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Isolation of *Alcaligenes* sp. Strain L6 at Low Oxygen Concentrations and Degradation of 3-Chlorobenzoate via a Pathway Not Involving (Chloro)Catechols

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Isolations of 3-chlorobenzoate (3CBA)-degrading aerobic bacteria under reduced O_2 partial pressures yielded organisms which metabolized 3CBA via the gentisate or the protocatechuate pathway rather than via the catechol route. The 3CBA metabolism of one of these isolates, L6, which was identified as an *Alcaligenes* species, was studied in more detail. Resting-cell suspensions of L6 pregrown on 3CBA oxidized all known aromatic intermediates of both the gentisate and the protocatechuate pathways. Neither growth on nor respiration of catechol could be detected. Chloride production from 3CBA by L6 was strictly oxygen dependent. Cell-free extracts of 3CBA-grown L6 cells exhibited no catechol dioxygenase activity but possessed protocatechuate 3,4-dioxygenase, gentisate dioxygenase, and maleylpyruvate isomerase activities instead. In continuous culture with 3CBA as the sole growth substrate, strain L6 demonstrated an increased oxygen affinity with decreasing steady-state oxygen concentrations.

Chlorinated benzoic acids are common metabolites in the degradation of many chlorinated environmental pollutants, such as polychlorinated biphenyls. Some chlorinated benzoic acids, e.g., 2,5-dichloro-3-aminobenzoic acid and 2,3,6-trichlorobenzoic acid, have been used in agriculture as pesticides (1, 14, 38, 39, 45). Therefore, it is not surprising that the biodegradation of chlorinated benzoic acids has been studied extensively (4, 11, 21, 22, 31, 38). The general picture that emerged from these studies is that under aerobic conditions, 3-chlorobenzoate (3CBA) is metabolized via (chloro)catechol by initial dioxygenations. This implies that oxygen is needed not only as an electron acceptor in the respiratory electron transfer chain but also as a substrate for dioxygenases which incorporate oxygen atoms from molecular oxygen into hydrocarbons. It is known that dioxygenases have half-saturation constants with relatively high values for oxygen. Dorn and Knackmuss (9) obtained values for the Michaelis-Menten constants (K_ms) of pyrocatechases I and II from *Pseudomonas* sp. strain B13 ranging from 73 to 33,000 μM O_2. Shaler and Klečka (41) estimated a half-saturation constant for oxygen of 37.5 μM during growth of an enrichment culture on 2,4-dichlorophenoxyacetic acid. They also summarized half-saturation constants for oxygen of dioxygenases of various organisms, which ranged from 10 to 55 μM. Cell extracts of *Pseudomonas putida* consuming O_2 in the presence of catechol yielded a K_m for O_2 of 80 μM (49). The K_m for O_2 during growth of a *Mycobacterium* sp. on pyrene in a fermentor at different oxygen concentrations was 5.9 μM. However, growth at low oxygen concentrations (3.4 μM) was slower than expected from the kinetic data, probably because of limitation by an oxygenase for pyrene degradation (12). Washed-cell suspensions of *Pseudomonas aeruginosa* JB2 and two variants of this organism indicated apparent K_m values for oxygen in the range of 10 to 31 μM during respiration of (chlorinated) benzoates (47). Finally, *Pseudomonas* sp. strains H1 and H2 grown on 3CBA or benzoate possessed K_m values for O_2 of 110 to 2,000 μM during respiration of the growth substrates (23). These observations result in an interesting paradox, as in nature many of these organisms and activities are found at oxic-anoxic interfaces, where the oxygen concentration is generally low (16–18, 27, 52). Therefore, organisms living at such oxic-anoxic interfaces are expected to play an important role in determining the fate of haloaromatic compounds in nature, for example, in the top layers of aquatic sediments in lakes, rivers, and estuaries. At low oxygen concentrations, one would expect the existence of organisms with oxygenases possessing improved O_2 affinities, possibly with alternative metabolic pathways, involved in the metabolism of aromatic compounds. Olsen et al. (37) reported the disappearance of BTEX contaminants (benzene, toluene, ethylbenzene, and xylene) under oxygen-limiting conditions. The Michaelis-Menten constants for oxygen of the ring fission enzyme catechol dioxygenase indeed appeared to be significantly lower for those strains that were active under oxygen-limiting conditions than for a few other *Pseudomonas* strains that were less active under hypoxic conditions. These results suggest that such organisms have adapted to growth and metabolism in low-oxygen environments. In a recent study on the effect of oxygen on the biodegradation of gasoline in soil (54), the rate of degradation of gasoline at lowered oxygen concentrations (10% O_2) in the presence of nitrate was almost three times higher than that under air-saturating conditions. However, no isolations or O_2 kinetic studies were reported. With respect to 3CBA degradation, hydroxylations catalyzed by hydrolases or monooxygenases, or initial reduction of 3CBA, may be alternative mechanisms which do not require low-O_2-affinity dioxygenases in the initial metabolic steps (5, 11, 21). In 1972 Johnston et al. reported the degradation of 3CBA by a *Pseudomonas* sp., in which they detected as intermediates some phenolic compounds which resembled 3-hydroxybenzoate and gentisate (24). This is the only reported example in which a dioxygenase is not involved in the initial step of aerobic degradation of

