed no or little growth in the presence of benzene, whereas their parental strain *Rhodococcus* sp. 33 showed good growth in either the presence or absence of benzene (Table 1). By the addition of the purified 33 EPS the rough strains showed good growth ($OD_{600} > 0.5$) even in the presence of benzene. Without the addition of carbon sources all of the strains showed no or little growth ($OD_{600} < 0.1$) on PAS medium containing the purified 33 EPS at 0.1 mg/ml. These data suggest that the rough mutants have no or few defects in their benzene-assimilation system.

Next, we examined the effects of EPS on the toxicity of benzene toward the rough strain, R0, under the resting conditions described in the Materials and Methods (Table 2). After exposure to benzene for 1 h, R0 showed great sensitivity to benzene under the resting conditions (0.19% survival rate). In the presence of 33 EPS at a final concentration of 0.1 mg/ml, the survival rate of R0 increased to 91 % after exposure to benzene for 1 h, suggesting the protective effect of 33 EPS against benzene toxicity. Although after 1-h incubation in the presence of benzene the cell numbers of strain 33 were slightly higher than in its absence, the cells showed no or little growth even in the presence of benzene after longer period of incubation time.

Effects of chemically modified 33 EPS on the benzene tolerance

Reduced or partially-hydrolyzed 33 EPS was examined for its protective effect against benzene toxicity under the

Table 1. Effects of 33 EPS on the growth of *Rhodococcus* sp. 33 and its rough mutants in the presence of benzene^a

Strain	EPS ^b	growth ^c		
		mannitol ^d	mannitol + benzene ^d	benzene ^d
Strain 33	(-)	+++	+++	+++
	(-)	+++	-	-
Rough mutants	native 33 EPS	+++	+++	+++
	reduced 33 EPS	+++	-	-
	partially-hydrolyzed 33 EPS	+++	-	-
	S-2 EPS	+++	+++	+++

^a Benzene tolerance test under growth conditions was performed as described in the Materials and Methods section.

^b EPS concentration used in this test was 0.1 mg/ml. The symbol (-) means no addition.

^c The symbols +++ and - indicate good growth ($OD_{600} > 0.5$) and no growth ($OD_{600} < 0.1$), respectively.

^d Mannitol was added to PAS medium at 1% as its final concentration, and benzene was allowed to diffuse into media as a vapor into the medium. The cultures, in 250-ml shake flasks, were incubated with shaking for 3 days at 28°C.

Table 2. Effects of 33 EPS on the survival of resting cells of *Rhodococcus* sp. 33 and its rough mutant treated with benzene^a.

Strain	33 EPS ^b	Benzene	
		—	+
Strain 33	(—)	65 (2)	97 (1)
	(+)	73 (3)	110 (0)
Rough mutant R0	(—)	38 (1)	0.19 (0.03)
	(+)	86 (5)	91 (11)

^aThe 33 EPS concentration used in this test was 1 mg/ml. The values are percentages of the initial viable cell number ($(3.5 \pm 0.1) \times 10^7$). The values in parentheses are standard deviations.

^bEPS concentration used in this test was 0.1 mg/ml. The symbol (—) means no addition.

growth conditions described in Materials and Methods (Table 1). After the addition of either reduced or partially-hydrolyzed 33 EPS the rough strains showed no growth in the presence of benzene. Finally, we examined the effect of S-2 EPS on the benzene toxicity under growth conditions and found this polysaccharide to protect the rough strains against the benzene toxicity.

DISCUSSION

In this study we showed that the rough strains derived from *Rhodococcus* sp. 33 were more sensitive to benzene than the parental mucoidal strain under either the growth or the resting conditions that contained up to 200 p.p.m benzene¹. These rough strains did not produce EPS, and the addition of 33 EPS improved their growth and survival in the presence of benzene (Tables 1 and 2). These data suggest that 33 EPS plays an important role in benzene tolerance of *Rhodococcus* sp. 33, especially in its ability to survive an initial challenge with benzene.

Even in the presence of purified 33 EPS the rough strains showed no or little growth on PAS medium saturated with benzene (1.8 g/l) by the addition of liquid benzene (data not shown), whereas *Rhodococcus* sp. 33 grew well under such conditions¹. The data suggest that the protective effects to benzene of endogenous 33 EPS might be higher than those of the added 33 EPS.

The mechanism of tolerance to benzene associated with 33 EPS is unknown. Since rough strains of *Rhodococcus* have strongly hydrophobic surfaces, the direct contact between such cells and benzene may enable the microorganisms to take up the hydrocarbon rapidly. It may be that in the presence of 33 EPS the influx of benzene into the cells might be decreased due to either covering of benzene with 33 EPS micelles and/or a change in cell-surface hydrophobicity.

