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Growth of Aerobic Bacteria on Alkali-Solubilized Lignite

Scientific Note

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INTRODUCTION

Coal contains a complex mixture of organic compounds, the variety of which depends on the particular type of coal (*1,2*). There is a general agreement that coal is composed of a macromolecular fraction and a lower-mol-wt fraction that are noncovalently associated with each other (*1,2*). Huttinger and Michenfelder (*3*) have proposed a structural unit for the macromolecular portion of a lignite coal that comprises 2 and 3-ring fused aromatics, paraffin, terpene, cycloaliphatics, hydrocarbon bridges, several carboxyl moieties, straight-chain saturated hydrocarbons, branched-chain hydrocarbons, sulfur heterocyclics, ether linkages, alcohol groups, nitrogen heterocyclics, and chelated metals (*3*). Low-mol-wt compounds found in coal can be separated from macromolecules by extraction with organic solvents, such as tetrahydrofuran (*1,2*). Low-mol-wt organic compounds that have been revealed by such extractions include straight-chain (C_{13}–C_{33}), branched, and cyclic alkanes; aryl and aryl alkyl compounds with 1–6 rings; and phenolic compounds (*1*). In low-ranked coals, branched alkanes predominate over straight chain (*1*).

Various interactions of microorganisms with coal have been described. Quigley et al. (*4*) have determined that certain fungi can solubilize untreated

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coal by producing alkaline substances and by chelating coal-associated metal ions (*4,5*). Other investigators have proposed that lignin peroxidases may use coal as a substrate (*6*). Recently, Gupta et al. (*7*) presented evidence that *Pseudomonas cepacia* is able to use lignite as a sole carbon and energy source (*7*).

Several studies have indicated that coal contains antimicrobial substances (*8–11*). Olsson et al. (*10*) have reported that growth of *Sulfolobus* is affected by compounds leached from coal with water (*10*). Such compounds would include salts, trace elements, and low-mol-wt organic compounds (*10,11*). Certain species of *Sulfolobus* were inhibited by the leached compounds (*10*), and growth of one species of *Sulfolobus* was enhanced by the leached compounds (*10*). There have been other reports that leached coal compounds inhibit microbial growth (*11*). We reported recently that, in general, the growth of microorganisms is enhanced, rather than inhibited, by low-mol-wt organic compounds in coal (*12*). The organic compounds used for that study were extracted from lignite with tetrahydrofuran. The same extracts were used in the present study as a growth substrate for a bacterial strain that was isolated from an aerobic coal enrichment culture.

Our research is primarily concerned with isolating and characterizing microbes that are able to convert coal to liquid fuels and chemical feedstock. The problems that must be considered in approaching such research derive mainly from the heterogeneity of coal. Because coal is so complex, many different types of metabolic capabilities are probably necessary to significantly convert it to a homogenous product. This report describes the enrichment for, and isolation of, aerobic microorganisms that are capable of significantly modifying lignite. Because low-mol-wt coal molecules are probably more easily metabolized than macromolecular coal by most microbes, growth of a promising strain, KPA16, was tested in the presence of different molecular fractions of lignite.

MATERIALS AND METHODS

Media

Medium formulas are shown in Table 1. Basal salts contained 0.02% NH_4Cl, 0.0001% NaCl, 0.001% K_2HPO_4, 0.001% $MgSO_4 \cdot 7H_2O$, 0.0001% $CaCl_2 \cdot 2H_2O$, 0.01% sodium EDTA, 0.0035% $FeSO_4 \cdot 7H_2O$, and 5 mL/L of medium of a trace-element solution containing 0.1% $ZnSO_4 \cdot 7H_2O$, 0.1% $MnSO_4 \cdot H_2O$, 0.1% $CuSO_4 \cdot 5H_2O$, 0.1% $Na_2B_4O_7 \cdot 10H_2O$, 0.1% $CoCl_2 \cdot 6H_2O$, and 0.1% $Na_2MoO_4 \cdot 2H_2O$. The composition of the basal-salt solution is derived from a solution described elsewhere (*13*). In all experiments concerning strain KPA16, Tris buffer was added to basal salts to give a final concentration of 25 m*M*. All media were adjusted to pH 7.5 prior to autoclaving or filter-sterilization. The final concentrations of different coal

fractions that were used for the different media (Table 1) were stoichiometric with respect to the yield of THF-soluble material that resulted from the tetrahydrofuran Soxhlet extraction of coal (*see below*). Semisolid media contained 1.5% of either agar or agarose.

