Degradation of alkanes and highly chlorinated benzenes, and production of biosurfactants, by a psychrophilic *Rhodococcus* sp. and genetic characterization of its chlorobenzene dioxygenase

Peter Rapp and Lotte H. E. Gabriel-Jürgens

*Rhodococcus* sp. strain MS11 was isolated from a mixed culture. It displays a diverse range of metabolic capabilities. During growth on 1,2,4-trichlorobenzene, 1,2,4,5-tetrachlorobenzene (1,2,4,5-TeCB) and 3-chlorobenzoate stoichiometric amounts of chloride were released. It also utilized all three isomeric dichlorobenzenes and 1,2,3-trichlorobenzene as the sole carbon and energy source. Furthermore, the bacterium grew well on a great number of n-alkanes ranging from n-heptane to n-triacontane and on the branched alkane 2,6,10,14-tetramethylpentadecane (pristane) and slowly on n-hexane and n-pentatriacontane. It was able to grow at temperatures from 5 to 30 °C, with optimal growth at 20 °C, and could tolerate 6 % NaCl in mineral salts medium.

Genes encoding the initial chlorobenzene dioxygenase were detected by using a primer pair that was designed against the α-subunit (TecA1) of the chlorobenzene dioxygenase of *Ralstonia* (formerly *Burkholderia*) sp. strain PS12. The amino acid sequence of the amplified part of the α-subunit of the chlorobenzene dioxygenase of *Rhodococcus* sp. strain MS11 showed >99 % identity to the α-subunit of the chlorobenzene dioxygenase from *Ralstonia* sp. strain PS12 and the parts of both α-subunits responsible for substrate specificity were identical. The subsequent enzymes dihydrodiol dehydrogenase and chlorocatechol 1,2-dioxygenase were induced in cells grown on 1,2,4,5-TeCB. During cultivation on medium-chain-length n-alkanes ranging from n-decane to n-heptadecane, including 1-hexadecene, and on the branched alkane pristane, strain MS11 produced biosurfactants lowering the surface tension of the cultures from 72 to ≤29 mN m⁻¹. Glycolipids were extracted from the supernatant of a culture grown on n-hexadecane and characterized by ¹H- and ¹³C-NMR-spectroscopy and mass spectrometry. The two major components consisted of α,α-trehalose esterified at C-2 or C-4 with a succinic acid and at C-2' with a decanoic acid. They differed from one another in that one 2,3,4,2'-trehalosetetraester, found in higher concentration, was esterified at C-2, C-3 or C-4 with one octanoic and one decanoic acid and the other one, of lower concentration, with two octanoic acids. The results demonstrate that *Rhodococcus* sp. strain MS11 may be well suited for bioremediation of soils and sediments contaminated for a long time with di-, tri- and tetrachlorobenzenes as well as alkanes.

INTRODUCTION

Numerous Gram-negative bacteria are known to utilize halogenated benzenes as sole carbon source (de Bont et al., 1986; Schraa et al., 1986; Spain & Nishino, 1987; van der Meer et al., 1987; Haigler et al., 1988; Oltmanns et al., 1988; Oldenhuis et al., 1989; Sander et al., 1991; Brunsbach & Reineke, 1994; Spiess et al., 1995; Potrawfke et al., 1998). However, only very few Gram-positive bacteria, mainly rhodococci, have been described as having this capability (Goulding et al., 1988; Stoecker et al., 1994; Zaitsev et al., 1995). Among these few Gram-positive bacteria utilizing chlorinated benzenes, none hitherto been found able to metabolize tri- and tetrachlorobenzenes. Most of the key enzymes of the Gram-negative bacteria involved in the degradation pathways, as well as the coding genes and regulatory elements, have been described (van der Meer, 1997; Sander et al., 1991; van der Meer et al., 1991a, b, c; Beil et al., 1997, 1998, 1999). It was shown that degradation of chlorobenzenes in *Pseudomonas* sp. strain P51 and *Ralstonia* sp. strain PS12 is initiated by the non-haem-iron-containing
TcbA and TecA dioxygenases (Werlen et al., 1996; Beil et al., 1997). These class IIB chlorobenzene dioxygenases are multicomponent enzyme systems consisting of a reductase and ferredoxin and a catalytic terminal dioxygenase composed of a large \( \alpha \) - and a small \( \beta \)-subunit, which are possibly in an \( \alpha_2 \beta_3 \) configuration (Kauppi et al., 1998). The \( \alpha \)-subunit can be divided into two distinct domains: a Rieske domain which contains the [2Fe–2S] centre, and the catalytic domain containing the mononuclear Fe(II) centre. Beil et al. (1998) showed that the \( \alpha \)-subunit of the chlorobenzene dioxygenase from *Ralstonia* sp. strain PS12 is responsible for its substrate specificity. In contrast to these data for the enzymes of Gram-negative bacteria involved in the degradation of chlorinated benzenes, no corresponding data have been reported up to now from Gram-positive bacteria.

A well-known property of Gram-positive bacteria, however, especially the rhodococci, is the capability to degrade alkanes (Rehm & Reiff, 1981; Finnerty, 1992). This property is often accompanied, especially among rhodococci, by the ability to produce biosurfactants (Hommel, 1990; Finnerty, 1992). Among these biosurfactants, glycolipids are the most important group (Hisatsuka et al., 1971; Rapp et al., 1979; Inoue & Ito, 1982; Ristau & Wagner, 1983; Passeri et al., 1992). They show a high surface or interfacial activity as well as pH and temperature stability, low toxicity and good biodegradability.