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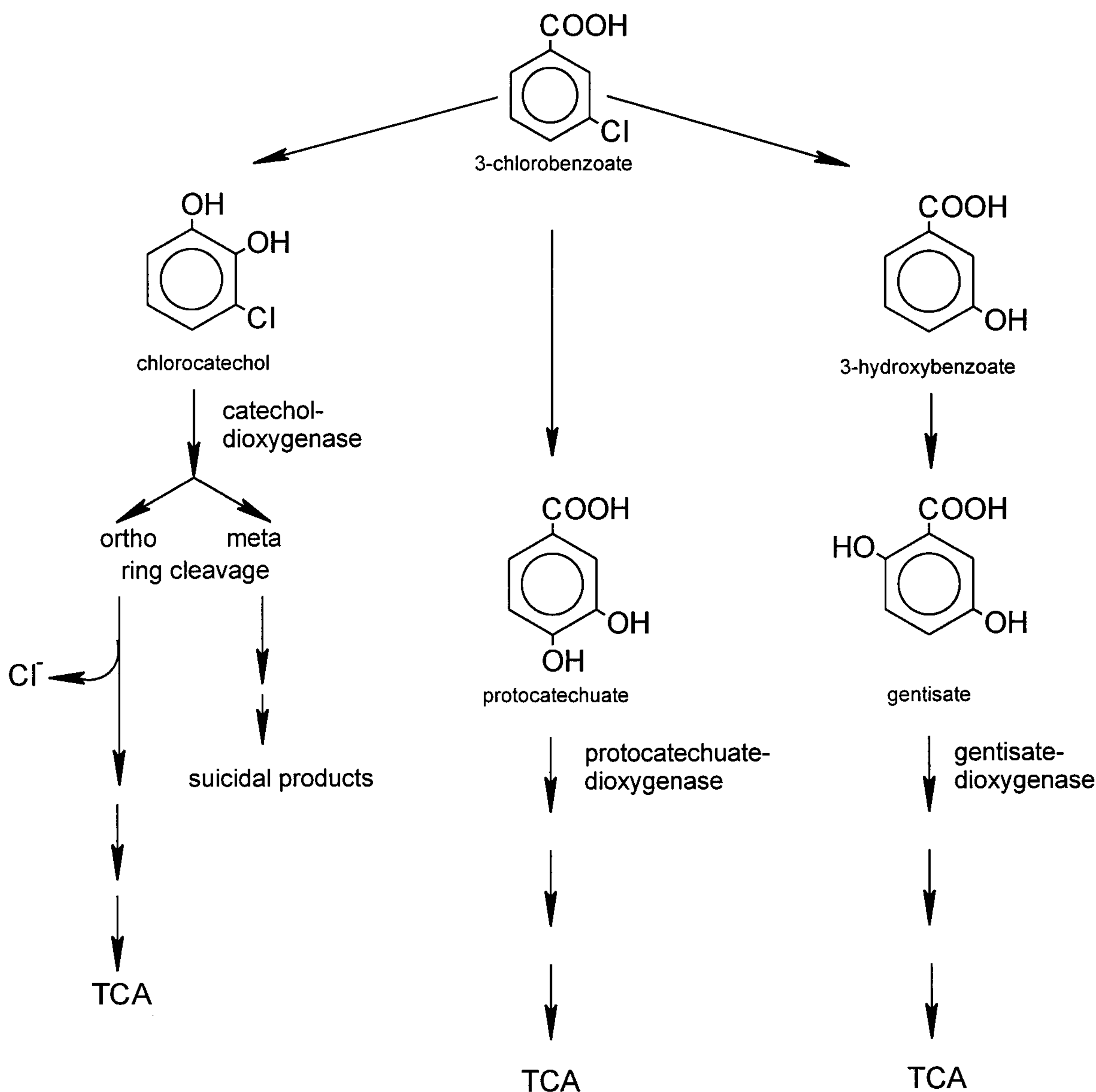

FIG. 1. Brief summary of various metabolic pathways for the degradation of 3CBA via catechol, protocatechuate, and gentisate, based on existing literature. TCA, tricarboxylic acid cycle.

3CBA. Another example in which 3CBA is not degraded via catechol was described recently by Nakatsu and Wyndham (33). This organism, an *Alcaligenes* sp., degraded 3CBA via protocatechuate as an intermediate. Those authors suggested, however, that 3CBA was probably attacked by an initial dioxygenase specific for positions 3 and 4 on the aromatic ring, leading to 3,4-dihydroxybenzoate. This same pathway probably is used by *Alcaligenes* sp. strain CPE3, as proposed by Fava et al. (10). However, these strains were not isolated under reduced partial pressures of oxygen. The metabolic pathways of 3CBA via catechol, protocatechuate, and gentisate are shown in Fig. 1.

In this report we describe the major physiological characteristics of a new isolate, *Alcaligenes* sp. strain L6, selectively enriched on 3CBA at reduced partial pressures of oxygen in the gas phase. The organism exhibits properties distinctly different from those observed with 3CBA-degrading aerobes isolated at air-saturating concentrations.

