sources of this coal (*1,2*). One of the problems associated with the utilization of such coal is the emission of SO_2 during its direct combustion. Physical and chemical methods of coal desulfurization either are not cost-effective or result in a loss of fine coal particles (*3–5*). The sulfur content of Appalachian and midwestern coal varies from 3.0 to 5.5% (*1,2*). The sulfur in these compounds consists of various thiol, sulfide, disulfide, and thiophene groups (*4,6*). At present, both *Thiobacillus* and *Sulfolobus* have been shown to be capable of converting the inorganic sulfur in coal into soluble sulfate, which can then be washed out. Our intention is to complement this by developing bacteria capable of degrading the organic sulfur components into sulfate or H_2S.

There are relatively few examples of bacteria capable of degrading sulfur-containing heterocycles, such as thiophenes, which predominate in the organic fraction of coal. In some cases, partial degradation of thiophenes, without release of the sulfur, has been observed (*7–10*). In others, sulfur was released (as H_2S) only under strictly anaerobic conditions in the presence of hydrogen (*11*). Recently, Isbister et al. (*12*) reported the removal of ^{35}S from dibenzothiophene by strain CBI without utilizing the organic component of the molecule as a source of energy for growth. Oxidation, but not desulfurization, of *n*-octyl sulfide, benzylmethyl sulfide, thioanisole, and 1-benzothiophene by this strain was also reported (*13*). Strain CBI was also reported to achieve 20–30% desulfurization of a low-organic-sulfur coal after 8–9 h of incubation. However, ultrahigh levels of inoculation (4×10^9–10^{10} cells/g) were required to treat the coal (*14*). Complete degradation of thiophenes under aerobic conditions has been described for two poorly characterized organisms (*15,16*). This suggests that thiophene-degrading bacteria are rare in nature. Thiophene-degrading bacteria and the status of microbial desulfurization of fossil fuels have been reviewed by Monticello and Finnerty (*11*).

Alternative methods for the desulfurization of coal are currently needed. The microbial removal of organic sulfur from coal, when complemented by the inorganic desulfurization system described by Detz and Barvinchak (*17*), should result in efficient bacterial removal of total sulfur from coal.

We have addressed this issue by attempting to construct, by genetic means, strains of bacteria that can degrade thiophenes and related organic sulfur compounds. Our first attempts in this direction have resulted in the isolation of a series of mutant strains of *Escherichia coli* with successively increased oxidizing ability towards furan and thiophene compounds (*18*). Three novel genes involved in thiophene oxidation, *thdA*, *thdC*, and *thdD*, were identified and mapped on the *E. coli* chromosone (*18*). In addition, mutations in two previously known regulatory genes, *fadR* and *atoC*, were also required. Further work resulted in more accurate mapping of *thdA* and *thdD* (*19*) relative to known chromosomal genes and in the isolation of a further mutation, *thdE*, so far unmapped (*19*). This conference presentation reviews some more recent findings, including the cloning of several genes involved in thiophene metabolism.

MATERIALS AND METHODS

Bacterial Strains and Culture Media

Bacterial strains were all derivatives of *Escherichia coli* K-12 and are listed in Table 1. Rich broth contained (/L) 10 g of tryptone, 5 g of NaCl, and 1 g of yeast extract. The minimal medium used was M9 for growth tests or medium E for genetic manipulations (*18*). Sugars, succinate, glycerol, and the like were used at concentrations of 0.4% as carbon sources, whereas aromatic and heterocyclic substrates were used at 0.1% (w/v), since many of these are moderately toxic at higher concentrations. Amino acids (50 mg/L) and vitamins (5 mg/L) were used where appropriate. Solid media contained 1.5% (w/v) Difco Bacto-agar. Antibiotics were used at 100 mg/L for ampicillin, 10 mg/L for tetracycline, 25 mg/L for kanamycin, and 30 mg/L for chloramphenicol.

