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0273-2289/88/1800-0393/$02.00

Successive Mutation of *E. coli* for Improved Thiophene Degradation

Scientific Note

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Index Entries: *Escherichia coli*; thiophene carboxylate; microbial coal desulfurization; furans.

INTRODUCTION

Utilization of domestic coal within the US has increased dramatically since 1970. Coal production is expected to double by the year 1990, with Appalachian and Midwest fields as the largest single sources (*1,2*). One problem associated with the utilization of such coal is the emission of sulfur dioxide during its direct combustion. Physical and chemical methods of coal desulfurization are either expensive or result in a loss of fine coal particles (*3–5*). The sulfur content of Appalachian and Midwestern Coal varies from 3.0–5.5% (*1,2*). This sulfur is a mixture of inorganic sulfur (mostly pyrites) and organic sulfur, including thiols, sulfides, disulfides, and thiophene groups (*5,6*).

Microbial desulfurization of coal before combustion should cost less and be more energy efficient than high-temperature chemical processes (*1–7*). The removal of sulfur compounds from coal via microbial catalysis can also be accomplished without any loss of fine coal particles. The large deposits of high-sulfur coal east of the Mississippi River and the presence of eastern markets provide economic incentives for the removal of sulfur from coal.

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Both *Thiobacillus* and *Sulfolobus* are capable of converting the inorganic sulfur in coal into soluble sulfate that can be washed out. Our intention is to complement this by developing bacteria capable of degrading organic sulfur into sulfate or sulfide.

MATERIAL AND METHODS

Bacterial Strains and Media

All strains used were *Escherichia coli* K-12 and are listed in Table 1. Rich broth contained, per liter, 10 g of tryptone, 5 g of NaCl, and 1 g of yeast extract. Minimal medium was M9 (*8*). Fatty acids and aromatic compounds were added to 0.1% (wt/vol) when used as carbon sources, whereas sugars, succinate, glycerol, and so on, were used at 0.4%. Amino acids were provided to auxotrophic strains at 50 mg/L, except that cysteine and the aromatic amino acids were used at 25 mg/L. Solid media

Table 1
Bacterial Strains

Strain	Relevant Markers	Source/Ref
DC625	*nalA atoC fadR adhC mel-1 supF58*	D. Clark
NAR10	first stage thiophene-degrading mutant of DC625	(*8*)
NAR20	second stage thiophene-degrading mutant of DC625	(*8*)
NAR30	third stage thiophene-degrading mutant of DC625	(*8*)
NAR40	fourth stage thiophene-degrading mutant of DC625	(*8*)
NAR41	fourth stage thiophene-degrading mutant of DC625	See text
SG20253	*zba-300*: :Tn*10*	S. Gottesman[a]
SK2257	*zbc-279*: :Tn*10*	S. Kushner[b]
RG139	*lon*: :Tn*10*	R. Gennis[c]
SG1095	*lon*: :Tn*10*	S. Gottesman
X2844	*tsx*: :Tn*10*	B. Bachmann[d]
JP2781	*zij*: :Tn*10 tyrR366*	J. Pittard[e]
N43	*acrA*	B. Bachmann
AX727	*dnaZ*(Ts)	B. Bachmann
NAR60	*zij*: :Tn*10 tyrR366* of NAR30	P1 (JP2781) X NAR30
NAR70	*zig*: :Tn*10 tyrR366* of NAR41	P1 (JP2781) X NAR41
NAR80	*zij*: :Tn*10 tyrR366* of DC625	P1 (JP2781) X DC625
NAR450	*zba*: :Tn*10 dnaZ*	P1 (SG20253) X AX727
NAR470	*zba*: :Tn*10 acrA*	P1 (SG20253) X N43

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contained 1.5% agar. Tetrazolium redox indicator plates were as described previously (*8*).

Mutagenesis

An overnight culture was diluted 1 in 10 into fresh rich broth and grown at 37°C to mid-exponential phase. Ethyl methane sulfonate was then added to a final concentration of 1.0% (v/v). 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. The mutagen treated culture was grown overnight to allow segregation before use in mutant screening procedures.