MATERIALS AND METHODS

Enrichment and isolation of strain L6. The inoculum used for the enrichment on 3CBA in batch culture under a 2% O_2 atmosphere consisted of a freshwater sediment originating from the Biesbosch in The Netherlands, an area in direct contact with the industrially polluted river Merwede. Ten grams (wet weight) of this sediment was suspended in 50 ml of mineral medium (LMM [see below]) and shaken for 2 h. After sedimentation, 10 ml was filtered through a 5-μm-pore-size filter to remove protozoa and used as an inoculum in 100 ml of LMM medium with 2.5 mM 3CBA as a growth substrate under a 2% O_2 atmosphere. After 7 days of incubation at 30°C in a rotary incubator, further enrichment was obtained by a dilution series in liquid medium with 2% O_2 in the gas phase. Strain L6 was isolated from this dilution series by plating on agar plates with 2.5 mM 3CBA at reduced partial pressures of oxygen.

Media and growth conditions. The medium used for isolation and cultivation of strain L6 is a low-chloride minimal medium (LMM medium) described previously by Gerritse and Gottschal (15). Yeast extract (10 mg/liter) was added to the medium before autoclaving. After autoclaving, 1 ml of vitamins (filter sterilized) (7) per liter, the carbon and energy source (from autoclaved 100 mM stock solutions), and 25 mM $K(NH_4)PO_4$ (pH 7) (from an autoclaved 1 M stock solution) were added. Cultures were incubated at 30°C in a rotary incubator under either a 2 or 20% O_2 atmosphere. Various chlorinated benzoates were used at a concentration of 2.5 mM; fatty acids and sugars were used at concen-

trations of 15 mM carbon. Chemostat experiments were carried out in a culture vessel (500 ml) with automatic O_2 regulation by coupling the stirring rate to continuous O_2 monitoring with a polarographic electrode (Ingold, Urdorf, Switzerland). Automatic additions of KOH maintained the pH at a constant value of 7.0.

Analytical procedures. Chloride measurements were done colorimetrically by the method of Bergmann and Sanik with NaCl as the standard (2). Chlorinated benzoates were detected gas chromatographically after methylation with methanol and extraction with chloroform (15). To separate the methylated chlorobenzoates, a linear temperature gradient from 100 to 250°C (10°C/min) was used. 2-Bromobenzoate was used as an internal standard. Growth was monitored by measuring the optical densities at 433 nm. Cell carbon was analyzed with a Shimadzu TC-500 carbon analyzer with biphthalate as a standard for dissolved organic carbon. Protein concentrations in cell suspensions were detected according to the method of Lowry et al. with bovine serum albumin as the standard (29). Protein concentrations in cell-free extracts (CFEs) were detected with the Bradford reagent (3).

Oxygen uptake rates. Respiration rates were measured in a biological oxygen monitor, using an oxygen YSS polarographic electrode. Exponentially growing cells were centrifuged for 10 min at 4°C and 11,000 × *g* and washed twice in LMM buffer (pH 7) containing 25 mM $K(NH_4)PO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$ per liter, and 0.05 g of $Ca(NO_3)_2 \cdot 4H_2O$ per liter. Cell pellets were resuspended in LMM buffer and stored on ice. Substrates were added to the cell suspensions from 100 mM stock solutions to final concentrations of 1 mM. Apparent K_m values for O_2 were obtained from both direct linear and Eadie-Hofstee plots.

Enzyme assays. Cell suspensions for CFEs were made by washing 250 ml of mid-log-phase culture (optical density at 433 nm, 0.50) twice in 25 mM $K(NH_4)PO_4$ buffer (pH 7) (11,000 × *g*, 10 min, 4°C) and concentrating the cell suspension to a final volume of 5 ml. Crude cell extracts were made by use of a French press (three times at 1,000 lb/in^2). Subsequently, CFEs were obtained after centrifugation of the crude cell extracts (11,000 × *g*, 10 min, 4°C). These extracts were stored on ice until use (within 2 h). Gentisate dioxygenase activity was detected in 1-ml quartz cuvettes by monitoring the formation of maleylpyruvate from gentisate at 340 nm (ε = 10,200 $M^{-1} \cdot cm^{-1}$) (50). The assay mixture contained 0.2 mM gentisate, 0.7 mM $FeSO_4$, 33 mM Tris buffer (pH 8), and 5 to 50 μl of CFE. Protocatechuate 3,4-dioxygenase activity was measured at 290 nm (ε = 2,300 $M^{-1} \cdot cm^{-1}$), where a net decrease in absorbance could be detected because of consumption of protocatechuate and formation of 3-carboxy-*cis,cis*-muconaat (42). The assay mixture contained 0.2 mM protocatechuate, 33 mM Tris buffer (pH 8), and 5 to 50 μl of CFE. Protocatechuate 2,3- and 4,5-dioxygenase activities were measured in the same assay mixture at 260 and 410 nm, respectively. Catechol dioxygenase activity was detected at 260 nm (ε = 16,800 $M^{-1} \cdot cm^{-1}$) in the presence of 1.33 mM EDTA, 0.2 mM catechol, 33 mM Tris buffer (pH 8), and 5 to 50 μl of CFE (8).

Genomic DNA isolation, PCR amplification, and determination of 16S rRNA gene sequence. Genomic DNA was isolated from approximately 0.1 g (wet weight) of cells by using the cetyltrimethylammonium bromide miniprep protocol for bacterial genomic DNA preparations (51). Nearly complete 16S rRNA genes were amplified by PCR (32, 40), using the forward primer 5′-AGAGTTTGATCMTGGCTCAG-3′, hybridizing at positions 8 to 27, and the reverse primer 5′-AAGGAGGTGWTCCAGCC-3′, hybridizing at the complement of positions 1541 to 1525 (*Escherichia coli* 16S rRNA gene sequence numbering). PCR was performed with a GeneAmp PCR System 9600 (Perkin-Elmer Corp., Norwalk, Conn.) and the reaction conditions reported previously (26).