Chlorinated benzenes are poorly soluble in water, and therefore less available to micro-organisms. Particularly in old polluted sites, a considerable fraction of the contaminants appears to be unavailable for biodegradation. This decrease of bioavailability of pollutants in the course of time is often designated as ‘ageing’. It may result from slow diffusion into very small pores, where micro-organisms are absent, and/or from absorption into organic matter of soils and sediments (Bosma et al., 1997). This decrease of bioavailability of contaminants in the course of time by ageing may be overcome to some extent by applying biosurfactants. These have the ability to desorb and disperse or dissolve such very hydrophobic compounds, i.e. alkanes or chlorinated benzenes. However, the bioremediation of contaminated soils and sediments by the addition of biosurfactants is restricted by their high production costs. Current efforts are therefore directed towards the isolation or design of micro-organisms that exhibit the desired degradative capabilities together with the simultaneous production of suitable biosurfactants. Gallardo et al. (1997) described the construction of such a recombinant micro-organism. They combined the production of biosurfactants with enhanced biodesulfurization of dibenzothiophene in a recombinant *Pseudomonas* strain. To our knowledge, however, no naturally occurring micro-organism has been isolated which combines the two properties, i.e. the degradation of hydrophobic chlorinated aromatic compounds and the production of suitable biosurfactants.

In the present study, the isolation of a psychrophilic, slightly halotolerant *Rhodococcus* sp. from a mixed culture is reported. The mineralization of tri- and tetrachlorobenzenes by this bacterium is described, as well as its ability to degrade alkanes of variable chain length coupled with the production of biosurfactants. Finally, its chlorobenzene dioxygenase is genetically compared with related benzene, chlorobenzene, toluene and biphenyl dioxygenases.

**METHODS**

Isolation and growth of bacteria. Strain MS11 was isolated as a contaminant from a liquid batch culture of *Burkholderia* sp. strain PS14 (Rapp & Timmis, 1999). Pure cultures of strain MS11 were obtained by serial dilutions on nutrient agar (Difco) and retransfer of single colonies onto plates consisting of mineral salts medium (Rapp & Timmis, 1999) and 12 g l\(^{-1}\) of agar no. 1 (Oxoid) with 1,2,4-trichlorobenzene (1,2,4-TCB) provided from the headspace. Liquid cultures were grown in 500 ml Erlenmeyer flasks containing 100 ml mineral salts medium on a rotary shaker (120 r.p.m.) at 20 °C. 1,2,4,5-tetrachlorobenzene (1,2,4,5-TCB) and other solid substrates were added as finely mortar-ground powder after sterilization to a concentration of 5 mM. 1,2- and 1,3-dichlorobenzene (DCB), 1,2-TCB and short-chain n-alkanes ranging from n-hexane to n-nonane as well as other volatile toxic substrates were added via the vapour phase also to a concentration of 5 mM (from a tube with two small openings inside the shake flask, which was mounted in the seal of its screw cap). Less volatile medium- and long-chain alkanes were added directly to the mineral salts medium to a concentration of 1 % (w/v). For growth on agar plates, solid substrates were placed in the lids of inverted Petri dishes and liquid volatile substrates were provided via the vapour phase.

Characterization of strain MS11. The pure culture was characterized by 16S rRNA sequence analysis (Woese et al., 1983; Gutell et al., 1985). It was classified additionally on the basis of Bergey's *Manual of Systematic Bacteriology* (Goodfellow, 1989). Tests were performed as described in the manual or by Smibert & Krieg (1981).

Preparation of resting cells and cell extracts. Cells grown for 48 h on 1,2,4-TCB, 1,2,4,5-TeCB or glucose were centrifuged at 12 000 \( \times \) g for 20 min at 4 °C and the pellets were washed twice with 33 mM Tris/HCl buffer, pH 8-0, and resuspended in 1 ml of the same buffer. Crude cell extracts were prepared by passing cell suspensions twice through a chilled French pressure cell (Amicon) at 90 MPa. After centrifugation at 25 000 \( \times \) g for 30 min at 4 °C, the clear supernatant was used as cell extract. Soluble protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Determination of cell growth with alkanes as carbon sources. A 10 ml volume of culture, grown on alkanes, was mixed with 10 ml ethanol/butan-1-ol/chloroform (10:1:1, by vol.) and centrifuged at 10 000 \( \times \) g for 30 min; the pellet was washed with 10 ml water and dried at 60 °C to a constant weight.

Oxygen uptake determinations. Assays for determination of the oxygen uptake rate were carried out with resting cells suspended in 54 mM phosphate buffer, pH 7-4, to an OD\(_{578}\) of 1-0 in a volume of 1 ml. The oxygen uptake rate was measured polarographically as described previously (Potrawke et al., 1998). Substrates were dissolved in dimethyl sulfoxide and their final concentration in the assay mixture was 0-1 mM. Protein concentrations of cell suspensions were determined by the method of Schmidt et al. (1963).

Chloride release. Chloride concentrations were determined by the mercury(II) thiocyanate method (Bergmann & Sanik, 1957;
Chauhan et al., 1998) and protein concentrations of cell suspensions by the method of Schmidt et al., 1963). Specific rates of chloro-
emission were determined by using washed cells suspended in
54 mM phosphate buffer, pH 7-4. Chloride concentration after
degradation of 1,2,4,5-TeCB, 1,2,4-TCB and 3-chlorobenzoate of
increasing concentration was determined in cultures with mineral
salts medium, in which chloride salts had been replaced by the cor-
responding sulfate salts. Standards were prepared by dissolving
appropriate amounts of sodium chloride in this chloride-free min-
eral salts medium.

### Nucleic acid extraction. Cells grown to an OD₅₇₈ of about 1 were
centrifuged and the pellet was suspended in phosphate buffer supplied with
the components of the FastDNA Spin kit for soil (Bio 101 Systems).
The subsequent extraction was carried out by the manu-
facturer’s protocol.

### Oligonucleotides. The designation, sequence (5’–3’) and priming
direction of the oligonucleotide primers used for amplification of
DNA fragments by PCR (Saiki et al., 1988) are as follows: pr-LG-Tec-RI
(forward), CCACAGGAGGACGAGCTCATC; pr-LG-Tec-R (reverse),
CCAGTCGAGCAGGTCATC. These primers were designed against TecC1, the a-subunit of the chlorobenzeno-
dioxygenase from Ralstonia sp. strain PS12 and the c-subunit of the chlorobenzene dioxygenase from Ralstonia sp. strain PS12.