Plasmids and Recombinant DNA Procedures

Chromosomal DNA was isolated and purified as described by Maniatis et al. (*20*). Plasmids pUC19, pKA10, and pKA15 were isolated by the alkaline lysis protocol followed by ethidium bromide/CsCl density centrifugation as described by Maniatis et al. (*20*). The rapid plasmid isolation of Birnboim and Doly (*21*) was used for screening clones by restriction analysis. Ligations using T4 DNA ligase and restriction enzyme digests were performed under conditions recommended by the manufacturers (Bethesda Research Laboratories, Bethesda, MD). DNA fragments were separated by electrophoresis on 0.7% agarose gels in 89 m*M* Tris-borate, 89 m*M* boric acid, and 2 m*M* EDTA. Transformation procedures were described by Hanahan (*22*).

Analysis of Proteins

Plasmid-encoded proteins were labeled by the maxicell technique of Sancar et al. (*23*). One milliliter of culture (approximately 2×10^8 cells/mL) was labeled for 90 min with 10μCi of ^{35}S-methionine. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the methods of Laemmli (*24*). Whole-cell proteins were prepared by boiling in sample buffer for 5 min. Samples were electrophoresed on 12.5% polyacrylamide-SDS gels. Molecular weight markers (97,400–14,000 daltons) were obtained from Bio-rad.

Methylene Blue Linked Oxidase Assay

Whole cells or samples of cell extract were incubated in 40 m*M* potassium phosphate buffer (pH 7.2) containing methylene blue (27.5 μM). Substrates were added at final concentrations from 1 to 50 m*M*. Cyanide (1 m*M* final) was added to eliminate operation of the respiratory chain. The absorbance was monitored, at 660 nm, in a recording spectrophotometer.

RESULTS

Cloning of Thiophene Degradation Genes

Chromosomal DNA from a derivative of the thiophene-degrading mutant NAR30 was digested with PstI, and the resulting fragments were ligated into the plasmid pUC19. Hybrid plasmids were transformed into a *recA* derivative of the parental strain DC625, and transformants were selected on furfuryl alcohol/tetrazolium indicator plates containing ampicillin. Colonies that were ampicillin resistant and gave a positive response (i.e., pink color) on the indicator plates were further analyzed. The plasmids were extracted from such isolates digested with PstI, and run on agarose gels.

We eventually found two distinct inserts of chromosomal DNA, both of which conferred the properties expected of the *thdA* gene. This raised the questions of which chromosomal fragment was the "real" *thdA* and what the other fragment could be. For example, if *thdA* were a regulatory gene, then one fragment would be *thdA* itself and the other would be some of the genes controlled by *thdA*.

As it turned out, the plasmid that we investigated first, pKA10, turned out to carry two genes, *thdF* and *thdG*, located at about 85 min on the *E. coli* chromosome. It would appear that the other DNA fragment, in pKA15, is *thdA* itself.

The plasmid pKA10 has a 3.8 kb insert of chromosomal DNA from NAR30 inserted into the PstI site of the multicopy vector pUC19. The plasmid pKA10 has been digested with a variety of restriction endonucleases, both singly and in combination. This yielded a list of cut sites and fragment sizes for each enzyme, and the double digestion experiments allowed the construction of a restriction map (Fig. 1).

Insertion Analysis of pKA10

The 3.8 kb insert of pKA10 is about four times as long as an average gene. We therefore wished to locate the *thd* genes more accurately in this segment of DNA. Our approach was to cut open the plasmid at a single restriction site and insert a segment of DNA specifying resistance to an antibiotic, in this case, kanamycin. This procedure was repeated for a selection of restriction sites around the target plasmid. The insertion derivatives were transformed back into a *recA* wild-type host bacterium and transformants selected by resistance to ampicillin (on the original plasmid) plus kanamycin (on the insert). The insertion derivatives were then examined for the presence of an active *thd* gene(s).