PCR DNA products were purified with Centricon 100 microconcentrators (Amicon GmbH, Witten, Germany) and sequenced directly with an Applied Biosystems 373A DNA sequencer and the protocol of the manufacturer (Perkin-Elmer, Applied Biosystems, GmbH, Weiterstadt, Germany) for *Taq* cycle sequencing with fluorescent-dye-labeled dideoxynucleotides. The sequencing primers have been described previously (28). Sequence data were aligned with reference rRNA (and rRNA gene) sequences (34, 36) by using evolutionarily conserved primary sequence and secondary structure as references (20, 53). Evolutionary distances were calculated from sequence pair similarities, including a correction factor for silent mutations (25). Dendrograms were generated by using a weighted least-squares distance method (35).

Nucleotide sequence accession number. The sequence for the 16S rRNA gene of *Alcaligenes* sp. strain L6 has been deposited with the EMBL under accession number X92415.

RESULTS

Isolation and identification of strain L6. Enrichments were done in batch culture with 3CBA as the sole substrate under an atmosphere of 2% oxygen. From these enrichments several organisms were isolated via further enrichment in dilution series followed by plating on agar media, always at reduced partial pressures of oxygen. Strain L6, one of the isolates, was an aerobic, gram-negative, motile rod (0.8 to 2.5 μm) with peritrichously attached flagella (Fig. 2). No reserve material was detected by electron microscopy during growth on 3CBA. Growth on 3CBA occurred at 18 to 37°C, whereas the optimum temperature for growth was between 30 and 32°C. The optimal pH for growth on 3CBA was 6.8.

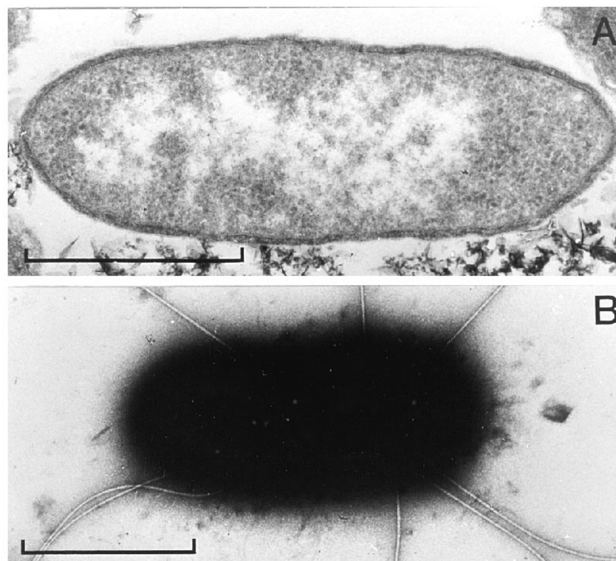

FIG. 2. Electron micrographs of log-phase cells of strain L6 grown on 3CBA in batch culture. (A) Ultrathin section (fixed with 3% glutaraldehyde, postfixed with 1% OsO_4–5% $K_2Cr_2O_7$, and stained with 1% uranyl acetate) showing the overall morphology of the cell (gram-negative rod). The central electron-light part represents the cellular DNA. (B) Intact cell, showing the peritrichously attached flagella (negatively stained with 1% uranyl acetate). Bars, 1 μm.

Sequencing of the PCR-amplified 16S rRNA gene of strain L6 allowed the determination of an estimated 97% (1,484 nucleotide positions) of the complete gene. Sequence comparison demonstrated that strain L6 clustered with bacteria of the beta subclass of the class *Proteobacteria* and most closely with species of the genus *Alcaligenes* (Fig. 3). Table 1 presents the sequence similarities determined from comparisons of the 16S rRNA gene sequences of strain L6 and reference organisms for which 16S rRNA sequence data exist.

Substrate range of *Alcaligenes* sp. strain L6. Growth of *Alcaligenes* sp. strain L6 under a 20% O_2 atmosphere was observed with the following substrates: acetate, propionate, lactate, pyruvate, succinate, 3CBA, 3,4-dichlorobenzoate, 3,5-dichlorobenzoate, 3-chloro-4-hydroxyphenylacetate, 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 3,4-dihydroxybenzoate, and 2,5-dihydroxybenzoate. No growth occurred on formate, malate, fumarate, glucose, fructose, 2-chlorobenzoate, 4-chlorobenzoate, 2,3-, 2,4-, 2,5-, or 2,6-dichlorobenzoate, 2,3,6-trichlorobenzoate, 2,3,5-trichlorobenzoate, 5-chloro-salycilic acid, phenol, or 3-chlorophenol. Anaerobic growth on lactate and pyruvate with nitrate as an electron acceptor did not occur.

Growth on 3CBA was studied in more detail. 3CBA (2.5 mM) was degraded to CO_2, H_2O, and cell carbon, with a maximum specific growth rate of 0.15 h^{-1} and stoichiometric release of chloride (Fig. 4). The yield for growth on 3CBA was 17.8 g of cell carbon per mol of substrate utilized. Washed-cell suspensions incubated in the presence of 3CBA released chloride only in the presence of oxygen.

