A Rhodococcus species that thrives on medium saturated with liquid benzene

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A bacterium isolated from a contaminated site in Sydney, Australia, utilized benzene in the liquid phase as a sole carbon source at levels toxic to other micro-organisms. The organism was a short Gram-positive rod which grew at 6% NaCl, 0-37 °C and pH 2-10. Biochemical tests, fatty acid analysis, and 16S rDNA sequencing identified the organism as a member of the genus Rhodococcus. Vapour-phase addition of benzene to the medium in batch and continuous systems resulted in initial concentrations averaging 200 p.p.m. Under these conditions, 95% of the benzene was degraded. In separate experiments, medium spiked with liquid benzene resulted in concentrations of up to 2789 p.p.m. and supported good growth of the organism. To confirm utilization of benzene at levels known to be toxic to other micro-organisms, continuous cultures were used; benzene added at 2% (v/v) per day resulted in growth and 89% degradation, which was maintained for more than 30 d. Rhodococcus sp. strain 33 appears to be the only organism known that can grow at these levels of benzene.

Keywords: benzene degradation, Rhodococcus sp., bioremediation

INTRODUCTION

The persistence of benzene, toluene and xylene in contaminated sites is indicative of the lack of natural systems that can efficiently degrade these compounds. Studies have been carried out on the degradation of benzene in aerobic (Bayly & Barbour, 1984; Alvarez et al., 1991) denitrifying (Kuhn et al., 1988; Hutchins et al., 1991) and anaerobic conditions (Vogel & Grbic-Galic, 1986; Grbic-Galic & Vogel, 1987). It is of great significance to completely mineralize benzene as this compound is a proven carcinogen even at low levels (1-12.4 p.p.m.) (Tompa et al., 1994). The most common pathway in the aerobic degradation of benzene is through the production of cis-benzene glycol, which is converted to catechol and further catabolized to either cis,cis-muconic acid or 2-hydroxymuconic semialdehyde depending on the micro-organism concerned.

Biodegradation studies for benzene are usually monitored in closed systems with initial concentrations of benzene supplied as vapour at around 10-100 p.p.m. in anaerobic systems, and up to 5% (v/v) of the medium in aerobic systems. Aerobic organisms used in these studies utilize available air in the headspace to carry out aerobic degradation but this results in low efficiency because the initial levels of benzene used are below the aqueous-phase solubility. Vapour-phase benzene was used in most of the studies because liquid benzene proved to be toxic to some micro-organisms when added directly (Gibson et al., 1970; Davis et al., 1981; Alvarez et al., 1991). A limiting factor in degradation studies is that benzene is toxic to most micro-organisms at levels as low as 0.1% (v/v).

Most of the petroleum hydrocarbons, including benzene, are soluble in water at low concentrations. However, most oil spills release petroleum hydrocarbons in concentrations exceeding their solubility limits (Boylan & Tripp, 1971; Harrison et al., 1975). Benzene has a solubility of 1780 mg l⁻¹ in water. Moriya & Horikoshi (1993) attempted to isolate a benzene-tolerant bacterium to improve biodegradation of petroleum contaminants in marine environments. They isolated a Flavobacterium strain, DS-711, that could tolerate up to 5% (v/v) benzene but was, however, unable to degrade it. Shirai (1986) used 1% (v/v) benzene in the vapour phase to isolate new strains of micro-organisms capable of producing catechol from benzene, and Winstanley et al. (1987) cultivated Acinetobacter calcoaceticus in 0.4% (v/v) liquid benzene. No organism has been reported to degrade benzene at concentrations higher than 1% (v/v) as liquid.

This paper describes biodegradation by a new isolate...
(identified as a *Rhodococcus* sp.) of benzene in the liquid phase at concentrations toxic to most of the microorganisms described in previous reports.