We opened pKA10 using the enzymes BamHI, BglII, or HpaI. Digested pKA10 was run on an agarose gel. The band corresponding to linearized pKA10 was cut out and the opened plasmid removed from the gel by the Gene-Clean procedure. The kanamycin resistance cassette was cut out of plasmid pUC4 by digestion with BamHI or SmaI. The cassette was then

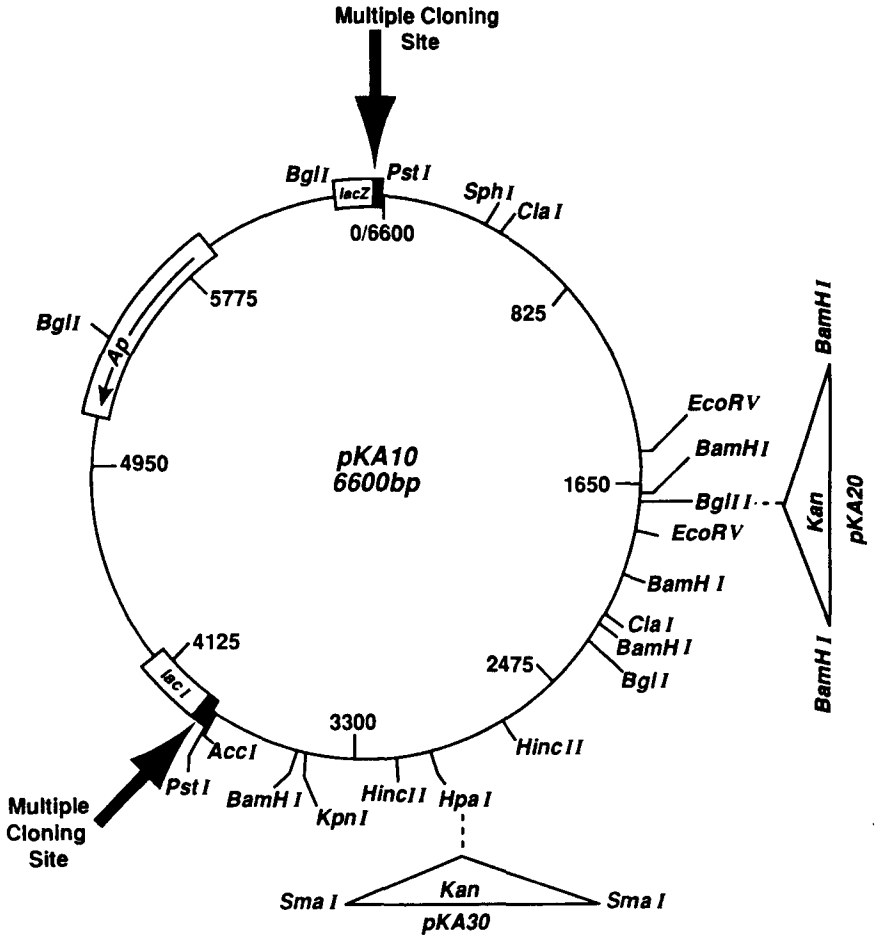

Fig. 1. pKA10 (pUC19 vector with 3800 bp insert including *thdFG*).

ligated into the linearized pKA10. After transformation and selection for ampicillin and kanamycin resistance, we isolated several derivatives of pKA10 carrying the kanamycin cassette. Insertions into pKA10 opened by the restriction enzyme BamHI resulted in derivatives carrying Kan, mostly in the BamHI site between Kpn and Pst at about one o'clock (Fig. 1). These retained full activity of the *thd* genes. In addition, derivatives were found that had lost one or more of the small Bam fragments (about three–four o'clock) and that were defective in *thd* activity. We then inserted the kanamycin cassette into the unique BglII site (about four o'clock) and isolated a plasmid, pKA20, with no *thd* activity. This plasmid was subjected to restriction analysis to confirm its structure. Since it contains a single insertion that inactivates the *thd* genes, these must lie around the BglII site, approximately in the middle of the chromosomal portion of pKA10. Insertions of Kan into the unique HpaI site (at five o'clock) did not inactivate the *thd* genes of pKA10. The various strains and plasmids