Genetic Procedures

A spontaneous streptomycin resistant derivative of the thiophene degrading mutant NAR30 was selected on rich broth agar containing 100 mg/L streptomycin, and was designated NAR33. This derivative was then crossed with each of a set of Hfr strains with various origins of transfer and each carrying a Tn10 insertion proximal to its origin of transfer (*9*). Conjugational mating was carried out by standard procedures, as detailed previously (*10*). Exconjugants were selected on rich broth agar containing streptomycin (100 mg/L) and tetracycline (10 mg/L), and then tested on tetrazolium indicator plates containing 0.1% thiophene-2-carboxylic acid. White colonies on these plates indicated the loss of ability to degrade thiophenes and hence cotransfer of the *thd* gene(s) with the Tn*10* insertion from the Hfr donor. Red colonies indicated retention of thiophene degrading ability. The Hfr::Tn*10* set was constructed by B. Wanner (*9*) and provided by B. Bachman of the Coli Genetic Stock Center.

Cotransduction using bacteriophage P1*vir* was performed as before (*8*). Tetracycline-resistant transductants were selected on medium E containing glucose (0.4%), casein hydrolysate (0.1%), and tetracycline (10 mg/L). The transductants were then tested for degradative ability toward thiophenes, as described above.

RESULTS

Isolation and Mapping of E. coli *Mutants*

Our previous work has resulted in the isolation of successive mutants of *E. coli*; NAR10, 20, 30, and 40, which gained the ability to use an increasing variety of aromatic compounds including furans and thiophenes as the sole source of carbon and energy (Table 2). *Escherichia coli* NAR30 can degrade thiophenes, furans, and assorted other aromatic compounds, as a result of three successive cycles of mutation and selection (*8*). The mutations in NAR30 were mapped by use of an Hfr::Tn*10*

Table 2
Aromatic Compounds as Sole Carbon Source

Carbon Source[a]	Strain				
	DC625	NAR10	NAR20	NAR30	NAR40
Benzoate	+	+	+	+ +	+ +
Phenylacetate	−	+ +	+ +	+ +	+ +
3-Hydroxyphenylacetate	−	+/−	+	+	+
Furfuryl alcohol	+	−	+/−	+	+
Furan-2-carboxylate	−	−	+/−	+	+
Ascorbic acid	−	−	+ +	+ +	+ +
Thiophene-2-carboxylate	−	−	+/−	+	+
Thiophene-2-acetate	−	−	−	+	+
Amino-3-thiophene-acetate	−	+/−	+	+	+
Thioproline	−	−	−	−	+/−
Homocysteine thiolactone Hydrochloride	−	+/−	+	+ +	+ +
Picolinic acid	−	−	−	−	−

[a]M9 minimal agar (pH 7.5) containing 0.1% of the carbon sources listed was incubated at 33°C for six days. − indicates no growth, +/−, + and + + indicate increasingly vigorous growth.

bank constructed by Wanner (*9*), which we have used previously to map anaerobic gene fusions (*10*). The results of the Hfr mapping indicated that genes involved in thiophene degradation are found in three regions of the *E. coli* chromosome (Fig. 1). We designated the genes for thiophene degradation *thdA* (5–15 min), *thdB* (30–45 min), *thdC* and *thdD* (85–100 min). For details see (*8*).

In order to define the map positions more closely we cotransduced each of *thdA, thdB, thdC,* and *thdD* with Tn*10* insertions in the appropriate regions of the chromosome. We found that *thdA* cotransduced with Tn*10* insertions at 10–13 min. The "*thdB*" gene was identical to *fadR* and/or *atoC*. The *fadR* gene, together with *atoC*, controls the breakdown of 4- and 5-carbon carboxylic acid derivatives. Although the pathway for thiophene degradation is unknown, it is clear that ring-fission will produce a 4-carbon, carboxylic acid-derivative containing sulfur. It is likely that further degradation would involve the *fadR*/*atoC* control systems and the pathways which these genes regulate. The *fadR* gene maps at 26 min and the *atoC* gene at 48 min. Mutations in *fadR* and *atoC* which result in constitutive expression of the 4-carbon pathway were incorporated into a parental strain from which the thiophene-degrading mutants were isolated. Thus the "*thdB*" gene at around 30–40 min is an "average" map position for *fadR* or *atoC* and indicates that loss of either *fadR* or *atoC* constitutive mutations results in loss of ability to degrade thiophenes. This has been confirmed by transducing NAR30 to $fadR^+$ (i.e., nonconstitutive) by means of a Tn*10* located close to the *fadR* gene. Replacement of *fadR* (constitutive) by $fadR^+$ results in loss of the ability to degrade thiophenes. A similar experiment involving *atoC* gave similar results. The *thdC* and *thdD*