### Sequence analysis. Sequences were assembled using Sequencher
version 4.0.5. They were submitted unaligned to the European
Molecular Biology Laboratory (EMBL: http://dove.embl-heidelberg.
de/Blast2) to determine the most similar sequence.

### Enzyme assays. Dihydrodiol dehydrogenase activity was deter-
mined by monitoring NAD⁺ reduction at 340 nm (Reineke &
Knackmuss, 1984). Catechol 1,2-dioxygenase activity was quantified
by measuring the formation of cis,cis-muconic acids at 260 nm
(Dorn & Knackmuss, 1978a). Interfering catechol 2,3-dioxygenase
activity was eliminated by preincubation of the assay mixture
with 0.01% (v/v) H₂O₂ for 10 min as described by Nakazawa &
Yokota (1973). Catechol 2,3-dioxygenase (EC 1.13.11.2) activity was
determined by monitoring the formation of 2-hydroxyxymuconic
semialdehyde at 375 nm (Nozaki, 1970). Absorption coefficients for
chloromononates were reported by Dorn & Knackmuss (1978b) and
Sander et al., 1991).

### Determination of 1,2,4,5-TeCB concentration. Two parallel
100 ml shake flask cultures with 1,2,4,5-TeCB as carbon source were
treated with 100 ml n-hexane. The extracts were dried over anhy-
drous sodium sulfate, and concentrated by evaporation to 1 ml at
1·4 x 10⁻³ Pa at 30 °C. 1,2,4,5-TeCB concentration was analysed by
GC as described previously (Rapp & Timmis, 1999). Peaks were
identified and quantified by comparing injections with authentic
external standards, prepared by dissolving defined amounts of
1,2,4,5-TeCB in mineral salts medium. These aqueous solutions
were extracted and the extracts concentrated in the same way as the
samples to be analysed.

### Analysis of fatty acids in the culture broth. A 100 ml shake
flask culture grown on n-dodecane was acidified with conc. HCl to a
pH of approximately 2-0 and extracted with 50 ml ethyl acetate. The
extract was dried over anhydrous sodium sulfate and evaporated to
dryness under reduced pressure. The residue was dissolved in 5 ml
CH₂Cl₂/CH₃OH/conc. HCl to a volume of 10 ml and heated for 90 min
at 100 °C. After cooling, the fatty acid methyl esters were recovered
in CH₂Cl₂, dried under nitrogen and dissolved in 1 ml n-octane for
analysis by GC and GC/MS. Capillary GC was performed on an
HP5890 Series II gas chromatograph with a fused-silica capillary
column (50 m x 0.11 μm) with cross-linked 5% phenylmethyl mili-
cone (RILS thickness 0.11 μm). The instrument was equipped with a
flame ionization detector and H₂ was used as carrier gas. The oper-
ating temperatures of the injector and detector were 250 and 300 °C,
respectively. The oven temperature programme was as follows:
100 °C for 2 min, increase to 290 °C at a rate of 4 °C min⁻¹, with an
isothermal period of 14 min at the end. The GC/MS analyses were
run on a similar chromatograph as described above (He was
the carrier gas), connected to an HP 5989 A quadrupole mass spec-
meter. The electron-impact ion source was maintained at 200 °C,
while the quadrupole temperature was 100 °C. The electron energy
was set at 70 eV.

### Determination of surface tension. The surface tension of cul-
tures was determined with a Du Nouy-ring tensiometer (K6, Krüss,
Hamburg, Germany) at room temperature (Weser, 1980).

### Thin-layer chromatography (TLC). This was performed on silica
gel 60 F₂₅₄ nano-plates (0·2 mm) (Macherey-Nagel). Trehalose
lipids were chromatographed with the solvent system CHCl₃/
CH₃OH/H₂O (65:15:2, by vol.) and mono- and disaccharides with
the solvent system CHCl₃/CH₃OH/H₂O (65:15:2, by vol.). Sugars and
trehalose lipids were detected by spraying with anisaldehyd/sulfuric acid
reagent (Krehb et al., 1967).

### Isolation and partial characterization of trehaloselipids. One
litre of mineral salts medium with 1% (w/v) n-hexadecane as sole
carbon source in a 3 l shake flask was inoculated with strain MS11,
grown on 1,2,4-TCB. The culture was grown at room temperature
at 120 r.p.m. for 168 h. After centrifugation at 12 000 g at 4 °C,
the supernatant was extracted with CH₂Cl₂/CH₃OH (2:1, v/v).
The extract was concentrated under reduced pressure and applied
to a silica gel 60 column (70-230 mesh, ASTM, Merck). Residual
n-hexadecane and other compounds less polar than the trehalose
lipids were eluted with CHCl₃/CH₃OH/HCl (5:1:0.01, by vol.).
Trehalose lipids were eluted with CHCl₃/CH₃OH (5:1:5, v/v)
followed by CHCl₃/CH₃OH (5:2, v/v), Euton was followed by
TLC with the solvent system CHCl₃/CH₃OH/H₂O (65:15:2, by vol.).
Fractions containing trehalose lipids were further purified
by thick-layer chromatography on silica gel 60 F₂₅₄
(2 mm) using the solvent system CHCl₃/CH₃OH/H₂O (65:15:2, by vol.). Trehalose lipids were obtained as a white and wax-like
substance and their examination by TLC on silica gel showed a single spot with an RF value of 0.41 in the solvent system CHCl₃/CH₃OH/H₂O (65:15:2, by vol.). This RF value and the green colour after spraying with anisaldehyde/sulfuric acid reagent (Krebs et al., 1967) suggested that the biosurfactants were glycolipids. They were saponified and the water-soluble fraction was analysed for reducing sugars by the 3,5-dinitrosalicylic acid method as described by Rapp et al. (1979). Acidic hydrolysis of the non-reducing carbo-
hydrate moiety (Rapp et al., 1979) delivered d-glucose as determined by TLC and enzymatic analysis (Boehringer Mannheim Biochemica, 1994), suggesting that trehalose may be the sugar moiety of the biosurfactants.