Table 1
Bacterial Strains and Plasmids

	Properties
Bacterial strains	
DC625	*fadR adhC gyrA*
DC679	*fadR atoC*
NAR11	*thdA* of DC625
NAR30	*thdA, thdC, thdD* of DC625
NAR420	*zba-300*::Tn*10 thdA*$^+$of NAR30 (retains *thdC* and *thdD*)
NAR710	*recA* of DC625
NAR955	*zic-501*::Tn*10*
NAR966	*thdFG*::Kan of DC679
BW6159	*ilv*::Tn*10*
18404	*zie-296*::Tn*10*
JC1552	*str leu trp his argG metB*
SG20253	*zba-300*::Tn*10*
Plasmids	
pUC19	cloning vector
pKA15	pUC19 plus 900bp PstI fragment (presumably the real *thdA* gene)
pKA10	pUC19 plus 3800bp PstI fragment carries *thdF* and *thdG*
pKA20	pKA10 plus Kan inserted in BglII site
pKA30	pKA10 plus Kan inserted in HpaI site

Table 2
Responses of Plasmid-Bearing Strains

Host strain	Plasmid	Substrate				
		FfOH	TmOH	TCA	TMSO	$TMSO_2$
DC625	None	–	–	–	–	–
	pKA10	+	++	++	++	+
	PKA20	–	+	–	–	–
	PKA30	++	+++	++	++	++
	pKA15	++	+++	++	+	+++
NAR11	None	+++	+++	+++	+++	++
NAR30	None	+++	+++	+++	+++	++
NAR420	None	++	+	++	+	+
	PKA10	++	+++	+	+	++
	pKA15	+++	+++	+++	+++	+++

FfOH = furfuryl alcohol; TmOH = thiophene methanol; TCA = thiophene-2-carboxylic acid; TMSO = tetramethylene sulfoxide; $TMSO_2$ = tetramethylene sulfone. Responses: –, no reaction, +, ++, +++, increasing color on tetrazolium indicator medium, with the specified substrate.

are described in Table 1; Table 2 summarizes the responses of host cells with various plasmids towards furan and thiophene substrates.

Proteins of Plasmid pKA10

We have run SDS-polyacrylamide gels to analyze the proteins produced by plasmid pKA10 and its derivatives pKA20::Kan and pKA30::Kan. Several gels have been run, and we have found several differences between the parental strain and plasmid-carrying derivatives. A maxicell experiment (*23*) was done to show plasmid-specific proteins. The gel shown (Fig. 2) compares DC625 *recA* (parental strain) with the same strain carrying pUC19 (the vector), pKA10, and pKA20. Plasmid pKA10 expresses three prominent protein bands that are largely absent in DC625 *recA* or pUC19/DC625*recA*. Two of these, the 48 Kd and 30 Kd bands, are lost in pKA20::Kan, implying that they might be the product of genes involved in thiophene degradation. The third band (27 Kd) is retained in pKA20. Perhaps this protein is encoded by an unrelated nearby gene that is also carried on pKA10, but not inactivated in pKA20. A DNA insert of 3.8 kb could code for 1250 amino acids, or a total of 144,000 molecular weight units of protein.

We have designated the two genes that code for the 48 Kd and 30 Kd proteins *thdF* and *thdG*. If these two proteins were both coded by the same operon, then both would be absent if Kan were inserted in whichever is nearest the promoter. This implies a cluster of at least two coregulated *thd* genes. In addition, there is a prominent protein produced only by pKA20. This is presumably the neomycin/kanamycin phosphotransferase protein, product of the *npt* gene, which is responsible for resistance to kanamycin and neomycin.

Location on Chromosome of *thdFG*

In order to decide whether pKA10 actually carried *thdA* or other *thd* genes controlled by *thdA*, it was necessary to find on the *E. coli* chromosome the source of the DNA inserted in pKA10.

The plasmid pKA20 contains Kan inserted into the *thd* gene of plasmid pKA10, so causing loss of the thiophene-positive phenotype and of two protein bands correlated with the presence of pKA10. We therefore recombined pKA20 with the chromosome in order to insert the Kan marker at the original location of the gene(s) carried on pKA10/pKA20. Plasmid pKA20 was transformed into the *recA*$^+$ *gyr*$^+$ strain, DC679, and transformants selected for resistance to both ampicillin and kanamycin. The pKA20-bearing derivative was then grown for many generations in rich broth in the presence of kanamycin, but without ampicillin. At appropriate time intervals, aliquots were diluted and plated on rich broth agar. A hundred colonies were picked and tested vs kanamycin and ampicillin.