Preparation of Coal Fractions

Alkali-Solubilized Whole Coal

Mississippi Wilcox lignite (−100 mesh) was stirred in 0.2*N* NaOH overnight. The mixture was centrifuged at 10,000*g* (10°C) and the supernatant was isolated. The supernatant was adjusted to pH 1.4 with concentrated HCl and maintained at this pH for several hours, while being stirred. The resulting suspension was centrifuged at 30,000*g* (14°C), and the precipitate was isolated, washed with 40 m*M* HCl, and lyophylized.

THF-Insoluble and THF-Soluble Fractions

Wilcox lignite (−100 mesh) was extracted with THF (tetrahydrofuran) by Soxhlet extraction for 24 h. The fraction of coal extracted into the THF was defined as THF-soluble; that which remained behind was defined as THF-insoluble. THF was removed from the THF-soluble coal fraction by evaporation at reduced pressures. THF-insoluble coal was freed of THF by air-drying and was then solubilized in alkali, as described above for whole coal. The yield of THF-soluble material from the 24-h extraction was approx 10% (w/w). Another extraction was performed later, in which the extraction period was extended to 7 d and the yield of THF-soluble material was the same. All experiments were performed with the material from the 24-h extraction, unless otherwise noted.

Aerobic Enrichments

Environmental samples were collected from clover leaves, river water and sediment material, human sputum, and cedar fronds. Samples were used to inoculate separate flasks of COAL+medium. Cultures were incubated stationary, in darkness (29°C, 50% humidity). Four days later, samples of the cultures were used to inoculate COAL+agar plates. These were incubated under the same conditions. After 2 d, many isolated colonies of various colors and morphologies were visible. Colonies were removed using sterile toothpicks and transferred in replicate to three types of solid media: CON+, COAL+, and THFS+. Plates were incubated under the same conditions for 4 d. Colony size was then compared for each isolate with respect to medium type. Five bacterial isolates, which showed enhanced colony size in the presence of coal when compared to the control, were selected for use in this study. Strains designated KPA16, KPC6, and KPC8 came originally from river water and sediment material, KPC9 was obtained from sputum, and KPC16 from a mix of clover leaves and cedar fronds.

Table 1
Media Compositions

Media	BS[a]	CA[b]	YE[c]	Whole Coal[d]	THFi Coal[e]	THFs Coal[f]
CON-	+	-	-	-	-	-
COAL-	+	-	-	+	-	-
CON+	+	+	+	-	-	-
COAL+	+	+	+	+	-	-
THFI+	+	+	+	-	+	-
THFS+	+	+	+	-	-	+
THFS-	+	-	-	-	-	+

[a]Basal salts.

[b]Casamino acids, 0.02% final conc.

[c]Yeast extract, 0.02% final conc.

[d]Alkali-solubilized whole Mississippi Wilcox lignite, 0.02% final conc.

[e]THF-insoluble portion of Wilcox lignite, 0.0185% final conc.

[f]THF-soluble portion of Wilcox lignite, 0.0028% final conc.

Initial Growth Analysis of Aerobic Isolates

Single colonies of each of the five isolates were resuspended in small amounts of CON+ medium, and aliquots of this were introduced to CON+ and COAL+ media (50 mL of medium/125-mL flask). These cultures were incubated stationary, in darkness (29°C, 50% humidity). After 3 d, measurements of absorbance (A_{660}) against an abiotic blank and of total protein were taken. For protein analyses, culture samples were centrifuged and cell pellets were dissolved in 2*N* NaOH. Protein was analyzed by a method described elsewhere (*14,15*).