There are several reports demonstrating that the level of tolerance to hydrocarbons is elevated by the production of various surface-active compounds^{14,15}. Emulsification was observed when either 33 EPS or S-2 EPS was mixed with various oils. Therefore, one of the possible mechanisms

Fig. 1. Ion-exchange chromatography of 33 EPS. Forty milligrams of 33 EPS were dissolved in 40 ml of 10 mM Tris-HCl buffer, applied to a DEAE-Toyopearl column (180 mm $\times$ 25 mm ϕ), and eluted with a 600-ml linear gradient (0–1 M) of NaCl.

Fig. 2. Gel filtration column chromatography of 33 EPS. One milligram of 33 EPS (closed circles) was applied to a Sephacryl S1000 gel filtration column (850 mm $\times$ 15 mm ϕ), and Dextran T2000 (open circles) was employed as a size marker. The arrow indicates the total volume of the gel (V_t).

Fig. 3. HPLC patterns of neutral sugars and uronic acid from 33 EPS. Panel A, standard samples; and panel B, 33 EPS. Standard samples: a-1, D-glucuronic acid; a-2, D-galacturonic acid; a-3, D-galactose; a-4, D-mannose; a-5, D-glucose; a-6, L-arabinose; and a-7, D-xylose.

for tolerance is that the surfactant activity of these EPS molecules renders cells resistant to benzene. Some *Rhodococcus* strains produce biosurfactants, and these molecules are predominantly glycolipids¹⁶. 33 EPS and S-2 EPS are high-molecular-weight acidic polysaccharides and, therefore, are different from previously reported *Rhodococcus* biosurfactants.

Sunairi *et al.* reported earlier that S-2 EPS lowered the cell-surface hydrophobicity of rough strains of *Rhodococcus*, indicating that S-2 EPS functions as a hydrophilin⁹. Aono and Kobayashi reported that a low cell-surface hydrophobicity serves as a defense mechanism against organic solvents¹⁷. It is thus possible that 33 EPS lowers the cell-surface hydrophobicity and establishes tolerance to benzene in the same way as S-2 EPS.

Rough strains showed good growth in the presence of benzene by the addition of native 33 EPS but not by that of the reduced or the partially-hydrolyzed 33 EPS (Table 1). The S-2 EPS also conferred the benzene tolerance to rough strains under the growth conditions (Table 1), which was reported to be a high-molecular-weight acidic polysaccharide consisting of D-glucose, D-galactose, D-mannose, D-glucuronic acid, stearic acid, and palmitic acid⁷. These data suggest that acidic residues and the high molecular weight of these rhodococcal EPSs may contribute to the protective effects of these EPSs against benzene toxicity.

Chemical analysis indicated that 33 EPS is an acidic polysaccharide containing 3 kinds of neutral sugars (66.0% W/W), uronic acid (18.4% W/W), and pyruvic acid (15.6% W/W), yielding a sum of their contents of 100%. The molar ratio of neutral sugars and uronic acid was estimated to be the following: D-galactose: D-glucose: D-mannose: D-glucuronic acid: pyruvic acid = 1: 1: 1: 1: 1. Richards' research group reported the chemical structures of EPS produced by 5 different serotypes of the equine pathogen *R. equi*^{18–22}, and the presence of pyruvic acid in its acetal form in EPS of serotypes 1, 3, 4, and 7. Although the molar ratio of neutral sugars, uronic acid, and pyruvic acid of serotype 3 EPS are the same as those of 33 EPS, lactate-substituted hexose was not detected in 33 EPS; whereas serotype 3 EPS was reported to contain lactate-substituted mannopyranose²⁰.

The reduced viscosity of the dilute aqueous solution of the EPS was 15.6 dl/g, ($1.56 \text{ m}^3 \text{ kg}^{-1}$) representing a higher viscosity than that of widely used thickeners, such as carboxymethyl cellulose (3.9 dl g^{-1} ($= 3.9 \times 10^{-1} \text{ m}^3 \text{ kg}^{-1}$)) or hydroxyethyl cellulose (8.0 dl g^{-1} ($= 8.0 \times 10^{-1} \text{ m}^3 \text{ kg}^{-1}$)), as measured under the same experimental conditions¹¹, thus suggesting the possibility of its application as a thickener.

Thus 33 EPS plays an important role in benzene tolerance of *Rhodococcus* sp. 33, especially helping the cells to survive an initial challenge with benzene, and its chemical composition affords some insight into the relationship between its chemical structure and function. We are cur-

rently studying its chemical structure in terms of the structure/function relationships.

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