**METHODS**

**Culture medium.** The basal medium (PAS) contained the following: 10 ml 1 M K$_2$HPO$_4$, 5 ml 1 M KH$_2$PO$_4$, 16 ml 1 M NH$_4$Cl and 365 ml Milli-Q water. This medium was sterilized at 121 °C for 15 min. A stock mineral salts solution containing the following was filter-sterilized: 37.5 g MgSO$_4$.7H$_2$O, 50 g MnSO$_4$.5H$_2$O, 50 g FeSO$_4$.7H$_2$O, 0.3 g CaCl$_2$.2H$_2$O and 1 g ascorbic acid per litre of Milli-Q water. Four millilitres of this salts solution was added to 400 ml basal medium prior to use. For solid media, 2% agar was added to the basal medium.

For batch experiments, the reaction vessel was a 250 ml flask containing 50 ml culture medium, with an empty test tube placed inside the flask. Benzene was added to the test tube to allow its vapour to diffuse into the medium and the headspace. The flask was covered with cotton to allow free diffusion of air into the system.

**Enrichment and isolation of benzene-degrading organisms.** Soil samples were taken from a chemical plant in Sydney, Australia. Approximately 1 g of the soil was dispersed in 9 ml sterile water and allowed to stand for 30 min. Five millilitres of this soil suspension was added to 50 ml culture medium and 2 ml benzene was added to the small tube inside the flask. The flask was incubated with shaking (100 r.p.m.) at 25 °C for 3 d.

A loopful of the cell suspension was streaked onto PAS plates and incubated in a desiccator with a beaker containing liquid benzene. Colonies obtained from PAS plates after incubation were reinoculated into the liquid medium to confirm utilization of benzene. Pure cultures utilizing benzene as a sole carbon source were kept on nutrient agar (NA) slants. The cultures were also deposited at the School of Microbiology Culture Collection (University of New South Wales) in a freeze-dried state.

**Identification of the isolates.** Morphological and biochemical tests were performed on the isolate. Growth at different temperatures, pH and NaCl concentrations was also determined. The isolate was further characterized by GC of the fatty acids of the cell wall against the MIDI Microbial Identification System (MIS) TSBA library version 3.8. The culture was submitted for fatty acid analysis to the Biological and Chemical Research Institute of the New South Wales Department of Agriculture, Australia.

**Extraction of amplification template, 16S rDNA PCR and DNA sequencing.** The cell suspension (500 µl) was treated to release sufficient genetic material for several DNA amplifications. Lysis of the pelleted cells was achieved by a modified incubation with an Instagene matrix (Bio-Rad) or by total lysis of the pelleted cells using the methods of Holmes & Quigley (1981) and Sambrook et al. (1989), without success. The absence of plasmids was confirmed by the caesium chloride gradient technique (Svenson, 1995).

**Analytical methods.** Concentrations of benzene were measured by GC analysis of the headspace. Triplicate samples (0.1 ml) were transferred to 2 ml vials with screw caps and Teflon-lined rubber septa. The vials were equilibrated at 60 °C for at least 4 h. A 10 µl headspace sample drawn with a 10 µl gas-tight Hamilton syringe was injected into the gas chromatograph (Perkin Elmer Autosystem GC) equipped with an Alltech Econo-cap Carbowax column (30 m × 0.32 mm i.d.) and a flame ionization detector. The operating conditions were as follows: column temperature 70 °C, injector at 250 °C, detector at 300 °C, and carrier gas He (1.06 ml min$^{-1}$). Catecho1 was assayed by the aminoantipyrine method (Shirai, 1986).
RESULTS
Isolation and characterization of Rhodococcus sp. strain 33
Six isolates (four Gram-positive, two Gram-negative) utilizing benzene as the sole carbon and energy source were obtained from the soil samples. Isolate 33 was chosen for the succeeding experiments because in the preliminary studies it never lost its ability to utilize benzene and it was the fastest-growing isolate. The Gram-negative isolates occasionally lost their benzene-utilizing ability. Isolate 33 was a Gram-positive, non-motile, short rod which grew between 0 and 37°C, but not at 42°C, and tolerated 6% NaCl and pH 2–10 in NA. It was not acid-fast and was negative for spore stain, metachromatic granules and oxidase; it did not grow in Winogradsky medium. It was catalase positive and utilized the following carbon sources: arabinose (with gas production), fructose, glucose, lactose, inositol (with gas production), mannitol, maltose (with gas production), sucrose (with gas production), sorbitol, salicin, urea and phenylalanine. After more than 36 h of growth in PAS medium, the cell shape changed from rod to coccoid. The test results placed the organism tentatively in the genus Rhodococcus, Arthrobacter or Mycobacterium. Fatty acid analysis by GC of isolate 33 identified it as Rhodococcus globorulus using the MIDI Microbial Identification System (MIS) TSBA library version 3.8. The fatty acid analysis revealed a relatively low similarity index of 0.2; this may have reflected poor database coverage for this genus.

