APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1990, p. 3179–3185
0099-2240/90/103179-07$02.00/0

Vol. 56, No. 10

Thiophene-Degrading *Escherichia coli* Mutants Possess Sulfone Oxidase Activity and Show Altered Resistance to Sulfur-Containing Antibiotics

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Received 12 March 1990/Accepted 6 August 1990

We have previously isolated mutants of *Escherichia coli* which show increased oxidation of heterocyclic furan and thiophene substrates. We have now found that strains carrying the *thdA* mutation express a novel enzyme activity which oxidizes a variety of substrates containing a sulfone (SO_2) moiety. Both heterocyclic sulfones (e.g., tetramethylene sulfone) and simple aliphatic sulfones (e.g., ethyl sulfone) were oxidized. The *thdA* mutants were more resistant than wild-type strains to aromatic sulfone antibiotics such as dapsone. In contrast they showed increased susceptibility to thiolutin, a cyclic antibiotic containing sulfur at the sulfide level of oxidation. Several new *thdA* mutant alleles were isolated by selecting for increased oxidation of various aliphatic sulfur compounds. These new *thdA* mutants showed similar sulfone oxidase activity and the same map location (at 10.7 min) as the original *thdA1* mutation. The constitutive *fadR* mutation was required for the phenotypic expression of *thdA*-mediated oxidation of sulfur compounds. However, the *thdA*-directed expression of sulfone oxidase activity was not *fadR* dependent. The *thdC* and *thdD* mutations probably protect against the toxicity of thiophene derivatives rather than conferring improved metabolic capability.

Although compounds containing thiophene rings are almost never found in modern-day living organisms, they form a substantial part of the organic sulfur fraction of fossil fuels such as coal and oil (4, 35). The major quinone of the archaebacterium *Sulfolobus* sp. is caldariellaquinone, which contains a thiophene ring fused to a benzoquinone moiety (36). Conceivably the fused thiophenes of coal are the metabolic fossils of archaebacterial metabolism. Although modern-day eubacteria neither produce thiophenes nor degrade them naturally under normal circumstances (31), we have been interested in developing bacterial strains capable of thiophene degradation (1, 5). Since approximately two-thirds of the organic sulfur found in high-sulfur Illinois coals consists of thiophene rings (2), such thiophene-degrading bacteria could be of practical value in coal biodesulfurization. Removing the sulfur before combustion is one approach to combatting the release of sulfur dioxide, which is largely responsible for acid rain. Since physical and chemical coal-cleaning procedures are expensive, waste fine coal particles, and also generate noxious chemical wastes (14, 21, 35), the alternative of using bacteria to desulfurize coal has recently received increasing attention (10, 13, 19, 24).

Our previous work (1, 5, 19) has generated several strains of *Escherichia coli* capable of partial degradation of thiophenes and carrying mutations in a series of novel genes designated *thdA*, *thdC*, *thdD*, and *thdE* (*thd* indicates thiophene degradation). The *thdB* mutation proved to be the same as the previously known *fadR* mutation, which results in constitutive expression of fatty acid transport and degradation (1, 25, 26).

Our objectives in the further investigation of the *thd* system were as follows. Firstly, we hoped to unravel the individual contributions of the various *thd* genes and of *fadR*. Secondly, we hoped to find at least some biochemical basis for the oxidative response towards thiophenes which had previously been observed solely as a phenotypic alteration on tetrazolium indicator plates (1, 5). Thirdly, the thiolutin resistance genes *tlnA* and *tlnB* (30) mapped close to the positions of the *thd* genes. Since thiolutin is a cyclic sulfur-containing molecule, we felt that there was probably some relationship between the *tln* and *thd* genes and that this might shed light on the thiophene oxidation pathway.

We show here that the *thdA* gene appears to be responsible for the expression of a novel activity which oxidizes sulfones and sulfoxides. The *thdC* and *thdD* genes improve the phenotypic response towards thiophenes, but probably as a result of increased resistance to toxicity rather than improved metabolism. We also believe that the *tln* genes are indeed closely related to the *thd* genes, since *thdA* strains show altered sensitivity to sulfur-containing antibiotics, including thiolutin.

MATERIALS AND METHODS

Bacterial strains and media. All strains used were *E. coli* K-12 and are listed in Table 1. Rich broth contained the following ingredients (per liter): 10 g of tryptone, 5 g of NaCl, and 1 g of yeast extract. The minimal medium was M9 (23). Fatty acids and aromatic compounds were added at a concentration of 0.1% (wt/vol) when used as carbon sources, whereas sugars, succinate, glycerol, etc., were used at 0.4%. Amino acids were provided to auxotrophic strains at 50 mg/liter, except that cysteine and aromatic amino acids were used at 25 mg/liter. Solid media contained 1.5% agar. Tetrazolium (TTC) redox indicator plates were as described previously (1). A red response on TTC plates indicates oxidation of the substrate, whereas a white response is negative.