Rates of oxygen uptake by cells of *Alcaligenes* sp. strain L6. An indication of the pathway involved in 3CBA degradation was obtained from measuring oxygen uptake rates with possible intermediates of known metabolic pathways. Batch culture-grown cells of *Alcaligenes* sp. strain L6 on 3CBA respired all

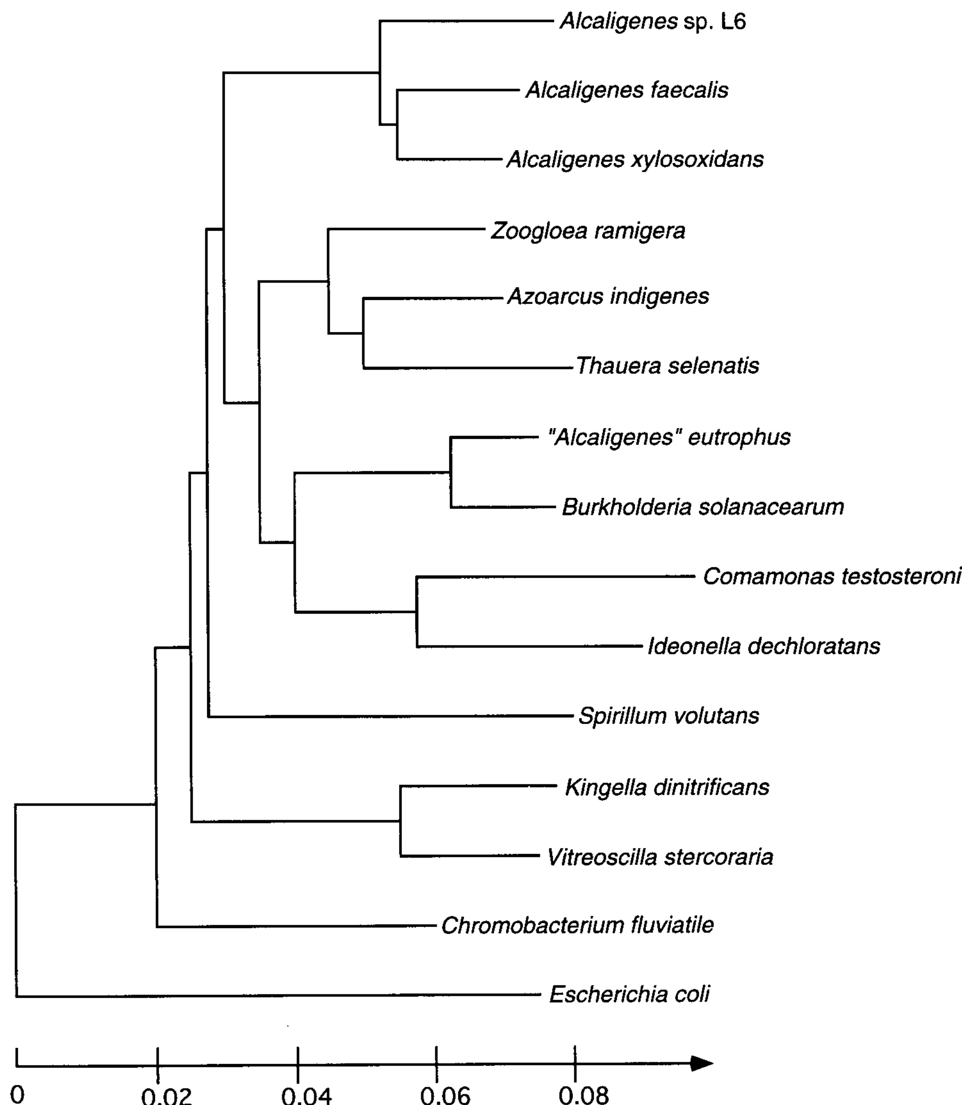

FIG. 3. Unrooted dendrogram showing the derived phylogenetic-taxonomic placement of strain L6 among reference species of the beta subclass of the *Proteobacteria*. The scale indicates the calculation of evolutionary distance.

known aromatic intermediates of the gentisate and protocatechuate pathways (Table 2). No catechol was respired. The rates of respiration of the primary growth substrates, 3CBA and benzoate, were significantly higher than those of all other intermediates tested (Table 2).

Chemostat-grown cells showed the same pattern of oxygen uptake as batch culture-grown cells. However, the maximum specific oxygen uptake rates ($Q_{O_2}{}^{max}$) for the monohydroxylated benzoates 3-hydroxybenzoate and 4-hydroxybenzoate were somewhat lower (Table 2). Interestingly, the $Q_{O_2}{}^{max}$ on the growth substrate 3CBA increased after the shift from 12 to 2% O_2 and eventually to oxygen limitation (<0.01% O_2) in the chemostat culture (Table 3). Apparent K_m values were determined with washed-cell suspensions. For each steady state at 12 or 2% O_2 or O_2 limitation, Eadie-Hofstee plots resulted in two different K_m values, one in the nanomolar range (≈65 nM) and another in the micromolar range (7 to 17 μM). Addition of 0.5 mM KCN, a known inhibitor of the respiratory chain, to the cell suspensions at high oxygen concentrations (>100 μM) did not significantly influence the oxygen uptake rate. However, at oxygen concentrations lower than 20 μM, the oxygen uptake was strongly (>80%) inhibited. These results suggest that the low K_m values represent those for oxygen consumption via the respiratory chain. The high K_m values thus reflect those for use of O_2 by oxygenases: 17, 7, and 8 μM for steady states at 12% O_2, 2% O_2, and O_2 limitation, respectively (Table 3). A decrease in the apparent K_m values for oxygen was observed, which, in combination with the increased $Q_{O_2}{}^{max}$ values, resulted in an improved affinity for oxygen from 11 to approximately 30 ml · min^{-1} · mg of protein^{-1} (Table 3).