The 16S rDNA sequence determined for isolate 33 was compared to previously published near-complete 16S rRNA gene sequences for the high-G+C Gram-positive bacteria and related micro-organisms; the alignment of the primary structure of the 16S rRNA gene for these bacteria provided the sequence identity values shown in Table 1. Comparison of the 16S rRNA sequence of isolate 33 with those in the GenBank, EMBL, and Ribosomal Database Project databases revealed a consistently high similarity with species of the genera Rhodococcus, Mycobacteria and Nocardia (Table 1); and isolate 33 showed 88% sequence similarity with R. globorulus, indicating the most probable taxonomic placement of the isolate to at least the genus Rhodococcus. A distance matrix was calculated and a tree constructed to support the placement of this strain within the high-G+C Gram-positive phylogenetic line as shown in Fig. 1. The presented tree topology and maximum likelihood phylogenies (data not shown) revealed the clustering of isolate 33 with a heterogeneous group of Gram-positive bacteria and with a monophyletic line of Rhodococcus species.

Benzene utilization by isolate 33
Vapour-phase benzene. GC analyses confirmed the solubility of liquid benzene in water and in PAS to be approximately 1800 p.p.m. When benzene vapour was allowed to diffuse into PAS in an open system, the saturated solution contained only 160–200 p.p.m. benzene and no further increase in concentration was detected even after 96 h of exposure to the vapour. Thus, solubility levels of benzene could not be introduced into the medium using this technique. When the system was entirely closed, maximum solubility levels of benzene were detected.

Fig. 2(a) shows that maximum cell count was obtained after 36 h when the organism was grown in PAS medium.