Mutagenesis. An overnight culture was diluted 1:10 into fresh rich broth and grown at 37°C to mid-exponential phase. Ethyl methanesulfonate was then added to a final concentration of 1.0% (vol/vol). Incubation of the culture at 37°C was continued for 60 min, after which the cells were harvested by centrifugation, washed, and resuspended in fresh rich broth.

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TABLE 1. Bacterial strains

Strain	Relevant characteristic(s)	Source or reference
DC271	*fadR mel-1 supF58*	8
DC272	*fadR adhC mel-1 supF58*	D. Clark
DC625	*nalA atoC fadR adhC mel-1 supF58*	1
DC675	*fadR mel-1 supF58 atoC*	8
DC698	*fadR mel-1 supF58 atoC adhC adhR*	8
DC757	*zcf*::Tn*10 fadR*$^+$	D. Clark
DC861	*mel-1 supF58*	D. Clark
DC987	*thdA2* mutant of DC272	See text
DC988	*thdA3* mutant of DC272	See text
DC989	*thdA4* mutant of DC272	See text
GV102	*cyo*::Kan^r	R. Gennis
NAR41	*thdA1 thdD thdC thdE* of DC625	5
NAR10	First-stage thiophene-degrading mutant of DC625, *thdA1*	1
NAR11	Spontaneous Cys$^+$ revertant of NAR10	See text
NAR12	*zba*::Tn*10* mutant of NAR10	P1 (SG20253) × NAR10
NAR13	*zba*::Tn*10 thd*$^+$ mutant of DC625	P1 (NAR12) × DC625
NAR14	*zba*::Tn*10 thdA1* mutant of DC625	P1 (NAR12) × DC625
NAR20	*thdA1 thdD* mutant of DC625	1
NAR30	*thdA1 thdD thdC* mutant of DC625	1
NAR41	*thdA1 thdD thdC thdE* mutant of DC625	5
MW1	*zch*::Tn*10 adh*$^+$ mutant of NAR30	P1 (JW380) × NAR30
MW3	*zch*::Tn*10 adh*$^+$ mutant of DC625	P1 (JW380) × DC625
MW5	*zba*::Tn*10 thdA1* mutant of DC271	P1 (NAR12) × DC271
MW11	*zba*::Tn*10 thdA1* mutant of DC625	P1 (NAR12) × DC625
MW13	*zba*::Tn*10 thdA1* mutant of DC698	P1 (NAR12) × DC698
MW15	*zba*::Tn*10 thdA1* mutant of DC675	P1 (NAR12) × DC675
MW17	*zba*::Tn*10 thdA1* mutant of DC272	P1 (NAR12) × DC272
MW90	*zba*::Tn*10 thdA2* mutant of DC625	P1 (NAR104) × DC625
MW94	*zba*::Tn*10 thdA1* mutant of DC861	P1 (NAR12) × DC861
MW96	*fadR*$^+$ *zcf*::Tn*10* mutant of NAR11	P1 (DC757) × NAR11
MW99	*zba*::Tn*10* mutant of DC988	P1 (SG20253) × DC988
MW100	*zba*::Tn*10 thdA3* mutant of DC625	P1 (MW99) × DC625
MW104	*zba*::Tn*10* mutant of DC987	P1 (SG20253) × DC987
JW380	*zch*::Tn*10*	J. Wechsler
SG20253	*zba-300*::Tn*10*	S. Gottesman
W1485	Wild type	B. Bachmann

The mutagen-treated culture was grown overnight to allow segregation before use in mutant-screening procedures.

Transduction. Cotransduction with bacteriophage P1 *vir* was performed as described before (1). Tetracycline-resistant transductants were selected on medium E containing glucose (0.4%), casein hydrolysate (0.1%), and tetracycline (10 mg/liter). The transductants were then tested for oxidative ability toward thiophenes on TTC indicator plates.

Antibiotics. The experimental antibiotic thiolutin (Fig. 1) was kindly provided by Nathan Belcher of Pfizer Inc. Dapsone and thiazolsulfone (Fig. 1) were provided by Parke, Davis & Co. Other antibiotics were purchased from Sigma Chemical Co.

Assay of inorganic sulfur. Sulfide was assayed by its incorporation into *N,N*-dimethyl-*p*-phenylenediamine in the presence of ferric chloride to form methylene blue, whose appearance was monitored at 650 nm (29). Sulfite was assayed by its reaction with tetrachloromercurate to give disulfitomercurate. This then reacts with *p*-rosaniline hydrochloride to produce a red-violet color at 520 nm (34). Sulfate was assayed by precipitation with barium chloride and by measuring the turbidity at 360 nm of the precipitate suspended in a gelatin cloud (12).