NMR spectroscopy. NMR 1D (1H and 13C), 2D 1H COSY (correla-
ted spectroscopy) and 2D 1H, 13C COSY spectra were recorded at 300 K on a Bruker ARX 400 NMR spectrometer locked to the major deuterium resonance of CD₃OD in the mixed CDCl₃/CD₃OH (7:3, v/v) solvent. All chemical shifts are given in p.p.m. relative to trimethylsilane and coupling constants in Hz.

1H NMR. Trehalose system δ = 5.44 [H-1, J = 3.6]; δ = 4.9 [H-2, J(2–3) 10.2]; δ = 5.54 [H-3, J(3–4) 9.8]; δ = 5.08 [H-4, J(4–5) 9.8]; δ = 3.75 [H-5, J(5–6) 2.2]; δ = 3.58 [H-6A, J(5–6) 4.8]; δ = 3.5 [H-6B, J(6A–6B) 12.4]; δ = 5.28 [H-1’(1’–2’) 3.6]; δ = 4.73 [H-2’, J(2’–3’) 9.9]; δ = 4.0 [H-3’, J(3’–4’) 9.5]; δ = 3.36 [H-4’, J(4’–5’) 9.2]; δ = 3.68 [H-5’]; δ = 3.83–3.69 [H-6A’]; δ = 3.83–3.69 [H-6B’]. Fatty acids δ = 0.89 [terminal CH₃’s (R₁, R₂, R₃)]; δ = 1.28 [CH₂n (R₂, R₃, R₄)]; δ = 2.7–2.45 [CH₂], while mitomycin (2 anhydroglucose decanoate) was isolated from strain MS11 which was obtained from a mixed culture utilizing decanoate as the carbon and energy source.

13C NMR. Trehalose system δ = 90.7 (C-1); δ = 91.2 (C-1’); δ = 73.1 (C-2); δ = 73.6 (C-2’); δ = 70.2 (C-3); δ = 71.2 (C-3’); δ = 68.9 (C-4); δ = 71.4 (C-4’); δ = 70.7 (C-5); δ = 73.4 (C-5’); δ = 60.8 (C-6); δ = 61.8 (C-6’). These assignments are interchangeable; fatty acids δ = 173.4 [CO (R₁)]; δ = 173.5 [CO (R₂)]; δ = 173.2 [CO (R₂ or R₃)]; δ = 34.4 [Cα (R₂, R₃, R₄ or R₅)]; δ = 32–32.3 [CH₂n CH₂, CH₃ (R₂, R₃, R₄ or R₅)]; δ = 30.0 [Cα (R₂ or R₃)]; δ = 29–29.7 [CH₂=CH (R₂ or R₃)]; δ = 25–25.3 [Cβ (R₂, R₃, R₄, or R₅)]; δ = 26–26.1 [Cα (R₁, R₂, R₃ or R₄)]; δ = 26–26.1 [Cβ (R₁, R₂, R₃ or R₄)]; δ = 22–22 [CH₃, CH₂CH₃ (R₂, R₃, R₄ or R₅); δ = 14.2 [terminal CH₃ (R₂, R₃, R₄ or R₅)].

Electrospray ionization tandem mass spectrometry (ESI-MS/MS). A quadrupole time-of-flight mass spectrometer (Micromass) equipped with a nanospray ion source was used and a voltage of approximately 1000 V was applied. For collision-induced dissociations, parents ions were selectively transmitted from the quadrupole mass analyser into the collision cell with argon as the collision gas. The kinetic energy was set at approximately ~30 eV. The resulting daughter ions were separated by an orthogonal time-of-flight mass spectrometer (Micromass).

Acquisition was performed in the negative ion mode. The spectra were acquired in the range m/z 80–1000 at a scan rate of 1 s/scan. The capillary and cone voltages were set to 25 and 25 V, respectively. All MS/MS spectra were acquired by collision-induced dissociation.

RESULTS

Isolation and characterization of strain MS11

Strain MS11 was obtained from a mixed culture utilizing 1,2,4,5-TeCB and 1,2,4-TCB as the sole carbon and energy source, by multiple streaking on nutrient agar. The isolate was aerobic, Gram-positive and non-motile. Its morphology changed from cocci to rods, which could elongate to longer ones or to branched filaments. It formed no aerial hyphae. Strain MS11 was catalase- and urease-positive and oxidase- and arylsulfatase-negative. It was not acid-fast, reduced nitrate to nitrite and its growth was not inhibited by 6% (w/v) NaCl. Strain MS11 grew at temperatures from 5 to 30°C; optimum growth was observed at 20°C. It grew well at 5°C on glucose and, after adaptation, also on the much less water-soluble n-hexadecane as the sole carbon source, thus demonstrating its psychrophilic character. Growth of strain MS11 on mineral salts medium with 5 g l⁻¹ of universal peptone (Merck) was not inhibited by penicillin G (10 µg ml⁻¹), while mitomycin C (2 µg ml⁻¹), gentamycin

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(25 μg ml⁻¹), chloramphenicol (5 μg ml⁻¹) and rifampicin (25 μg ml⁻¹) inhibited its growth. Strain MS11 utilized the following compounds as sole carbon and energy sources: D-glucose, D-fructose, D-mannose, D-ribose, D-xyllose, maltose, sucrose, cellobiose, starch, sorbitol, mannitol, myo-inositol, methanol, ethanol, n-butanol, butan-2,3-ol, glycerol, acetate, succinate, pimelate, citrate, L-glutamate, L-leucine, DL-norleucine, L-histidine, DL-phenylalanine, β-alanine, L-proline and L-asparagine. It did not utilize the following compounds as sole carbon and energy sources: D-galactose, L-arabinose, lactose, raffinose, gelatin, L-lactate, DL-mandelate, D-tartrate and DL-methionine. This nutritional versatility and antibiotic susceptibility of strain MS11 is typical of the genus Rhodococcus (Goodfellow, 1989; Zaitsev et al., 1995; Bizet et al., 1997; Goodfellow et al., 1998).