Growth Analysis of KPA16

Log-phase cultures of strain KPA16 grown in CON+ medium were used to inoculate the different media listed in Table 1. Cultures were incubated stationary, in darkness (29°C, 50% humidity). In later experiments, in which growth on different concentrations of THF-soluble material was compared, the following final concentrations were used: 1×THFS medium, 0.0028% (w/v) THF-soluble material; 0.5×THFS medium, 0.0014% (w/v) THF-soluble material; 2×THFS medium, 0.0056% (w/v) THF-soluble material. Culture samples were removed periodically, and absorbance (A_{660} against an abiotic blank), total protein, and total carbohydrate measurements were made. Samples were centrifuged, washed in 0.15% NaCl

Table 2
Growth of Aerobic Isolates on Alkali-Solubilized Whole Coal

Strain name	A_{660} values for different media		ug protein/ml culture	
	CON+	COAL+	CON+	COAL+
KPA16	0.130	0.215	47	60
KPC8	0.171	0.145	72	70
KPC16	0.082	0.085	36	46
KPC6	0.114	0.138	49	67
KPC9	0.319	0.303	58	55

and 10 m*M* Tris (pH 8.0), and pellets were resuspended in either 0.5*N* NaOH (for protein measurements) or deionized water (for carbohydrate analysis). Protein analysis was performed as described above and carbohydrates were determined by the phenol method reported elsewhere (*15*).

RESULTS AND DISCUSSION

Of the five aerobic strains tested for growth enhancement by alkali-solubilized whole coal, strains KPA16, KPC16, and KPC6 grew better in the presence of coal (Table 2). KPA16 exhibited the best growth overall (Table 2). Strains KPC8 and KPC9 were slightly inhibited by coal, which reflects the inaccuracy of the replica plating method for selecting microbes that grow on coal, since these two strains were initially selected based on larger colony size on COAL+ plates. Regardless, the method was sufficient to obtain three strains that exhibited enhanced growth on coal.

Growth of KPA16 on coal was studied in more detail, since it appeared to be the most promising strain. KPA16 grew to the same extent on stoichiometric amounts of different coal fractions (Fig. 1), and all fractions enhanced overall absorbance by approx 30%. Total protein and carbohydrate analyses also indicated that growth of KPA16 was increased over that of controls when any coal fraction was present in the growth medium (data not shown). Initial growth rates were not affected significantly, but were consistently lower ($n=6$) when any fraction of coal was present (Fig. 1). It appears that a nutrient that becomes limiting in the CON+ medium after about 20 h is supplied by the coal fractions and allows for more growth (Fig. 1). Growth did not occur on CON− or COAL−, suggesting that growth factors found in the supplemental carbon (casamino acids and yeast extract) were probably required by KPA16 (Fig. 1). The results imply that low-mol-wt THF-soluble material is a nutritional source. This is supplementary to our recent report that the growth of microbes, in general, is

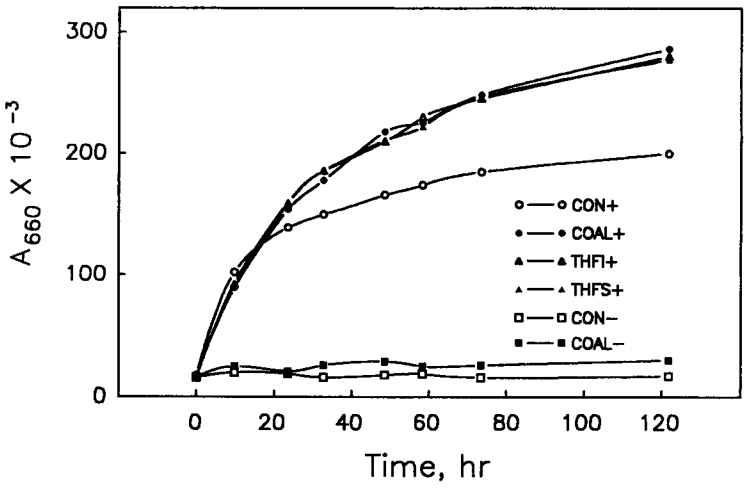

Fig. 1. Growth of strain KPA16 on different media. Turbidity of cell suspensions was measured at 660 nm. Media compositions are given in Table 1.