Enzyme assay. The sulfone oxidase activity was assayed spectrophotometrically by monitoring the reduction of methylene blue at 664 nm. The assay mixture contained methylene blue (11.3 mM) and whole-cell preparation (20 to 50 μl) in 40 mM potassium phosphate buffer (pH 7.2) for a total assay volume of 1.0 ml. Activity was initiated by the addition of substrate. Enzyme activity units are given as change in A_{664} per minute per milligram of protein.

RESULTS

Further mapping of the *thdA* gene. In our previous work we cotransduced the *thdA1* mutation of strain NAR11 with the *acrA* (10.6 min) and *dnaZ* (10.9 min) loci (3), as well as with the *zba*::Tn*10* insertion of strain SG20253 (5). We have also cotransduced *thdA* with the *cyo*::Kan^r mutation of Gennis (16) (Table 2). We have attempted to order *thdA* with respect to these neighboring genes by three-point crosses (5). However, we have found after many transductional crosses that such data, although reproducible for any given cross, are not reliable. The reasons are that (i) *thdA*-carrying strains are more sensitive to the toxic effects of thiophenes, and hence certain recombinant classes are underrepresented; (ii) strains carrying certain combinations of mutations, in particular *thdA* plus *acrA* or *thdA* plus *cyo*, grow poorly and are unusually sensitive to thiophene toxicity; and (iii) such toxic effects vary with the strain background. We have presented a compilation of cotransduction frequencies for *thdA* and nearby loci in Table 2. Some of these data are taken from previous work (5) and are included here for the sake of completeness.

New *thdA* mutations. The original *thdA1* mutation was isolated in strain NAR10 (1), which also carried a cysteine auxotrophic mutation. Strain NAR11 is a spontaneous derivative of NAR10 which no longer requires cysteine but retains *thdA1*. In the isolation of the original *thdA1* mutation, we selected a colony which showed increased oxidation of furfuryl alcohol on TTC indicator plates. Subsequently, this mutant was found to show increased oxidation of several other furan and thiophene derivatives (1). We have since isolated several new *thdA* mutants by screening for increased oxidation of various aliphatic sulfur compounds. The parental strain, DC272, was mutagenized with ethyl methanesulfonate (see Materials and Methods), and the survivors were screened on tetrazolium indicator agar containing 0.1% ethanesulfonic acid or 0.1% thiomalic acid. Colonies showing deep coloration were picked and purified. Three such mutants, DC987 (from ethanesulfonic acid), DC988 (from thiomalic acid), and DC989 (from thiomalic acid), were crossed with P1 grown on SG20253, which carries a *zba*::Tn*10* insertion cotransducible with the original *thdA1* mutation. The mutations of all three strains cotrans-

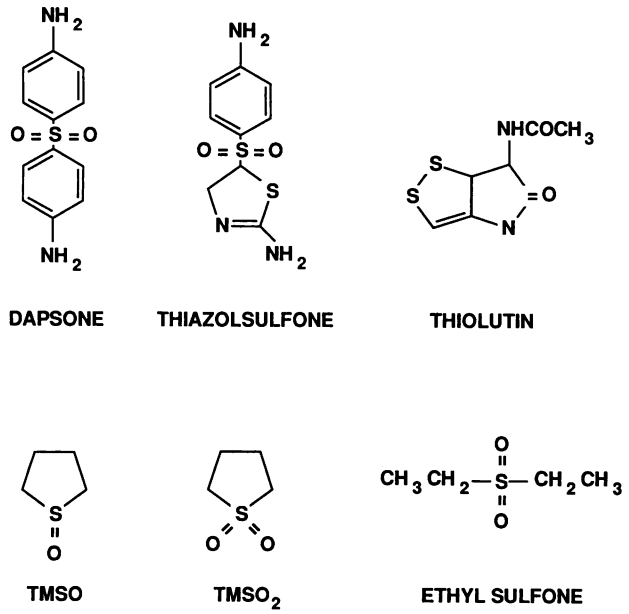

FIG. 1. Structures of sulfur compounds. Thiolutin, dapsone and thiazolsulfone are antibiotics. Tetramethylene sulfoxide (TMSO), tetramethylene sulfone ($TMSO_2$), and ethyl sulfone are substrates for the sulfone oxidase.

duced with *zba*::Tn*10* (17% for DC987, 43% for DC988, and 42% for DC989). We therefore designated these new mutations *thdA2* (DC987), *thdA3* (DC988), and *thdA4* (DC989).

In order to compare their properties directly with those of the original *thdA1* mutation, we cotransduced two of the new *thdA* alleles into strain DC625, the parent of NAR11 (*thdA1*), by using *zba*::Tn*10*. The new *thdA* mutations gave essentially the same response towards heterocyclic substrates as *thdA1*, despite being isolated on aliphatic sulfur compounds. In particular, all of the *thdA* strains showed a positive response towards benzyl alcohol, furoic acid, furfuryl alcohol, thiophene carboxylic acid, and thiophene methanol when tested on tetrazolium indicator agar. As described below, the new *thdA* mutations were also very similar to *thdA1* in their response to sulfur antibiotics and their enzyme activities.