16S rRNA of strain MS11 was partially sequenced and compared with known sequences in the database of the Gesellschaft für Biotechnologische Forschung, Division of Microbiology (Maidak et al., 1997; Stoesser et al., 1997). Its partial sequence (1390 bp) was 99.8% similar to that of type strain Rhodococcus erythropolis DSM 43066T.

On the basis of biological and biochemical characteristics as well as 16S rRNA sequence analysis, strain MS11 was classified as a Rhodococcus sp.

### Growth on alkanes and on halogenated and non-halogenated aromatic compounds

*Rhodococcus* sp. strain MS11 was able to utilize many alkanes as sole carbon and energy source (Table 1). It grew well on n-alkanes ranging from n-heptane to n-triacontane and slowly on n-hexane and on n-pentatriacontane. The products of the terminal oxidation of n-alkanes, in the form of 1-hexadecene, 1-hexadecanol and 1-hexadecanoic acid, as well as the branched alkane 2,6,10,14-tetramethylpentadecane (pristane) were also utilized by strain MS11 as sole carbon source. Fig. 1 illustrates the growth of strain MS11 in shake-flask cultures on alkanes: in this case with 1% (w/v) n-tetradecane as carbon source. With this substrate, a maximum specific growth rate of 0.042 h⁻¹ was determined. The values of μ_max determined in shake-flask cultures with other n-alkanes as carbon sources ranged from 0.03 h⁻¹ for n-decane to 0.08 h⁻¹ for n-hexadecane and from 0.06 h⁻¹ for n-heptadecane to 0.03 h⁻¹ for n-nonadecane.

A further ability of this bacterium was growth on halogenated and non-halogenated aromatic compounds (Table 1). Although it could not utilize any of the four monohalogenated benzenes as the sole carbon source, it was able to grow on all three isomeric dichloro- and dibromobenzenes as the sole carbon and energy source. 1,2-Dichlorobenzene, however, was utilized to a lesser extent than the other two dichloroisomers. Strain MS11 also utilized 1,2- and 1,4-diodobenzenes and to a lesser extent 1,4-difluorobenzene as its sole carbon source. It

![Table 1](http://mic.sgmjournals.org)
grew well on 1,2,4-trichloro- and 1,2,4-tribromobenzene and to a much lesser extent on 1,2,3-trichlorobenzene. Strain MS11 did not utilize 1,2,4-trifluoro- and 1,3,5-trichlorobenzene. From the three tetrachlorobenzenes only the 1,2,4,5-tetrachloro-isomer was utilized as a sole carbon and energy source by strain MS11. During growth on 1,2,4-TCB and 1,2,4,5-TeCB doubling times of 11 and 19 h, respectively, were determined. Finally, strain MS11 did not grow on higher chlorinated benzenes such as penta- and hexachlorobenzene. The ability of *Rhodococcus* sp. strain MS11 to grow on chlorinated benzenes is illustrated in Fig. 2, where 1,2,4,5-TeCB is the sole carbon source. Chloride was released stoichiometrically, suggesting complete mineralization of 1,2,4,5-TeCB. This release of chloride was concomitant with growth, and the 1,2,4,5-TeCB concentration decreased with the pH-value. The stoichiometric release of chloride during growth of strain MS11 on 1,2,4-TCB and 1,2,4,5-TeCB as the sole carbon source was confirmed by the data in Fig. 3, which shows the chloride concentration in parallel cultures after degradation of 1,2,4-TCB, 1,2,4,5-TeCB and 3-chlorobenzoate of increasing concentration. While strain MS11 grew well on the latter compound with a doubling time of 10 h, it was unable to utilize the 2- and 4-chloroisomers. Furthermore, it also utilized phenol, catechol, benzoic, salicylic, 3- and 4-hydroxybenzoic, gentisic and protocatechuic acid as the sole carbon and energy source. MS11 also grew on D- and L-2-chlorosuccinic acid as well as on m- and p-cresol, but not o-cresol. No growth was observed with benzene, toluene, ethylbenzene, styrene, naphthalene or biphenyl as carbon source.

**Specific rates of oxygen uptake with and chloride release from chlorinated benzenes by resting cells**

Table 2 shows the specific rates of oxygen uptake with chlorinated benzenes as carbon source by washed cell
suspensions of *Rhodococcus* sp. strain MS11. The highest rate was determined with 1,2,4-TCB, followed by 1,3- and 1,4-DCB. The specific oxygen uptake rates with 1,2-DCB, 1,2,4,5-TeCB and 1,2,3-TCB as substrates were within the range of 32–39 nmol min$^{-1}$ (mg protein)$^{-1}$, while the rates with 1,2,3,4-TeCB and 1,2,3,5-TeCB were only slightly above and that with pentachlorobenzene below the detection limit of 5 nmol min$^{-1}$ (mg protein)$^{-1}$.

The specific rates of chloride release from di-, tri- and tetrachlorobenzenes by resting cells of strain MS11 is shown in Table 3. The highest rate was determined with 1,2,4,5-TeCB followed by 1,4-DCB and 1,2,4-TCB. The lowest specific rate of chloride release was measured with 1,2-DCB. This lower rate of chloride release corresponded to the lower growth of strain MS11 on these two carbon sources.