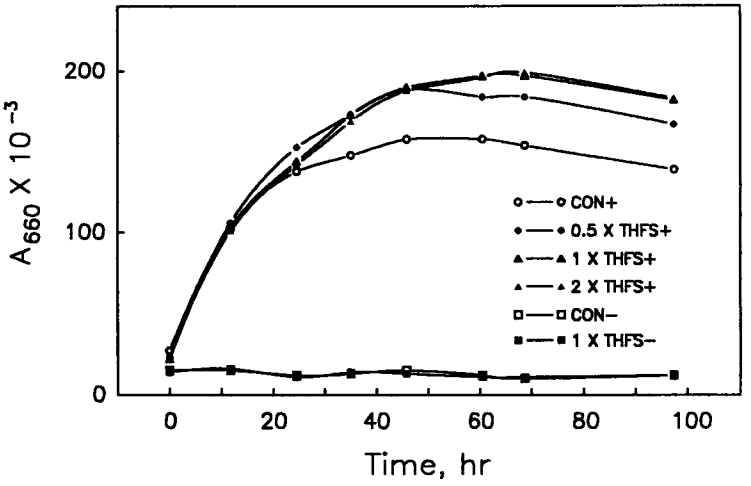

Fig. 2. Growth of strain KPA16 on different concentrations of THF-soluble material. Turbidity of cell suspensions was measured at 660 nm. Specific concentrations of THF-soluble material are described in Materials and Methods section.

enhanced by the low-mol-wt organic compounds in coal (*12*). KPA16 also grew well on THF-insoluble coal (Fig. 1), which had most of the extractable THF-soluble material removed from it. This suggests that KPA16 can grow at the expense of the macromolecular phase of coal, in addition to the low-mol-wt phase.

At 0.0028%, THF-soluble material was at a saturating concentration under these growth conditions, since twice that concentration showed the same growth pattern (Fig. 2). Half as much resulted in a lower overall yield, although higher than obtained with the control medium (Fig. 2). As

with the experiment depicted in Fig. 1, there is an implication that nutrients found in the THF-soluble material make up for a depletion of such nutrients in the control medium (Fig. 2). The most likely nutrients to become limiting in the CON+ medium are the carbon sources, which would be found in the small amounts of casamino acids and yeast extract. Extra growth factors supplied by the casamino acids and yeast extract are needed even with the THF-soluble material present, since KPA16 would not grow on THFS– medium (Fig. 2). Since our first Soxhlet extraction of coal was only 24 h long, we also tested the growth of KPA16 on material that was extracted for 7 d, to see if the duration of the extraction significantly affected the growth response of KPA16. This growth analysis indicated that KPA16 responded in the same manner to 24-h- and 7-d-extracted THF-soluble material (data not shown).

Preliminary respiratory experiments, in which oxygen consumption by KPA16 was measured in the presence of different coal fractions, were performed. The results indicated that coal does not promote oxygen consumption by KPA16 and that it may, in fact, inhibit oxygen consumption (data not shown). These observations imply that KPA16 does not use coal for the purpose of aerobic respiration and that coal may inhibit aerobic respiration in this organism. This suggests that KPA16 is able to grow on coal fermentatively or that it is able to use some element in coal (such as iron) as an anaerobic respiratory electron acceptor. Fermentation and anaerobic respiration are processes that are typically inhibited by oxygen. This would explain why the enhancement of growth that is observed occurs late in the growth phase, since this is when the culture medium would be most deficient in oxygen. Preliminary experiments indicate that carbon, not iron, is the most probable limiting nutrient under the growth conditions employed. This suggests that KPA16 may ferment the carbon in coal and that it does not use the iron in coal for anaerobic respiration. Future studies will be directed at determining whether or not KPA16 grows fermentatively at the expense of coal and what products may result from this.

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