| Table 1. Percentage 16S rRNA gene sequence similarity (above diagonal) and pairwise genetic distances for isolate 33 and members of the high-G+C Gram-positive prokaryotes and E. coli |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| 1. Isolate 33 | 88 | 85 | 84 | 83 | 82 | 81 | 79 | 78 | 75 | 74 | 63 | 63 |
| 2. R. globorulus | 96 | 98 | 93 | 95 | 91 | 92 | 89 | 88 | 88 | 87 | 71 | 73 |
| 3. Rhodococcus sp. | 0.65 | 0.01 | 92 | 93 | 90 | 90 | 87 | 87 | 87 | 71 | 71 |
| 4. R. equi | 0.71 | 0.70 | 0.69 | 93 | 90 | 91 | 88 | 88 | 88 | 71 | 73 |
| 5. Nocardia sp. | 0.66 | 0.33 | 0.32 | 0.70 | 92 | 92 | 89 | 88 | 88 | 71 | 72 |
| 6. M. aichense | 0.65 | 0.66 | 0.64 | 0.62 | 0.66 | 99 | 93 | 87 | 85 | 85 | 71 | 71 |
| 7. M. smegmatis | 0.67 | 0.60 | 0.60 | 0.70 | 0.66 | 0.29 | 94 | 87 | 86 | 86 | 72 | 71 |
| 8. M. flavescentis | 0.71 | 0.72 | 0.70 | 0.72 | 0.71 | 0.72 | 0.73 | 86 | 87 | 86 | 70 | 70 |
| 9. Frankia sp. | 0.70 | 0.70 | 0.69 | 0.38 | 0.69 | 0.43 | 0.42 | 0.72 | 87 | 86 | 71 | 71 |
| 10. S. coelicolor | 0.67 | 0.68 | 0.67 | 0.71 | 0.67 | 0.68 | 0.74 | 0.68 | 95 | 72 | 72 |
| 11. S. ornatus | 0.67 | 0.67 | 0.66 | 0.70 | 0.67 | 0.66 | 0.66 | 0.74 | 0.68 | 0.16 | 72 | 72 |
| 12. Mic. aeruginosa | 0.74 | 0.73 | 0.72 | 0.73 | 0.74 | 0.73 | 0.73 | 0.75 | 0.71 | 0.73 | 0.74 | 0.71 |
| 13. E. coli | 0.73 | 0.73 | 0.68 | 0.73 | 0.73 | 0.73 | 0.75 | 0.68 | 0.71 | 0.72 | 0.72 | 0.72 |
with benzene vapour as the sole carbon source. The mean generation time was calculated to be 3.03 h and no lag phase was observed. To correlate growth with benzene utilization, the change in benzene concentration was monitored. At zero time 174 p.p.m. of benzene was in the medium (Fig. 2b). Twelve hours after inoculation, as cell density started to increase, benzene concentration started to decrease. As the cells entered the exponential phase, benzene concentration was at its lowest (detection limits of 10 p.p.m.), with these levels in the culture lasting another 42 h. The OD₆₀₀ value at this time corresponded to the point where cell numbers started to reach a maximum.

The pH of the medium went down from an initial value of 6.8 to 5.0 after 12 h and eventually decreased to 3.0 after 18 h (Fig. 2c), even though the medium was strongly buffered. The organism produced catechol after 6 h of growth; this reached its maximum level at around 18 h and declined rapidly thereafter.

**Liquid benzene.** Liquid benzene (0.2 ml benzene per 50 ml medium) was added directly to PAS to increase the concentration of benzene in the medium. GC analyses revealed that benzene volatilized out of the open system after 4 h and thus a constant supply of benzene was not possible with this technique. The addition of liquid benzene every 4 h ensured that the compound was continuously available to the organism but at varying concentrations. Benzene detected in the medium ranged from 1100 to 2789 p.p.m. An estimated generation time of 5.56 h was obtained, indicating that the organism tolerated high levels of benzene and at the same time utilized maximum solubility concentrations for biomass production. No lag phase was observed. This system, however, did not allow high levels of the compound to be constantly retained in the medium; continuous cultures were therefore used to allow the constant supply of benzene as liquid in the medium.

**Benzene utilization in continuous culture of Rhodococcus sp strain 33.** Continuous flow of the medium into the culture was commenced when the batch culture had reached a cell density of OD₆₀₀ 0.5 and a pH of 5.5. In batch culture experiments, this cell density corresponded to the mid-exponential growth phase, where catechol production was at its maximum. Steady-state conditions were attained when OD₆₀₀ was maintained between 0.5 and 0.6 and the pH was maintained at pH 5.5 due to the buffering capacity of the medium.

Initial benzene concentration supplied as vapour in the reactor was 174±7 p.p.m.; 95% removal was observed.
Benzene degradation by a Rhodococcus sp.

when cultures reached steady state and was maintained for 30 d (Fig. 3). In a separate experiment, liquid benzene was allowed to equilibrate in the reactor at 0.833 ml h\(^{-1}\) for 24 h before inoculation. When cell density increased to OD\(_{600}\) 0.5 after 48 h, feeding of the medium and liquid benzene (20 ml per day) commenced. A steady cell density was obtained and residual concentrations of 80–500 p.p.m. benzene were detected in the medium over a period of more than 30 d (Fig. 4).