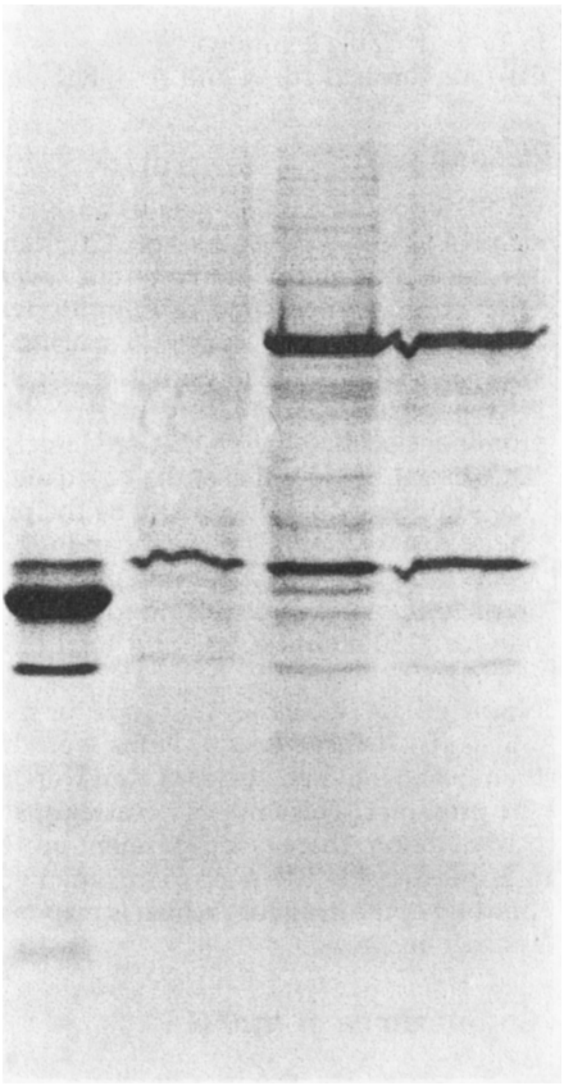

Fig. 2. Protein analysis. Autoradiogram of plasmid-encoded proteins labeled with ^{35}S-methionine. Lane 1—pKA10, labeled for 90 min; Lane 2—pKA10, labeled for 24 h; Lane 3—pUC19, labeled for 90 min; Lane 4—pKA20, labeled for 90 min. Protein a is the 48 Kd protein (*thdF* protein), b is beta- lactamase (31 Kd), c is neomycin phosphotransferase (29 Kd), and d is the 26 kD protein. In this gel, the 30 Kd (*thdG*) protein cannot be separated from beta-lactamase.

After approximately 100 generations, 9% of the colonies had lost the plasmid, as evidenced by sensitivity to ampicillin, yet retained kanamycin resistance. After 200 generations, 93% were Amp^S yet Kan^R. The Kan fragment has presumably inserted into the host chromosome by reciprocal recombination between the chromosomally derived sequences surrounding Kan on pKA20 and their chromosomal homologs. Several such

Table 3
Mapping of *thdFG*::Kan

HfrC *thdFG*::Kan×JC1552			
Map position	Gene tested	Colonies tested	Cotransfer %
2	*leu*	71	0
28	*trp*	100	1
44	*his*	58	0
69	*argG*	53	9
89	*metB*	13	23
Transductions of Tn*10* with DC679*thdFG*::Kan			
Map position	P1 donor	Colonies tested	Cotransduction %
84.5	BW6159 *ilv*::Tn*10*	100	16
84	18404 *zie*-296::Tn*10*	100	27
83	NAR955 *zic*-501::Tn*10*	100	0

Kan^R derivatives of strain DC679 were purified, crossed with P1 grown on SG20253 that carried the *zba*::Tn*10* insertion close to *thdA*. No cotransduction was observed between *zba*::Tn*10* and Kan. We also tried Tn*10* insertions highly linked to *thdC* and *thdD* and again found no cotransduction.