TABLE 2. Cotransduction of *thdA* alleles[a]

P1 donor (relevant characteristic[s])	Recipient (relevant characteristic)	% Cotransduction (no. of colonies scored)
SG20253 (*zba*::Tn*10*)	DC987 (*thdA2*)	17 (200)
SG20253 (*zba*::Tn*10*)	DC988 (*thdA3*)	43 (200)
SG20253 (*zba*::Tn*10*)	DC989 (*thdA4*)	42 (200)
MW104 (*zba*::Tn*10 thdA2*)	DC272	15 (100)
MW104 (*zba*::Tn*10 thdA2*)	DC625	22 (100)
MW99 (*zba*::Tn*10 thdA3*)	DC272	18 (100)
MW99 (*zba*::Tn*10 thdA3*)	DC625	20 (100)
GV102 (*cyo*::Kan^r)	DC987 (*thdA2*)	21 (188)
GV102 (*cyo*::Kan^r)	DC988 (*thdA3*)	29 (177)
GV102 (*cyo*::Kan^r)	NAR11 (*thdA1*)	26 (300)
SG20253 (*zba*::Tn*10*)	NAR30 (*thdA1*)	49 (100)

[a] Tetracycline was the selected marker in all crosses involving Tn*10*, and kanamycin was the selected marker in those involving *cyo*::Kan^r.

Sensitivity to sulfur-containing antibiotics. We tested our *thdA* mutants against various sulfur-containing antibiotics. We were particularly interested in thiolutin-resistant mutants have been isolated by other workers and were found to map very close to *thdA* (30). We found that our *thdA* mutants were hypersensitive to thiolutin, relative to their parental strains, when assayed in minimal medium. There was little difference in sensitivity in rich broth agar.

The multiple mutant, NAR41 (*thdACDE*) was as sensitive as the single mutant NAR11 (*thdA*). Thus, the *thdC*, *D*, and *E* mutations do not affect the response to thiolutin. *E. coli* is much less sensitive to thiolutin in rich medium than in minimal medium (30). Although the reason is not known, it seemed possible that thiolutin might be a toxic analog of biotin, lipoic acid, or some other cyclic sulfur-containing vitamin or cofactor. We found that all our strains were more sensitive to thiolutin in minimal medium than in rich medium. However, addition of biotin or lipoate (or both together) to minimal medium made no difference (data not shown). We also tested the newly isolated *thdA2* and *thdA3* mutations. These also conferred hypersensitivity to thiolutin. In general *thdA* mutants were inhibited by 1 to 2 μg of thiolutin per ml, whereas wild-type strains were killed only by 5 μg or more per ml.

Since *thdA* mutants oxidize sulfones (see below), we tested the response of our strains towards the aryl sulfone antibiotics dapsone and thiazolsulfone (17, 28). In contrast to the results with thiolutin, we found that *thdA* mutants such as NAR11 were slightly more resistant to dapsone than the wild-type strain was. Whereas 60 μg of dapsone per ml inhibited the wild type, NAR11 was inhibited only by 100 μg/ml. The *thdA thdD* strain NAR20 responded similarly to NAR11 (*thdA*). However, strain NAR30, which carries the *thdA*, *thdD*, and *thdC* mutations, was more sensitive to dapsone (inhibited by 45 μg/ml), indicating an effect of *thdC*. Thiazolsulfone was somewhat less potent than dapsone but otherwise gave similar results.

In our earlier work we found that NAR30 was more resistant than its parent, DC625, to the toxic effects of thiophene and furan derivatives (1). More recently we have observed that NAR11 is more sensitive to these compounds than DC625 is. Thus, the *thdA* mutation generally confers sensitivity to reduced sulfur compounds, whereas the *thdC* mutation of NAR30 confers resistance.

Genes involved in furan and thiophene oxidation. The novel mutations *thdA*, *thdC*, and *thdD* of NAR30 were originally isolated in strain DC625; thus, NAR30 is also *fadR adhC atoC* (1). Therefore, genetic manipulations were carried out in order to determine which of these mutations were required to oxidize furans and thiophenes.

Thiophene degradation is thought to occur through a coenzyme A (CoA) thioester intermediate (1, 8). Formation of CoA thioesters can be carried out by the alcohol dehydrogenase complex of *E. coli*, which normally interconverts ethanol with acetyl-CoA (9). NAR30 carries the *adhC* mutation, which results in constitutive high-level expression of alcohol dehydrogenase. To see if *adhC* was required for furan and thiophene oxidation, P1 grown on strain JW380 (*zch*::Tn*10 adh*$^+$) was crossed with NAR30 (*thdACD adhC*). The *adh*$^+$ derivatives from these crosses were tested on TTC plates with furans and thiophenes (Table 3). The results indicated that *adhC* is not required in NAR30 in order to oxidize furans and thiophenes.