**Table 2.** Specific rates of oxygen uptake with chlorinated benzenes by resting cells of *Rhodococcus* sp. strain MS11

<table>
<thead>
<tr>
<th>Assay substrate</th>
<th>Specific oxygen uptake rate* [nmol min$^{-1}$ (mg protein)$^{-1}$]</th>
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<tbody>
<tr>
<td>Benzene</td>
<td>0-0</td>
</tr>
<tr>
<td>Chlorobenzene</td>
<td>0-0</td>
</tr>
<tr>
<td>1,2-Dichlorobenzene</td>
<td>38.9 ± 15.9</td>
</tr>
<tr>
<td>1,3-Dichlorobenzene</td>
<td>68.5 ± 9.2</td>
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<tr>
<td>1,4-Dichlorobenzene</td>
<td>68.5 ± 12.8</td>
</tr>
<tr>
<td>1,2,3-Trichlorobenzene</td>
<td>31.6 ± 7.5</td>
</tr>
<tr>
<td>1,2,4-Trichlorobenzene</td>
<td>85.6 ± 6.2</td>
</tr>
<tr>
<td>1,3,5-Trichlorobenzene</td>
<td>5.1 ± 3.5</td>
</tr>
<tr>
<td>1,2,3,4-Tetrachlorobenzene</td>
<td>7.4 ± 2.5</td>
</tr>
<tr>
<td>1,2,3,5-Tetrachlorobenzene</td>
<td>7.7 ± 0.8</td>
</tr>
<tr>
<td>1,2,4,5-Tetrachlorobenzene</td>
<td>32.4 ± 1.6</td>
</tr>
<tr>
<td>Pentachlorobenzene</td>
<td>&lt;5-0</td>
</tr>
</tbody>
</table>

*Results are shown as the mean±SD of four replicates from two independent experiments.

This is in agreement with the different growth of strain MS11 on these two carbon sources.

**Genetic characterization of chlorobenzene dioxygenase**

Genes from *Rhodococcus* sp. strain MS11 encoding the initial chlorobenzene dioxygenase were amplified by PCR using two specific primers designed against the β-subunit (TecA1) of the chlorobenzene dioxygenase of *Ralstonia* sp. strain PS12 (Beil *et al.*, 1998). The screening of the PCR clone library revealed only a single sequence (comprising 417 out of 459 amino acids) which was >99% identical with the amino acid sequence of the β-subunit (TecA1) of the chlorobenzene dioxygenase from *Ralstonia* sp. strain PS12. The regions of the amino acid sequences of both β-subunits responsible for substrate specificity (Beil *et al.*, 1998) were completely identical. The very close relationship between these two chlorobenzene dioxygenases is well reflected in the phylogenetic tree based on the DNA sequences of β-subunits of different initial dioxygenases (Fig. 4).

**Enzyme activities in cell extracts**

In almost all known dioxygenase gene clusters, the gene for the dihydriodiol dehydrogenase is directly downstream of the dioxygenase genes (van der Meer, 1997). The specific dihydriodiol dehydrogenase activity for the oxidation of benzene dihydriodiol was significantly induced in cells of strain MS11 grown on 1,2,4,5-TeCB, as shown in Table 4. This table shows additionally that in extracts of cells grown on 1,2,4,5-TeCB rather high chlorocatechol 1,2-dioxygenase activities were measured with 3,6-dichloro- and 3,4,6-trichlorocatechol as substrates, but also a high catechol 1,2-dioxygenase activity. In contrast, no catechol 2,3-dioxygenase activity could be detected in cells grown on 1,2,4,5-TeCB and glucose.

**Occurrence of intermediary products and biosurfactant formation during growth on alkanes**

During growth of strain MS11 on n-dodecane as sole carbon source, dodecanoic acid accumulated in the culture broth. It constituted approximately 50% of all fatty acids extracted from the culture. On the other hand, no dodecanedioic acid could be detected. These results suggest that degradation of n-alkanes by strain MS11 is initiated by monoterinal oxidation (data not shown).

Using n-alkanes ranging from n-decane to n-heptadecane, 1-hexadecene or pristane as sole carbon and energy sources, the surface tension of the cultures always strongly decreased. Fig. 1 illustrates the biosurfactant formation, measured by means of the decrease of surface tension, during cultivation of MS11 on n-tetradecane as sole carbon source. It was lowered from 72 to 42 mN m$^{-1}$. This decrease took place rapidly at the beginning of cultivation, when the cell mass was still low. However, the surface tension of the cultures was not lowered markedly when strain MS11 was grown at

**Table 3.** Specific rates of chloride release by resting cells of *Rhodococcus* sp. strain MS11

<table>
<thead>
<tr>
<th>Assay substrate</th>
<th>Rate of chloride release* [μmol h$^{-1}$ (mg protein)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-Dichlorobenzene</td>
<td>1.23 ± 0.26</td>
</tr>
<tr>
<td>1,3-Dichlorobenzene</td>
<td>1.35 ± 0.32</td>
</tr>
<tr>
<td>1,4-Dichlorobenzene</td>
<td>1.62 ± 0.03</td>
</tr>
<tr>
<td>1,2,3-Trichlorobenzene</td>
<td>1.44 ± 0.03</td>
</tr>
<tr>
<td>1,2,4-Trichlorobenzene</td>
<td>1.56 ± 0.04</td>
</tr>
<tr>
<td>1,2,4,5-Tetrachlorobenzene</td>
<td>2.18 ± 0.11</td>
</tr>
</tbody>
</table>

*Results are shown as the mean±SD of three replicates from two independent experiments.
room temperature on n-alkanes with chain lengths of n \geq 18 and n \leq 9 or on 1-hexadecanol and hexadecanoic acid as sole carbon source (data not shown).

**Characterization of trehaloselipids**

The structure of the glycolipids was established from the combined 1H- and 13C-NMR, and MS data (see Methods). The \( \alpha \)-glucopyranose moieties in the glycolipids were identified from the correlations in the 2D COSY spectrum and from the magnitude of the chemical shifts and vicinal coupling constants of the 1D 1H spectrum. In the 1H detected 13C–1H correlated spectrum recorded by the HMBC (heteronuclear multiple bond correlation) procedure, the anomeric proton of the glucopyranose moieties showed both a residual one-bond and three-bond correlations to the anomeric carbon, thus indicating an \( \alpha, \alpha \)-trehalose system. The 2D 1H COSY spectrum of the trehalose lipids indicated by the low-field shifts of the corresponding glucose ring protons next to the esterified hydroxyl groups, in the region of 4.7–5.5 ppm, that the hydroxyl groups at positions 2, 3, 4 and 2' were esterified with fatty acids. The 1H NMR data showed additionally that the fatty acids consisted of three medium-chain-length acids and one succinic acid. Long-range 13C–1H correlations in the 2D 1H, 13C COSY spectrum of the trehalose tetraesters between the trehalose ring and methylene protons of the fatty acids with their carbonyl carbons showed that the fatty acids at C-2 and C-2' were of medium chain length and that a succinic acid residue was at C-2 or C-4.