DISCUSSION

An organism identified as a Rhodococcus sp. by morphological and biochemical tests, fatty acid analysis of the cell wall and 16S rRNA gene sequencing grew in benzene in both vapour and liquid phases in batch and continuous cultures. Utilization of benzene was directly related to growth. The depletion of phosphate in batch cultures appeared to be the factor that limited growth (data not shown). The batch open reaction system contained a constant supply of vapour benzene at approximately 200 p.p.m. and benzene was efficiently degraded as long as the cells were viable. Closed systems have been employed to culture micro-organisms in previous isolation techniques (Gibson et al., 1968; Hogn & Jaenicke, 1972; Axcell & Geary, 1973), with concentrations usually in the order of only \(\mu\)g ml\(^{-1}\) or very rarely mg ml\(^{-1}\). Open systems closely resemble those present at surfaces and in near-subsurface environments, as an exchange of air and benzene vapour occurs in contaminated sites. After benzene liquid spills both vapour- and liquid-phase benzene remain in the area. Degradation in an open system, as described in this paper, therefore better simulates the situation found in contaminated sites exposed to air in the natural environment.

The levels of benzene used in this study in both vapour and liquid phases are considerably higher than those previously reported (Alvarez et al., 1991; Chang et al., 1992; Anid et al., 1993) employing levels of 0.1–50 p.p.m. Degradation rates of only 25 mg l\(^{-1}\) d\(^{-1}\) and as high as 45 mg l\(^{-1}\) d\(^{-1}\) with 2–4 d lag periods have been reported. Although benzene is soluble at 1780 p.p.m. in water this level has proved to be toxic to most micro-organisms (Gibson et al., 1970; Davis et al., 1981; Alvarez et al., 1991). The highest level of benzene studied to date is 5% (v/v), in experiments with a Flavobacterium isolate (Moriya & Horikoshi, 1993); however, tolerance of this Flavobacterium was demonstrated when the organism was grown on a complex medium but no degradation of benzene was observed (see Introduction). A very slight difference in growth of this organism was observed on the complex medium alone as compared with the complex medium with benzene. Thus it is possible that the growth of the Flavobacterium sp. in the presence of benzene was supported by the other organic constituents of the complex medium such as peptone.

Apart from benzene toxicity, limitations in degradation could also be attributed to oxygen limitation. Weber & Corseuil (1994) showed that growth of micro-organisms in benzene was terminated by the depletion of oxygen in
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a closed system. Twelve days were enough for indigenous micro-organisms to utilize all oxygen supplied, and when lower concentrations of benzene were employed, oxygen was completely used in 10 h, resulting in the decline in cell yield and degradation. The present study employed open systems to eliminate oxygen limitation, allowing the cells to grow at normal rates to confirm the organism’s tolerance to high levels of benzene.

The addition of liquid benzene directly into the medium introduced a new phase in the system in the form of water-insoluble micelles (insoluble interphase resembling oil droplets in water), which eventually resulted in higher concentrations of benzene even if the reactor was aerated. *Rhodococcus* sp. strain 33 utilized soluble benzene (1780 p.p.m.) in the medium and also tolerated the presence of concentrated benzene in the form of micelles. Residual benzene averaged 200 p.p.m., corresponding to 89% removal. *Rhodococcus opacus* has also been reported to grow on liquid benzene in batch cultures, but growth was strongly inhibited when benzene concentrations exceeded 200 p.p.m., resulting in lag phases of 26–88 h (Zaitsev et al., 1993). The degradation route of benzene by *R. opacus* has not been elucidated.