Strain NAR30 also carries *fadR* and *atoC* regulatory

TABLE 3. Effect of *adhC*, *atoC*, and *fadR* mutations

Strain	Characteristics				Oxidation with indicated substrate[a]					
					Bzt	BzOH	FCA	FfOH	TCA	TmOH
W1485	+	+	+	+	W	W	W	W	W	W
DC625	*fadR*	*atoC*	*adhC*	+	W	W	W	W	W	W
MW3	*fadR*	*atoC*	+	+	W	W	W	W	W	W
NAR30	*fadR*	*atoC*	*adhC*	*thdACD*	P	R	RR	RR	P	R
MW1	*fadR*	*atoC*	+	*thdACD*	P	R	RR	RR	P	R
NAR11	*fadR*	*atoC*	*adhC*	*thdA*	P	P	RR	RRR	R	R
MW11	*fadR*	*atoC*	*adhC*	*thdA*	P	R	RR	RRR	R	P
MW13	*fadR*	*atoC*	*adhC*	*thdA adhR*	R	R	RRR	RRR	RR	RR
MW15	*fadR*	*atoC*	+	*thdA*	NT	NT	P	RR	P	P
MW17	*fadR*	+	*adhC*	*thdA*	P	R	P	RR	P	P
MW5	*fadR*	+	+	*thdA*	R	P	P	RR	P	P
MW94	+	+	+	*thdA*	W	W	W	W	W	W
MW96	+	*atoC*	*adhC*	*thdA*	W	W	W	W	W	W

[a] Oxidation was assessed on TTC indicator plates. W, White colonies (i.e., no oxidation). Increasing levels of oxidation are indicated as follows: P, pink; R, red; RR, deep red; and RRR, very deep red. NT, Not tested; Bzt, benzoate; BzOH, benzyl alcohol; FCA, furan-2-carboxylic acid; FfOH, furfuryl alcohol; TCA, thiophene-2-carboxylic acid; TmOH, thiophene methanol.

mutations that are required for constitutive degradation of fatty acids (25, 27) and were previously thought to be required for furan and thiophene oxidation (1). To confirm and extend these observations, P1 phage grown on NAR12 (Tn*10* near *thdA*) was crossed into strains with various combinations of the *fadR*, *atoC*, *adhC*, and *adhR* mutations (6). Tetracycline-resistant transductants were then tested on TTC-furfuryl alcohol plates. Red *thdA* derivatives were found at frequencies between 13 and 22% with DC625, DC679, and DC698, all of which are *fadR atoC adhC*. Weakly positive (pink) *thdA* derivatives were found at frequencies between 8 and 14% with DC271, DC272, and DC675, all of which are *fadR*. The presence of the *thdA* mutation could not be detected by TTC indicator plates when transduced into the wild-type strain, DC861 (all transductants were white). The *thdA* derivatives of these strains were then tested on TTC plates with several other furans and thiophenes (Table 3). These tests demonstrated that *fadR* is absolutely required for furan and thiophene oxidation by *thdA* strains. The *adhC* and *atoC* regulatory mutations do not seem to make any difference by themselves; however, when they are both present they appear to enhance the *thdA*- and *fadR*-dependent oxidation of furans and thiophenes, as does the addition of the *adhR* mutation. Note that strain MW1 is essentially a reconstruction of NAR11, the original first-stage mutant (containing *thdA* but not *thdC* or *thdD*). Hence, these results confirm that the *thdA* mutation alone accounts fully for the oxidation of furans and thiophenes in the first-stage mutant (as long as *fadR* is present for phenotypic expression).

The *fadR* effect. Because the *fadR* regulatory mutation is required for *thdA*-dependent oxidation of furans and thiophenes as assessed on TTC indicator agar, it seemed worthwhile to investigate which *fad* gene(s) was required. Mutations in *fadAB*, *fadD*, *fadE*, and *fadL* (25–27) were therefore transduced (separately) into both NAR11 (*thdA*) and NAR30 (*thdACD*). Nearby Tn*10* insertions were used to move these defects into NAR11 and NAR30. Tetracycline-resistant transductants were screened for growth on oleic acid, and those unable to grow (i.e., *fad* mutants) were then tested on TTC plates with furans and thiophenes. No differences in oxidative ability, as compared to NAR11 and NAR30, were noted for any of the *fad* derivatives. Therefore, it appears that the *fad* gene required for *thdA*-dependent oxidation of furans and thiophenes is as yet uncharacterized.