Enzyme activities in CFEs of *Alcaligenes* sp. strain L6. On the basis of the oxygen uptake rates, as shown above, the most likely metabolic pathway used by *Alcaligenes* sp. strain L6 for growth on 3CBA proceeds via either gentisate or protocatechuate. To confirm this, the activities of the key enzymes of these pathways were measured. The activities of gentisate dioxygenase, protocatechuate dioxygenase, and catechol dioxygenase in 3CBA-, benzoate-, acetate-, and protocatechuate-grown cells were compared (Table 4). Gentisate dioxygenase was present during growth on 3CBA, whereas the protocat-

TABLE 1. 16S rRNA gene sequence similarities between strain L6 and reference species

Organism	Sequence similarity[a] with strain L6 (%)
Alcaligenes faecalis	93
Alcaligenes xylosoxidans subsp. *denitrificans*	94
Alcaligenes eutrophus	89
Azoarcus indigenes	88
Burkholderia solanacearum	89
Burkholderia cepacia	89
Comamonas testosteroni	85
Ideonella dechloratans	88
Kingella denitrificans	86
Spirillum volutans	87
Thauera selenatis	87
Zoogloea ramigera	88
Pseudomonas aeruginosa	81
Escherichia coli	81
Agrobacterium tumefaciens	76
Desulfovibrio desulfuricans	76
Dehalospirillum multivorans	76

[a] 16S rRNA gene sequence similarities derived from comparison with sequence data deposited with the EMBL (34) and with the Ribosomal Database Project (36).

echuate dioxygenase activity was lower than it was in protocatechuate-grown cells. Additionally, after conversion of 0.2 mM gentisate to maleylpyruvate by gentisate dioxygenase, this product was rapidly consumed following addition of 0.05 mM reduced glutathione, indicating activity of maleylpyruvate isomerase (6). Gentisate dioxygenase activity was also present during growth on protocatechuate. Benzoate-grown cells possessed a much higher gentisate dioxygenase activity. Cells grown on acetate or on benzoate possessed similar activities of both gentisate dioxygenase and protocatechuate dioxygenase, indicating that these enzymes are constitutive. Catechol dioxygenase activity could not be detected during growth on either 3CBA, benzoate, acetate, or protocatechuate (Table 4).

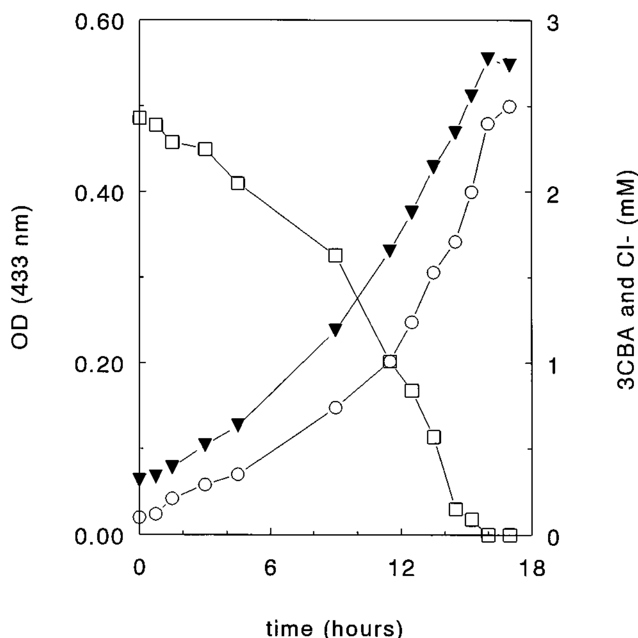

FIG. 4. Stoichiometric release of chloride (○) and increase in optical density (OD) (▼) due to degradation of 3CBA (□) by *Alcaligenes* sp. strain L6.

TABLE 2. Maximum oxygen uptake rates of batch- and continuously grown cells of strain L6

Substrate	Maximum oxygen uptake rate (nmol · min^{-1} · mg of protein^{-1}) of cells pregrown on:		
	3CBA (Batch culture)	3CBA (Continuous culture)	BA[a] (batch culture)
3CBA	171	187	51
BA	101	97	257
3-OH-BA	31	47	73
4-OH-BA	109	36	195
3,4-OH-BA	112	42	114
2,5-OH-BA	73	58	97
Catechol	0	0	0

[a] BA, benzoate.

DISCUSSION

Very few attempts to isolate bacteria on aromatic compounds under low oxygen tensions have been made so far. Mikesell et al. (30) isolated and compared BTEX-degrading organisms obtained under low-oxygen conditions (hypoxic) and under fully aerobic conditions. It was concluded that the conditions of isolation, rather than the nature of the substrate, predominantly influenced the apparent substrate utilization range of the isolates. However, they did not note any differences in metabolic pathways or O_2 kinetic parameters. In another study a nitrate-reducing strain of *Pseudomonas maltophilia* was isolated under a 2% O_2 atmosphere (43). This organism degraded toluene and xylene poorly under nitrate-reducing conditions. However, when 2% O_2 was present in the atmosphere, enhanced growth was obtained in both the presence and absence of nitrate.