The ESI-mass spectrum of the 2,3,4,2'-trehalose tetraesters showed, besides some very small signals, two prominent (M + Na)+ ion peaks at \( m/z \) 899.4 and 871.38 (see Methods), the first with a more than twofold higher relative abundance than the latter. The next highest peaks after the molecular ion peaks were those at \( m/z \) 583.25 and 555.24. Their appearance resulting from the removal of an anhydroglucose moiety esterified with a decanoic acid demonstrated that the medium-chain-length fatty acid at C-2' of both trehalose tetraesters was a decanoic acid. The fragmentation patterns of both molecular ions confirmed

**Table 4.** Specific catabolic enzyme activities in cell extracts of *Rhodococcus* sp. strain MS11

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Specific enzyme activity [units (mg protein)(^{-1})] after growth on 1,2,4,5-TeCB</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrodiol dehydrogenase</td>
<td>Benzene dihydrodiol</td>
<td>0.121 ± 0.035</td>
<td>( \leq 0.001 )</td>
</tr>
<tr>
<td>Catechol 1,2-dioxygenase</td>
<td>Catechol</td>
<td>0.609 ± 0.020</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>3,6-Dichlorocatechol</td>
<td>0.117 ± 0.009</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>3,4,6-Trichlorocatechol</td>
<td>0.252 ± 0.004</td>
<td>0.004</td>
</tr>
<tr>
<td>Catechol 2,3-dioxygenase</td>
<td>Catechol</td>
<td>( \leq 0.001 )</td>
<td>( \leq 0.001 )</td>
</tr>
</tbody>
</table>

*Data are shown as the mean ± SD of four replicates from two or three independent experiments.*
additionally that trehalose was, as already indicated by the NMR spectra, esterified at C-2 or C-4 with a succinic acid. However, the two fragmentation pathways also differed from one another. While an octanoic and a decanoic acid were split off from one glucose moiety of the higher molecular mass 876 was found to be linked at C-2, C-3 or C-4 with a decanoic acid. Furthermore, the trehalose lipid of molecular mass 848 with two octanoic acids. Structure of 2,3,4,2′-trehalose tetraester of molecular mass 848: R_2 or R_4, succinic acid; R_2, R_3 or R_4, two octanoic acids. Structure of 2,3,4,2′-trehalose tetraester of molecular mass 876: R_2 or R_4, succinic acid; R_2, R_3 or R_4, octanoic and decanoic acid.

**DISCUSSION**

Strain MS11 is, to our knowledge, the first Gram-positive bacterium described which is able to grow aerobically on 1,2,4-TCB and 1,2,4,5-TeCB, as well as on 1,2,3-TCB and all three isomeric dichlorobenzenes as the sole carbon and energy source. It was identified by morphological, biochemical and 16S rRNA sequence analysis as a member of the genus *Rhodococcus* closely related to *Rhodococcus erythropolis* DSM 43066^T_. From Gram-negative bacteria, only four strains are known to utilize aerobically tri- and tetrachlorobenzenes. These are *Pseudomonas* sp. strain P51, which could grow on 1,2,4-TCB as sole carbon source (van der Meer *et al*., 1987), *Pseudomonas chlororaphis* RW71, degrading 1,2,3,4-TeCB (Potrawförke *et al*., 1998), and *Ralstonia* (formerly *Burkholderia*) sp. strain PS12 as well as *Burkholderia* sp. strain PS14, both mineralizing 1,2,4-TCB and 1,2,4,5-TeCB (Sander *et al*., 1991; Beil *et al*., 1997; Rapp & Timmis, 1999). Comparing the substrate spectrum of *Rhodococcus* sp. strain MS11 relating to chlorinated benzenes with that of *Ralstonia* sp. strain PS12 and *Burkholderia* sp. strain PS14, it did not grow on monochlorobenzene, in contrast to these two strains. While all three strains were able to degrade the three isomeric dichlorobenzenes and could mineralize 1,2,4-TCB and 1,2,4,5-TeCB, strain MS11 seems to be somewhat superior to strain PS14 as measured by the release of chloride from 1,2,3-TCB. The utilization of 3-chlorobenzoate as the sole carbon and energy source is another property of *Rhodococcus* sp. strain MS11 that matches with the abilities of *Ralstonia* sp. strain PS12.

The chlorobenzene dioxygenase of strain MS11 can be grouped most probably into the class IIB dioxygenase subfamily. These Rieske non-haem-iron oxygenases are now classified into groups based on their substrate specificity and the sequence of their α-subunits (Gibson & Parales, 2000; Nam *et al*., 2001). The sequence of the α-subunit of the chlorobenzene dioxygenase of strain MS11 was >99 % identical with that of the chlorobenzene dioxygenase from *Ralstonia* sp. strain PS12 (Beil *et al*., 1997) and 98 % identical with that of the chlorobenzene dioxygenase from *Pseudomonas* sp. strain P51 (Werlen *et al*., 1996). These sequences cluster very closely together with sequences of the α-subunits of toluene, benzene and biphenyl dioxygenases assembled in group IV of the classification scheme of Nam *et al*., 2001 (Fig. 4). Additionally, the region of the amino acid sequence of the α-subunit of the two chlorobenzene dioxygenases from strains MS11 and PS12 responsible for substrate specificity (Beil *et al*., 1998) showed 100 % identity. It is well known that many catabolic genes are associated with transposons (Wyndham *et al*., 1994) and that transposition is a major mechanism for the acquisition of catabolic genes by bacterial genomes. However, such genes can also be acquired by site-specific integration (Poelarends *et al*., 2000). *Ralstonia* (formerly *Burkholderia*) sp. strain PS12 was isolated together with *Burkholderia* sp. strain PS14 from a soil sample of a waste disposal site (Sander *et al*., 1991). Both strains are able to degrade the same chlorinated benzenes, ranging from 1,2,4,5-TeCB to monochlorobenzene (Sander *et al*., 1991; Beil *et al*., 1997), and have plasmids of the same size (Sander, 1991). These correspondences of the two strains suggest that they have the same gene encoding chlorobenzene dioxygenase. Taking this into consideration as well as the similarity of >99 % among the sequences of the chlorobenzene dioxygenases of strains PS12 and MS11, one can possibly conclude that the gene encoding chlorobenzene dioxygenase of the Gram-positive strain MS11 may have been acquired from the Gram-negative strain PS14 by horizontal gene transfer.