Mutations affecting the Krebs cycle, cytochromes, or molybdenum cofactor. Mutations in *sdh* (succinate dehydrogenase), *sucCD* (succinyl-CoA synthetase), *icd* (isocitrate dehydrogenase), *cyo* (cytochrome *o*), and *cyd* (cytochrome *d*) were transduced into NAR11 and NAR30 by using nearby Tn*10* insertions for genetic selection. None of these mutations affected the response of either NAR11 or NAR30 towards thiophene or furan derivatives.

We selected derivatives of our *thd* mutants which were resistant to chlorate (0.2%) under anaerobic conditions. Two such *chl* mutants, one each of NAR30 and NAR41, were identified as *chlE* mutants by cotransduction with a Tn*10* known to be closely linked to the *chlE* locus (data not shown). Such *chl* mutants lack the molybdenum-pterin cofactor required for various anaerobic reductases, including those for nitrate, trimethylamine oxide, and dimethyl sulfoxide (18, 22). The *chl* derivatives of the thiophene-degrading mutants showed no difference in their oxidative ability towards furan and thiophene derivatives. Hence the Mo-pterin cofactor is not required by the oxidative system for thiophenes and furans.

The *thdC* and *thdD* mutations. The novel mutations in NAR30, *thdA*, *thdC*, and *thdD*, were the result of three successive rounds of mutagenesis with ethyl methanesulfonate. The effect of *thdA* alone has been shown above; however, the effects of *thdC* and *thdD* also needed investigation. Therefore, P1 phage grown on strains NAR700 (Tn*10* near *thdC*) and NAR695 (Tn*10* near *thdD*) was crossed with the same series of strains described above for *thdA*. Tetracycline-resistant transductants were then tested on TTC-furfuryl alcohol plates. All crosses yielded 100% white colonies. This indicated that *thdC* and *thdD* alone are insufficient for furan and thiophene oxidation, although they enhance the oxidative ability of strains already carrying the *thdA* mutation.

Mechanism of thiophene oxidation. Membrane-bound oxidases can often be assayed by coupling to certain redox-sensitive dyes. A methylene blue-coupled assay was chosen for use, as methylene blue is one of the least specific dyes. Whole cells grown aerobically or anaerobically were initially tested in this assay with 2-furoic acid as the substrate. The activity was monitored as substrate oxidation coupled to

methylene blue reduction. Sodium cyanide (1.5 mM) was also added to the assay mixture to eliminate background cytochrome activity. Strains DC625 (*fadR* parent) and NAR11 (*thdA* mutant of DC625) were assayed at two different protein concentrations (0.016 and 0.06 mg/ml) for a range of 2-furoic acid concentrations (0.02 to 0.5%). The results showed several interesting phenomena. First of all, the reaction did not exhibit Michaelis-Menten kinetics (Fig. 2). The loss of activity at high substrate concentrations could be due to standard substrate inhibition (20) or to competition with methylene blue binding by high concentrations of 2-furoic acid (11, 33). Higher protein concentrations required higher concentrations of 2-furoic acid before loss of activity was observed. Therefore, protein concentrations were always standardized for each assay. Other substrates that were tested and acted like 2-furoic acid were thiophene carboxylate, thiophene methanol, thiophene acetic acid, and mesaconic acid. Anaerobically grown cells showed the same activity as aerobic cultures. The results varied slightly from one strain to another. However, these differences were not significant and were probably due to small fluctuations in protein concentration. A wild-type strain (DC861) was also tested with 2-furoic acid and showed equivalent activity. This is the first time *E. coli* has been shown to oxidize heterocyclic substrates such as these. Substrates that were tested but showed no activity in this assay were 3-furoic acid, 5-bromo-2-furoic acid, benzoic acid, phenylacetic acid, and citraconic acid.

Certain other substrates, such as tetramethylene sulfoxide and tetramethylene sulfone, showed activity with cells of NAR11 (*thdA1*) but not with DC625 (*fadR* parent). Therefore, it appeared that activity with these substrates was conferred by *thdA* (Table 4). These substrates did obey Michaelis-Menten kinetics (Fig. 2). In order to confirm that this oxidase activity was specific to *thdA* strains, we tested a pair of transductants, NAR13 (*thd*$^+$) and NAR14 (*thdA1*), constructed by transducing DC625 with P1 grown on a derivative of NAR10 carrying a Tn*10* close to the *thdA1* mutation. Only the *thdA1* transductant, NAR14, showed sulfone and sulfoxide oxidase activity. Strains carrying the *thdA2* and *thdA3* alleles were also assayed and showed activity comparable to that of strains carrying the original *thdA1* allele (Table 4).