genes have been accurately located by transduction with Tn*10* insertions in the 90–100 min region. The *thdC* mutation is at 92 min and *thdD* is at 98 min. The map positions of *thdC*, and *thdD* are presently being further defined by three point crosses relative to known chromosomal genes. The *thdA* gene is close to *acrA* and *dnaZ* and the results of three point crosses using these markers and nearby Tn*10* insertions are given in Table 3. The probable gene order is *tsx, lon, thdA zba*::Tn*10 acrA dnaZ*.

Growth Conditions for Thiophene Degradation

We have also investigated the optimum growth conditions for thiophene degradation by strains of *E. coli*. We found that the addition of trace metals and a variety of vitamins had no effect. However, the pH of the medium had considerable influence when carboxylic acid derivatives of thiophene were used. Substantial growth inhibition by thiophene carboxylic acid was observed at mildly acidic pH, whereas growth was much better at pH 7.5–8.0. These data suggested that thiophenes are transported into the bacterial cell as the unionized Thph-COOH form. At neutral to alkaline pH, most of the thiophene is ionized as Thph-COO^-, transport is slower and consequently toxicity is reduced. We found that thiophene methanol was less toxic than the carboxylic acid derivatives and that there was no pH effect in this case.

Since thiophenes may be regarded as benzene analogs, we suspected that thiophene toxicity involved interference with the biosynthesis of aromatic (benzenoid) amino acids. In support of this theory we found that addition of the aromatic amino acids tyrosine and phenylalanine (25 mg/L each) partly overcame thiophene toxicity (Table 4).

The *tyrR* mutation results in the derepression of aromatic acid biosynthesis and *tyrR* mutants are, therefore, resistant to inhibitors of the aromatic pathway such as fluorotyrosine or thienylalanine (*11,12*). We introduced a *tyrR* mutation provided by J. Pittard into our thiophene degraders by cotransduction with the neighboring Tn*10* insertion. The *tyrR* derivatives were tested against fluorotyrosine, to which they were resistant, as was expected. However, the *tyrR* strains were no more resistant to thiophene carboxylate or thiophene methanol than their *tyr*$^+$ par-

Table 3
Three Point Crosses with thdA

P1 Donor[a]	Recipient	Recombinant Classes			
		acr$^+$ thd$^+$	acrA thd$^+$	acrA thdA	acr$^+$ thdA
NAR470	NAR30	5	47	40	0
		dna$^+$ thd$^+$	dnaZ thd$^+$	dnaZ thdA	dna$^+$ thdA
NAR450	NAR30	0	0	64	32

[a]P1 grown on donors containing the zba::Tn*10* insertion was crossed with the thiophene degrading mutant NAR30. Transductants receiving *zba*::Tn*10* were selected by resistance to tetracycline and scored for transfer of the *dnaZ* mutation (from NAR450) or *acrA* (from NAR470).

Table 4
Relief of Thiophene Toxicity

Addition	DC625	NAR20	NAR30	NAR41
None	+/−	+/−	+/−	+/−
Cysteine	−	+/−	+/−	−
Phe + Tyr	+/−	+/−	+/−	+/−
Proline	+/−	+	+	+/−
Pro, Phe, Tyr	+	+	+	+
Pro, Phe, Tyr, Cys	+ +	+ +	+ +	+ +
No thiophene carboxylate	+ +	+ +	+ +	+ +

Bacterial growth was tested on M9-minimal agar with succinate as growth substrate in the presence of .1% thiophene-2-carboxylate, which causes severe growth retardation. Amino acids were added at 25 mg/L. Cys = cysteine, Pro = proline, Phe = phenylalanine, Tyr = tyrosine. − through + + indicate successively increasing levels of growth.