It would appear that pKA10 carries genes that give a thiophene-positive phenotype, at least when present in multiple copies, but that are distinct from *thdA, C,* or *D*. In order to map this novel locus, we grew P1 on a Kan derivative of DC679 and transduced the Kan insertion into HfrC to give HfrC *thdFG*::Kan. The Kan derivative was crossed with JC1552, a multiply marked, streptomycin resistant, F$^-$ strain. Recombinants were selected for Str^R and Kan^R and tested for retention of the auxotrophic markers of JC1552 (Table 3). The results showed that *thdFG*, the novel *thd* genes, lay closest to *metB* at 89 min on the chromosome. We then used P1 to transduce Tn*10* insertions in this neighborhood of the chromosome into DC679 with the insert of *thdFG*::Kan (Table 3). The results indicate a location close to 85 min on the chromosome. It is clear from this that the *thdFG* genes on pKA10 are quite distinct from the *thdA* gene at 11 min.

A Second *thdA* Plasmid

As a result of our cloning attempts, we also isolated a plasmid, pKA15, that behaves much as pKA10 and confers a thiophene degradation (*ThdA*) phenotype on the host strain that carried it. Upon restriction analysis, we found pKA15 to possess a 900 bp Pst fragment of chromosomal DNA. This fragment has no restriction sites in common with the insert of pKA10. We suspect that pKA15 carries the true *thdA* gene, which is presumably a control gene for *thdF* and *thdG*. However, the confirmation of this theory and the analysis of pKA15 are still in progress.

Enzyme Activity of *thdA* Mutants

We have previously assayed various enzymatic activities as possible candidates for the product of the *thdA* gene. We previously tested NAD- and NADP-linked alcohol dehydrogenase reactions and NADH- and NADPH-linked hydroxylations. We also tested dye-linked oxygenase. We found that methylene-blue-linked activities were significant with many furan and thiophene substrates. At first many of the results were confusing, especially with regard to consistent differences between parental strains and mutants. We have found several factors that explain a lot (but not yet all!) of the confusion.

1. Growth phase of cells used in assay. Cells grown overnight into stationary phase work well. Those grown for only 3–4 h (exponential phase) do not and, in fact, often seem to die and lyse during the assay.
2. At the moment, we have obtained good results only with whole cells. Broken cell preparations do not react. We think this is in consequence of damage of a membrane-bound enzyme system or dilution of a needed cofactor upon breaking the cells. We have tested ATP, coenzymeA, NAD, NADH, NADP, NADPH, and FAD as possible cofactors, but none stimulated methylene-blue-linked activity. Thiol-protecting agents and activation procedures using reduced Fe had no effect.
3. Different substrates react differently.
   a) Class I. Reaction linked to methylene blue is seen with wild type and mutants. Class I includes 2-furoic acid, 2-thiophene methanol, mesaconic acid.
   b) Class II. Reaction with wild type is low or negligible. Reaction is much better with mutants, but actual reaction rates are still rather erratic. Class II includes cyclohexanone, tetramethylene sulfoxide (TMSO), and tetramethylene sulfone $TMSO_2$).
   c) Class III. No reaction is observed with 3-furoic acid, 5-bromo-2-furoic acid, benzoic acid, phenylacetic acid, or citraconic acid.
4. Inhibitory effects are seen with higher amounts of substrate. Thus, intermediate concentrations of, e.g., furoic acid give a good reaction rate, but lower or higher concentrations both give poor rates. In retrospect this is not surprising, since furan and thiophenes are severely toxic to the growth of bacterial cells at moderate concentrations.