To our knowledge the present report describes for the first time an organism selectively enriched and isolated on 3CBA under low-oxygen conditions. Determination and comparison of the 16S rRNA gene sequence demonstrated that strain L6 is most closely related to species of the genus *Alcaligenes*, that is, *A. faecalis* (the type species of the genus) and *A. xylosoxidans* ("*Alcaligenes*" *eutrophus* is misnamed and groups with species of the genus *Burkholderia*). The sequence similarities between strain L6 and *A. faecalis* and between strain L6 and *A. xylosoxidans* of 92.7 and 93.7%, respectively, are not, in themselves, confirmatory evidence that strain L6 is a species of *Alcaligenes*. However, such a close phylogenetic relationship between strain L6 and *Alcaligenes* spp., together with the morphological char-

TABLE 3. Apparent K_m values for O_2, maximum oxygen uptake rates on 3CBA, and oxygen affinities of washed cells of strain L6 grown in continuous culture on 3CBA with different concentrations of oxygen in the culture liquid[a]

O_2 (μM)[b]	K_m (μM)	$Q_{O_2}^{max}$ (nmol · min^{-1} · mg of protein^{-1})	Affinity ($Q_{O_2}^{max}/K_m$) (ml · min^{-1} · mg of protein^{-1})
143 (≈12)	17	187	11
24 (≈2)	7	238	34
<0.1 (<0.01)	8	240	30

[a] Cells were grown at pH 7 and 30°C (D = 0.025 h^{-1}). Oxygen-limited growth occurred at oxygen concentrations below the detection limit of the O_2 probes (<0.1 μM). In such cultures 3CBA was consumed incompletely.
[b] Percentages of dissolved oxygen are shown in parentheses.

TABLE 4. Enzyme activities in CFEs of strain L6 grown on 3CBA, benzoate, protocatechuate, and acetate

Growth substrate	Sp act ($nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$) of:		
	Catechol dioxygenase	Gentisate dioxygenase	Protocatechuate dioxygenase
3CBA	0	251	62
Benzoate	0	1,501	117
Protocatechuate	0	328	273
Acetate	0	1,327	171

acterization (gram negative, peritrichous flagella, rod shaped), suggests that strain L6 should be recognized as a species of *Alcaligenes.*

In general 3CBA is degraded via (chloro)catechol in aerobic organisms. The (chloro)catechols are formed by the initial action of dioxygenases (11, 13). Therefore, it can be expected that the availability of oxygen plays a significant role in the early steps of degradation of 3CBA to (chloro)catechol. Dorn and Knackmuss (9) reported the accumulation of chlorocatechols to toxic levels during growth of *Pseudomonas* sp. strain B13 on 3CBA under reduced oxygen tensions. Haller and Finn (23) reported that at low dissolved oxygen concentrations (<30 μM), 3CBA-utilizing pseudomonads showed a strong decrease in respiration of 3CBA. In addition, more recently it was reported that the induction of the catechol 1,2-dioxygenase of *P. putida* was strongly reduced at lower partial pressures of oxygen (49). On the basis of these apparent difficulties with oxidative metabolism of (chloro)benzoates and catechols in these examples, it could be expected that selective enrichment of bacteria on 3CBA at low oxygen concentrations would result in isolates either with initial dioxygenases with relatively low half-saturation constants for O_2, as reported for a catechol dioxygenase (37), or with other metabolic pathways not involving initial dioxygenases. Indeed, our results strongly indicate that *Alcaligenes* sp. strain L6 metabolizes 3CBA via an alternative route. The range of aromatic substrates used by this organism, the respiration of hydroxylated benzoates, and the enzyme activities detected indicate that 3CBA is metabolized via the gentisate or the protocatechuate pathway. Metabolism of 3CBA via the catechol pathway can be excluded, since *Alcaligenes* sp. strain L6 did not use catechol as a growth substrate, did not respire this substrate when grown on 3CBA, and did not possess any detectable catechol dioxygenase activity. So far the protocatechuate pathway has been described only for the aerobic degradation of 3CBA by *Alcaligenes* spp. (10, 33). These organisms were not isolated under reduced partial pressures of oxygen, indicating that the oxygen tension is not the only factor determining the metabolic pathway used for the degradation of (chloro)aromatics under aerobic conditions.

In *Alcaligenes* sp. strain L6 the gentisate pathway and the protocatechuate pathway appear to be activated simultaneously, as judged by the oxygen uptake rates with gentisate and protocatechuate and because of the observed activities of both gentisate dioxygenase and protocatechuate dioxygenase. Yet, CFEs of 3CBA-grown cells of *Alcaligenes* sp. strain L6 showed higher activity of gentisate dioxygenase than of protocatechuate dioxygenase, whereas in protocatechuate-grown cells the two activities were almost the same. Moreover, benzoate-grown cells of *Alcaligenes* sp. strain L6 possess a six-times-higher gentisate dioxygenase activity than 3CBA-grown cells, whereas the protocatechuate dioxygenase activity was only twice that of 3CBA-grown cells and was lower than that in protocatechuate-grown cells. These observations taken together suggest that the gentisate pathway is the most predominant one during degradation of benzoate and that this pathway is less induced by the substituted benzoate 3CBA. The observed activities of both gentisate dioxygenase and protocatechuate dioxygenase in acetate-grown cells indicate that these enzymes are constitutive. The phenomenon that two comparable pathways are induced at the same time whereas only one of the metabolic routes is used (gratuitous induction) was described previously for *Rhodococcus erythropolis* during growth on salicylate (44).