Although the *fadR* mutation was necessary for expression of the *thdA* phenotype as observed on TTC agar containing thiophene or furan derivatives, we found that *fadR* was not necessary for expression of the *thdA*-specific sulfone oxidase activity. Both MW96, which is a *fadR*$^+$ transductant of NAR11, and MW94, which was made by transducing the *thdA1* allele into the wild-type strain DC861, possessed the *thdA*-specific oxidase in levels comparable to those of strains with the *fadR* constitutive mutation (Table 4). Strains MW5, MW17, and MW13 (Table 3) also had similar levels of sulfone and sulfoxide oxidase activity to NAR11, indicating that the *adhC* and *atoC* mutations also had no effect on the expression of this enzyme (data not shown). Butadiene sulfone and ethyl sulfone were also tested as substrates against NAR11, and both showed substantial activity. Butadiene sulfone showed nonlinear kinetics, with substantial substrate inhibition at higher concentrations. At lower concentrations, butadiene sulfone was approximately as good a substrate as tetramethylene sulfone (data not shown). Ethyl sulfone yielded approximately four times the activity of tetramethylene sulfone.

The exact values for the sulfone and sulfoxide oxidase activity varied somewhat from culture to culture, even with the same strain. We grew a series of cultures of strain NAR11 for different periods. The oxidase activity varied substantially with growth phase and was expressed maximally in the late exponential to early stationary phase (data not shown). All of the activities described above were observed with whole washed cells in the assay. No activity could be detected in unfractionated cell lysates, even when cofactors such as ATP, CoA, NADP, NAD, NADPH, NADH, and flavin adenine dinucleotide were supplied in the reaction mixture. The enzyme reactivation procedure used for FeS enzymes such as aconitase (15), which involves ferrous ions and reduced thiol compounds, was attempted but had no effect.

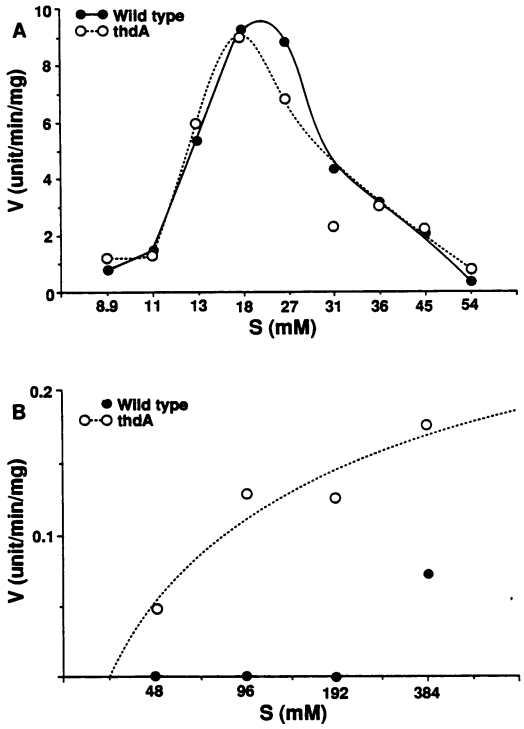

FIG. 2. Methylene blue hydroxylase assay. (A) Equal oxidation of furoic acid by wild-type strain DC625 and *thdA* mutant NAR11; substrate inhibition is believed to occur at approximately 30 mM. (B) Oxidation of tetramethylene sulfoxide by the same two strains. V, Reaction velocity; S, substrate concentration.

Sulfur is not released. Strains DC625 and NAR41 (*thdACDE* mutant of DC625) were incubated with thiophene-2-carboxylate (0.1%) for up to 10 days. The UV absorbance was greatly decreased, indicating reduction of the double bonds of the thiophene ring, and an unidentified product with a distinctive nuclear magnetic resonance spectrum accumulated (data not shown). However, although assays were performed for sulfate, sulfite, and sulfide, no inorganic sulfur was released. Thus, the vivo oxidation product of thiophene carboxylate retains organically bound sulfur.

We have tested several thiophene derivatives as well as tetramethylene sulfone, tetramethylene sulfoxide, diethyl sulfone, and dimethyl sulfoxide as possible sulfur sources for both wild-type and *thdA* strains. However, no strain was able to grow when any of these compounds was used as the sulfur source.

TABLE 4. Sulfone oxidase activities

Strain	Characteristic	Sulfone oxidase activity with[a]:		
		TMSO (0.5%)	TMSO (1.0%)	$TMSO_2$ (2.0%)
DC625	+	0	0	0
NAR11	*thdA1*	0.42	0.62	0.56
MW90	*thdA2*	0.25	0.68	0.37
MW100	*thdA3*	0.41	0.67	0.50
MW96	*thdA1*	0.64	0.72	0.38
NAR13	+	NT	0	0
NAR14	*thdA1*	NT	0.83	0.89
DC861	+	NT	0	0
MW94	*thdA1*	0.41	0.97	0.33

[a] Activity is expressed as A_{660} per minute per milligram of protein in whole cells. TMSO, Tetramethylene sulfoxide; $TMSO_2$, tetramethylene sulfone; NT, not tested.