While the gene encoding the α-subunit of the chlorobenzene dioxygenase and possibly also the other upper pathway genes of *Rhodococcus* sp. strain MS11 exhibit high sequence similarities to those of *Ralstonia* sp. strain PS12 and *Pseudomonas* sp. strain P51, such a correspondence between the lower pathway genes encoding chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase and dienolactone hydrolase seems to be doubtful and has to be examined. The reason for this assertion is that the chlorocatechol-degrading enzymes and especially the chloromuconate cycloisomerase of another *Rhodococcus*, *R. opacus* 1CP, have unusual biochemical properties and sequences with relatively little similarity to those of proteobacterial chlorocatechol-degrading enzymes, thus indicating a different origin (Schlömann, 2002).
The most striking difference, however, between the four above-mentioned Gram-negative, tri- and/or tetrachloro-benzene-degrading proteobacteria and the Gram-positive, tri- and tetrachlorobenzene-degrading *Rhodococcus* sp. strain MS11 is the ability of the latter to utilize an unusually broad range of alkanes as sole carbon and energy source. Strain MS11 was able to grow on straight-chain alkanes consisting of 7–30 carbon atoms, on its possible metabolites as demonstrated by means of 1-hexadecene, 1-hexadecanol and 1-hexadecanoic acid, and on the recalcitrant branched alkane 2,6,10,14-tetramethylpentadecane (pristane). It grew slowly on n-pentatriacontane and on n-hexane. The metabolism of alkanes is a not uncommon feature of rhodococci (Finnerty, 1992). There are strains able to grow on n-decane to n-triacontane (Milekhina et al. 1998), on n-hexadecane to n-dotriacontane (Whyte et al., 1998) or on n-hexane to n-eicosane and on pristane (Bej et al., 2000). The rhodococci, however, also include strains metabolizing only a rather narrow spectrum of n-alkanes ranging from n-decane to n-triacontane and on pristane (Milekhina et al. 1998). Like some other *Rhodococcus* species (Bej et al., 2000; Whyte et al., 1998), strain MS11 was psychrotolerant, since it could grow at 5°C, while its optimum temperature was 20°C.

It has been observed that strain MS11 degraded n-alkanes with chain lengths of C_{10}–C_{17} as readily as or even better than those of shorter chain length, although the water solubilities of the latter are higher (McAuliffe, 1969; Zhang & Miller, 1995). This may partly depend on the toxicity of n-alkanes with chain lengths of n ≤ 9. However, it may also indicate that an additional mechanism to the mere dissolution in water is responsible for the bioavailability of alkanes ranging from n-decane to n-heptadecane. It is well known that many hydrocarbon-degrading bacteria optimize the uptake of medium-chain-length alkanes by producing biosurfactants (Hommel, 1990). Among them, rhodococci are the most prominent group (Finnerty, 1992). Therefore, it was not surprising that *Rhodococcus* sp. strain MS11 also produced biosurfactants, lowering the surface tension of cultures from 72 mN m^{-1} to ≤ 29 mN m^{-1} during growth on n-alkanes ranging from n-decane to n-heptadecane, as well as on 1-hexadecene and the branched alkane pristane. The biosurfactants were extracted and purified from the supernatant of a culture grown on n-hexadecane as sole carbon source. \textsuperscript{1}H and \textsuperscript{13}C NMR spectroscopy as well as ESI-MS/MS showed that the two main biosurfactants were \( \alpha, \alpha \)-trehalose tetraesters linked at C-2 or C-4 with a succinic acid and at C-2′ with a decanoic acid. They differed in that one tetraester was esterified at C-2, C-3 or C-4 with an octanoic and a decanoic acid and the other one at the same positions with two octanoic acids. Similar \( 2,3,4,2′ \)-trehaloselipids, the main components of which were esterified with two decanoic, one octanoic and one succinic acid, were found in cultures of *R. erythropolis* (DSM 43215) grown on n-tetradecanoic and hexadecanoic acid (Uchida et al., 1989). Finally, Bartrakov et al. (1981) extracted from *Mycobacterium paraffinicum* grown on n-hexadecane, 2-O-octanoyl-3,2′-di-O-decanoyl-6-O-succinoyl-\( \alpha, \alpha \)-trehalose together with four other trehaloselipids.

*Rhodococcus* sp. strain MS11 is, to our knowledge, the first micro-organism described to degrade di- and tetra-chlorobenzenes and to produce biosurfactants during growth on a wide range of alkanes. Alkanes and chlorinated benzenes, especially the higher substituted ones, are hydrophobic and tend to sorb onto soils and sediments. Over a longer contact time they slowly diffuse into their inorganic or organic matrix. Furthermore, accumulation of these contaminants into fissures, cavities and pores also renders them inaccessible to micro-organisms (Bouwer & Zehnder, 1993). The extracellular surface- and interfacial-active trehalose tetraesters excreted by *Rhodococcus* sp. strain MS11 may be well suited for bioremediation of sites polluted in such a way. Moreover, strain MS11 was found to be psychrophilic and, according to Larsen’s classification (Larsen, 1986), also slightly halotolerant. This wide range of capabilities together with the known environmental persistence of rhodococci (Warhurst & Fewson, 1994) makes *Rhodococcus* sp. strain MS11 a good candidate for bioremediation of soils and sediments, contaminated for years or even decades with these recalcitrant compounds.

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**REFERENCES**