The respiration of 3- and 4-hydroxybenzoate by 3CBA-grown cells of *Alcaligenes* sp. strain L6 suggests that one of these monohydroxybenzoates is an intermediate in 3CBA degradation. Whether 3CBA is indeed converted to 3- or 4-hydroxybenzoate and this is followed by the formation of gentisate or protocatechuate by the action of hydrolases and/or monooxygenases or whether 3CBA degradation occurs by direct dioxygenations remains to be studied. Reductive dechlorination of 3CBA to benzoate followed by hydroxylation of benzoate is, theoretically, yet another possibility. It was shown by Van den Tweel et al. (46) that reductive dechlorination of 2,4-dichlorobenzoate to 4-chlorobenzoate by *Alcaligenes denitrificans* NTB-1 occurred under aerobic conditions. However, an initial reduction of 3CBA to benzoate by *Alcaligenes* sp. strain L6 seems unlikely, since chlorine release from 3CBA was

TABLE 5. Apparent K_m values for oxygen, $Q_{O_2}^{max}$, and affinity for oxygen ($Q_{O_2}^{max}/K_m$) of washed-cell suspensions of various organisms degrading (chlorinated) aromatics

Organism	Substrate	Cells[a]	Half-saturation constant for O_2 (μM)	$Q_{O_2}^{max}$ ($nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$)	Affinity ($ml \cdot min^{-1} \cdot mg$ of $protein^{-1}$)	Reference
P. aeruginosa JB2	2,5-dichlorobenzoate	RC	30	100–250	3.3–8.3	47
P. aeruginosa JB2 variety 2	2,5-dichlorobenzoate	RC	31	80–200	2.6–6.5	47
Pseudomonas sp. strain A3	3CBA	RC	24	84	3.5	Unpublished data
Pseudomonas sp. strain H1	Benzoate	RC	110	501	4.6	23
Pseudomonas sp. strain H2	3CBA	RC	2,000	3.2	1.6×10^{-3}	23
	Benzoate	RC	313	1.8	5.8×10^{-3}	23
Mycobacterium sp.	Pyrene	GC	5.9			12
Enrichment	2,4-dichlorophenoxyacetic acid	GC	37.5			41
P. putida	Catechol	CE	80			49
Alcaligenes sp. strain L6	3CBA					
	O_2 excess	RC	17	187	11	This paper
	O_2 limitation	RC	8	240	30	This paper

[a] RC, resting cells; GC, growing cells; CE, cell extract.

strictly oxygen dependent. Enzyme measurements to further elucidate the initial steps in the 3CBA degradation by *Alcaligenes* sp. strain L6 are now in progress.

From Eadie-Hofstee plots two K_m values were obtained, one reflecting a high-oxygen-affinity system (K_m for $O_2 \approx 65$ nM) and the other reflecting a low-affinity system (K_m for $O_2 = 7$ to 17 μM). Since at low O_2 concentrations the oxygen consumption of 3CBA-grown cells was blocked by KCN and no such inhibition was measured at high oxygen concentrations, we can conclude that the apparent K_m values in the micromolar range reflect the activity of (di)oxygenases. These K_m values fall within the lower range of K_m values for oxygen of some other strains utilizing aromatic substrates (Table 5). Olsen et al. (37) also found that a *Pseudomonas* sp. growing under hypoxic (i.e., O_2-limiting) conditions had lower K_m values for O_2 than those that grew poorly under these conditions. However, data supporting this conclusion were not shown. Similarly, the observed lowered K_m value probably permits good growth of *Alcaligenes* sp. strain L6 on 3CBA and a high rate of respiration of 3CBA not only under air-saturating conditions but also under conditions of strong O_2 limitation. This may be explained, at least partly, by the presence of pathways which probably do not require dioxygenation of the primary substrate. To establish whether this is a common property of bacteria selected for growth at reduced oxygen tensions with chlorobenzoates as growth substrates, other isolates, obtained under similar conditions, are now being studied. Interestingly, preliminary results from these studies indicate that the other isolates degrading (3-chloro)benzoates via a pathway not involving catechols seem to have significantly lower maximum specific growth rates than the organisms metabolizing (3-chloro)benzoates via catechol (data not shown). Moreover, results of competition experiments with *Alcaligenes* sp. strain L6 and *Pseudomonas* sp. strain A3, an isolate obtained under air-saturating conditions, revealed that *Alcaligenes* sp. strain L6 invariably outcompeted *Pseudomonas* sp. strain A3 at reduced oxygen tensions (data not shown). This may suggest that organisms like L6 belong to a group generally characterized by relatively low μ_{max} values on chlorinated aromatics with relatively high substrate affinities (also for O_2). In its general form, a distinction between low μ_{max}-low K_m and high μ_{max}-high K_m types of bacteria is based on numerous competition and enrichment studies (19, 48) with heterotrophic and autotrophic bacteria. If indeed this concept also applies to growth on chlorinated aromatics and to the use of molecular oxygen in (di)oxygenation reactions, organisms like *Alcaligenes* sp. strain L6 may be of greater importance than organisms obtained under air saturation for (micro)aerobic degradation of chlorinated aromatics in natural environments.

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