DISCUSSION

We originally isolated a thiophene-oxidizing mutant of *E. coli* designated NAR30 which had gained mutations at three novel loci, *thdA* (10.5 min), *thdC* (93 min), and *thdD* (99 min). In addition, a mutation at the *fadR* (*thdB*) locus was also required (1). Further mutagenesis yielded NAR41, which had gained the *thdE* mutation and which showed an improved oxidative response to thiophene derivatives (5). However, as shown here, none of these strains releases inorganic sulfur from thiophene derivatives. Instead, the thiophene derivatives are oxidized to presently unidentified intermediates which retain organic sulfur but have lost their double bonds, as indicated by decreased UV absorbance. The nuclear magnetic resonance spectrum of the degradation products suggests that the ring structure is retained. However, since relatively few thiophene and tetrahydrothiophene derivatives have been synthesized, nuclear magnetic resonance reference spectra to fully identify these intermediates are not available.

We have found that both mutant and wild-type strains of *E. coli* can oxidize furan, thiophene carboxylate, and alcohol derivatives by an enzyme which couples to methylene blue. Although previously unreported, the significance of this reaction is unclear.

We have also found an enzyme activity which is specific for strains carrying a *thdA* mutation. This is a methylene blue-linked sulfone oxidase activity which can use tetramethylene sulfone (or sulfoxide), butadiene sulfone, or ethyl sulfone as a substrate. Thus, a cyclic substrate is not essential. Two major alternatives exist. The insertion of oxygen between the sulfone sulfur and a neighboring carbon atom is the mechanism for cyclohexanone oxygenase, which oxygenates five- or six-membered rings containing a C=O moiety (32). If an analogous reaction occurred for tetramethylene sulfone, the product would be butane sultone, which is commercially available. We have tested butane sultone and found that it does act as a sulfur source for either wild-type *E. coli* or *thdA* mutants (data not given). Since tetramethylene sulfone is not a sulfur source, even for *thdA* strains, it seems unlikely that butane sultone could be its in vivo oxidation product. The other chemically plausible alternative is hydroxylation on the carbon atom next to the sulfur (33), which would yield a hydroxy-sulfone. Such hydroxylation on the neighboring carbon atom is involved in the metabolism of alkyl sulfonic acids by certain pseudomonads (7).

Although the phenotype of *thdA* mutants requires the presence of the *fadR* (25) and *atoC* (27) mutations, expression of the sulfone oxidase activity does not. Thus, the oxidative response to thiophene and furan derivatives observed on tetrazolium indicator medium involves at least one activity regulated by *fadR* and *atoC* in addition to the effect of the *thdA* mutation per se.

The fact that *thd* mutants oxidize a wide variety of thiophene and furan derivatives suggests that *thdA* may be a regulator responsible for controlling several genes, one of which codes for sulfone oxidase. This idea is supported by the hypersensitivity of *thdA* mutants to thiolutin, a cyclic sulfide antibiotic (30). One interpretation is that oxidation of thiolutin by a *thdA*-regulated enzyme activates thiolutin to the actual in vivo inhibitor. Previous work with thiolutin resulted in the discovery of two loci, *tlnA* and *tlnB*, which conferred thiolutin resistance (30). The *tlnA* locus was mapped to 10.2 min (approximately), which is very close to *thdA* (10.5 min). It seems plausible that *thdA* and *tlnA* are allelic. Presumably *tlnA* mutants have lost the ability to oxidize thiolutin to the active form, whereas *thdA* mutants have increased activity. We have recently isolated and cloned a new gene, *thdF*, which is involved in thiophene oxidation. The *thdF* gene maps at 85 min, very close to *tlnB* (1a). (Strains carrying the *thdF* gene on a multicopy plasmid do not overproduce sulfone oxidase. Hence, *thdF* is not the structural gene for sulfone oxidase.)

The antibacterial agent dapsone [bis(4-aminophenyl)sulfone] contains a sulfone moiety (17, 28). We found that *thdA* mutants were more resistant both to dapsone and to the closely related thiazolsulfone than isogenic wild-type strains were. The most plausible reason is that *thdA* strains oxidize dapsone and other aryl sulfone antibiotics just as they oxidize other sulfones. Unfortunately, the low solubility of aromatic sulfones prevented us from testing them satisfactorily in the sulfone oxidase assay (data not shown).

At present, our working hypothesis is that *thdA* is a regulator gene which controls several genes and enzyme activities involved in the oxidative metabolism of organic sulfur compounds. One of these is the sulfone oxidase described here; another is the *thdF* gene, which may be involved in the oxidation of cyclic organic sulfides, including thiolutin and thiophenes.

ACKNOWLEDGMENTS

This work was supported by contracts DE-FG22-87PC79912 and DE-FC01-83FE60339 from the U.S. Department of Energy, Pittsburgh Energy Technology Center.

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