*Rhodococcus* sp. strain 33 is an efficient benzene degrader considering its doubling time of only 3 h when grown on 200 p.p.m. benzene vapour and 5.6 h on liquid benzene. An *Alcaligenes* sp. exhibited slow growth on benzene, with a doubling time of 15 h (de Bont et al., 1986). Over the period of 2 years that *Rhodococcus* sp. strain 33 was cultivated solely on benzene vapour its doubling time was reduced to 1.8 h (data not shown). This remarkable efficiency in utilizing benzene as sole carbon source will find application in bioremediation studies. The ability of this strain to grow in 6% NaCl and at 0–37 °C will make it applicable for treatment of oil spills in marine environments. Ongoing studies using this isolate have also shown that it can co-metabolize toluene when benzene is present as the primary carbon source and utilize chlorinated benzenes as sole carbon sources (data not shown).

In a related study, it has been shown that *Rhodococcus* sp. strain 33 oxidized benzene to cis-benzene glycol, then to catechol and eventually to cis,cis-muconic acid (Paje & Couperwhite, 1996). The absence of metapyrocatechase activity confirmed that the product after the conversion of catechol is cis,cis-muconic acid. cis-Benzene glycol dehydrogenase and pyrocatechase were both inducible, as no activity was detected when the organism was grown in mannitol. The degradation of benzene therefore took place via the ortho pathway (the intra-diol cleavage) which was the same pathway as in *Pseudomonas* and *Moraxella* (Gibson et al., 1968; Hogn & Jaenicke, 1972). Catechol as an intermediate was also confirmed by NMR analysis (data not shown).

The most studied bacterial genus for benzene degradation is *Pseudomonas*, the enzymes of which have been purified and characterized and the pathways fully elucidated (Gibson et al., 1968; Axcell & Geary, 1973). Gram-positive organisms such as *Arthrobacter*, *Corynebacterium* and *Rhodococcus* have also been reported to utilize benzene as a sole carbon source but the metabolism of benzene has not been described (Axcell & Geary, 1973; Schraa et al., 1987; Warhurst et al., 1994; Zaitsev et al., 1995). *Rhodococcus* strains have been described to grow on a variety of complex carbon sources, such as short- and long-chain as well as halogenated hydrocarbons, aromatic compounds, polycyclic aromatic compounds, and steroids (Warhurst & Fewson, 1994), but rarely on reduced aromatic compounds such as benzene and toluene. Dugan & Golovlev (1982) isolated 56 strains of rhodococci from contaminated sites but none could grow on benzene. *Rhodococcus* sp. strain 33 appears to be the first Gram-positive organism shown to metabolize benzene. It illustrates the potential for these organisms, which occupy a significant niche in the environment, for use in the breakdown of hydrocarbons.

*Rhodococcus* sp. strain 33, being able to degrade solubility levels of liquid benzene and at levels 20 times higher than those previously studied, is therefore an ideal organism for the basic study of benzene uptake and transport into the cell. This is one of the most neglected aspects of biodegradation, which is not surprising, because all hydrocarbons are to a greater or lesser extent hydrophobic and most are toxic to cells of all types (Smith, 1994). Studies on solvent tolerance have focused on toluene using *Pseudomonas* strains (Inoue & Hori-koshi, 1989; Heipieper & de Bont, 1994; Li et al., 1995). To date there has been no detailed work on the specific mechanism by which benzene is toxic because of the lack of micro-organisms capable of growth in high concentrations of the compound. The exceptional tolerance of *Rhodococcus* sp. strain 33 to high levels of liquid benzene is also an important characteristic as this can form the basis of comparison of structures present in organisms tolerant to benzene and those that are readily lysed by this compound. It also opens the way to new genetic studies of benzene degradation. Finally, the isolation of this new strain of *Rhodococcus* from a contaminated site verifies that there are still organisms ‘waiting to be isolated’ from the environment that could enhance our capability to use such organisms to solve if not eliminate problems which, so far, limit bioremediation.

REFERENCES


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