Table 4 shows reaction rates for furoic acid, thiophene-methanol, and mesaconic acid vs W1485 (wild type), DC625 (*fadR atoC*), and NAR11 (*fadR atoC thdA*) as assessed by the methylene-blue assay. No strain differences are seen. For cyclohexanone and $TMSO_2$ we see differences be-

Table 4
Class I Substrate Oxidation

	Oxidation Rate[b]		
Strain[a]	2-Furoic acid	Thiophene methanol	Mesaconic acid
W1485	0.330	0.177	0.196
DC625	0.335	0.158	0.225
NAR11	0.350	0.210	0.264

[a]W1485 is wild type, DC625 is *fadR atoC* and NAR11 is a *thdA* mutant of DC625.
[b]The assay is linked to methylene blue; the units are absorbance change at 660 nm/mg protein/min.

Table 5
Methylene Blue Assay: Class II Substrates

	Activity, absorbance/min/mg				
			$TMSO_2$		
Strain	Cyclohexanone	TMSO	A	B	C
W1485 wild type	0	0	0	ND	ND
DC625 *fadR atoC*	0	0	0	0	0.072
NAR11 *fadR atoC thdA*	0.052	0.024	0.030	0.032	0.167
NAR13 *fadR atoC*	0	0	0	ND	ND
NAR14 *fadR atoC thdA*	0.031	0.107	0.036	ND	ND

TMSO = tetramethylene sulfoxide; $TMSO_2$ = tetramethylene sulfone; ND = not done. Experiments A and B were separate cultures using 15 μL $TMSO_2$/mL reaction mix, whereas experiment C used 30 μL $TMSO_2$ (vs the same cells as used for B).

tween mutants and wild types, but different experimental runs vary in activities, as shown in Table 5.

DISCUSSION

Our previous work resulted in the isolation of a series of mutants of *E. coli* with increasing ability to oxidize furan and thiophene derivatives. Mutations in three novel genes were involved, *thdA*, *thdC*, and *thdD*, together with mutations in *fadR* (*"thdB"*) and *atoC*. Of these, *thdA* gave the biggest observable effect, and we have therefore focused on this gene in our more recent work.

We thought it possible that *thdA* coded for a nonspecific cytochrome P450 type oxidation system. However, we could not find the characteristic spectral peak at 450 nm produced in the presence of carbon monoxide in our *thdA* strains (data not shown). Enzyme assays have suggested the

thdA may be a methylene-blue-linked oxygenase, most of which are flavoproteins. The methylene-blue-linked reactions observed appear to fall into two classes. Class I reactions were observed with furoic acid, thiophene, methanol, and related substrates. These reactions were found in both wild-type and *thd* mutants and do not appear to consume molecular oxygen. In contrast, Class II reactions occurred with tetramethylene sulfone, sulfoxide, and cyclohexanone. Class II reactions seem to be much greater in *thdA* strains than in wild-type bacteria.

We have also cloned several genes from our thiophene-degrading mutant of *E. coli*, NAR30 *thdA thdC thdD* (*18*). We isolated two plasmids carrying chromosomal fragments from NAR30, both of which conveyed the properties of a *thdA* mutation on the wild-type strain DC625. However, although there is only one *thdA* gene, the two clones carried distinct DNA segments. We chose to investigate first pKA10 carrying a 3.8 kb PstI fragment of DNA. We found that three proteins were produced by this plasmid in substantial amounts. When the production of the 48 Kd and 30 Kd proteins was abolished by insertion of a Kan element (to give pKA20), the plasmid lost its thiophene-degradation properties. Conversely, when the 26 kD protein was abolished by Kan inserted at a different position (to give pKA30), there was no loss of thiophene-degradative ability. We found that the DNA insert of pKA10 came from the 84–85 min region of the chromosome and was therefore not *thdA* (or *thdC* or *thdD*). We have called the two new genes *thdF* and *thdG* and propose that expression of these genes is increased by a *thdA* mutation.

The presence of *thdFG* in multiple copies because of being carried on the multicopy plasmid pUC19 (approximately 40 copies) would also increase their expression. Hence cloned *thdFG* gives much the same effect as a regulatory mutation in *thdA*. A second plasmid pKA15 with a small 900 bp insert may carry the genuine *thdA*. Future work is necessary to confirm this and to confirm or disprove whether *thdA* really is a regulatory gene.

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