Table 5
Effect of tyrR Mutation

		Growth in Presence of T2C			
Parent Strain	tyrR[a]	.1%	.15%	.2%	.25%
DC625	WT	+ +	+ +	+	−
DC625	*tyrR*	+ + +	+ +	+	−
NAR30	WT	+ +	+ + +	+ +	−
NAR30	*tyrR*	+ + +	+ +	+	−
NAR41	WT	+ +	+ +	+	−
NAR41	*tyrR*	+ +	+	−	−

[a]WT refers to wild type *tyrR* gene, *tyrR* refers to introduction of the *tyrR366* allele (*11,12*) by cotransduction with a nearly Tn*10* insertion. Cells were grown on minimal succinate medium with the concentration of thiophene-2-carboxylate indicated. − indicates no growth and +, + +, and + + + represent increasingly vigorous growth.

ents (Table 5). In some cases they were even somewhat more sensitive. This paradoxical result may be a result of increased transport of aromatic compounds (including thiophenes) in *tyrR* strains. Nonetheless, it was clear that other factors were involved in thiophene toxicity.

We found that the heterocyclic amino-acid proline also protected against thiophene toxicity (Table 4). A mixture of proline with tyrosine plus phenylalanine gave almost full protection against the toxic effects of thiophene derivatives. Although proline does not contain an aromatic ring system it has been shown that certain furan derivatives are potent inhibitors of some enzyme reactions involving proline (*13*).

Thiophene Specific Derivative of E. coli

The triple mutant NAR30 degrades many thiophene, furan, and benzene derivatives (*8*). Efficient microbial desulfurization of coal re-

quires the removal of sulfur from thiophene derivatives yet should leave the benzenoid carbocyclic matrix of coal largely unchanged if possible. Starting with NAR30, several fourth stage mutants showing improved thiophene degradation were isolated. Most of these, e.g. NAR40, showed moderately improved degradation of all types of aromatic substrate. However, we found one mutant, NAR41, which although showing superior degradation of thiophenes, had partially lost the ability to degrade the corresponding furan and benzene derivatives (Table 6). Thus NAR41 probably has an altered enzyme whose substrate specificity has changed so that it shows increased affinity toward the thiophene ring and decreased specificity for rings lacking sulfur. The novel mutation has been designated *thdE* and awaits genetic analysis.

DISCUSSION

Starting from an *E. coli* strain that showed poor but detectable degradation of phenylacetate, we isolated mutants able to grow on furan and thiophene derivatives after three rounds of selection. Until recently, the ability to degrade aromatic compounds was considered as proper for pseudomonads and related organisms, but thought to be largely absent from facultative anaerobes such as the enterobacteria. The pioneering work of Cooper and associates showed that some strains of *E. coli* could degrade hydroxyphenylacetate (*14*), and later work extended these observations to other derivatives of phenylacetate and phenylpropionate (*15*). Our parental strain DC625 could slowly degrade both phenylacetate and benzoate. Successive selection of mutants gave both an extended range of growth substrates and greater oxidative ability toward previously usable compounds. Our third and fourth stage mutants were able to degrade a wide range of both benzenoid and oxygen or sulfur con-

Table 6
Growth of Thiophene Specific Mutant, NAR41[a]

Growth Substrate	DC625	NAR30	NAR41
Benzoic acid	+	+ +	+
Benzyl alcohol	−	−	+
3-Hydroxy phenylacetate	−	+	+
4-Hydroxy phenylacetate	−	+	+
2-Furoic acid	−	+ +	+
3-Furoic acid	−	+ + +	+ +
Furfuryl alcohol	−	+ +	+
Thiophene-2-carboxylate	−	+ +	+ +
Thiophene-methanol	−	+	+ +
Thiophene-methylamine	−	+	+ +
Amino-thiophene-acetate	+	+ +	+ + +

[a]Bacterial strains were assessed both by use of tetrazolium indicator plates and by growth on minimal medium with the substrate indicated as sole carbon source. Those methods gave equivalent results and have been combined in this table.

taining heterocyclic aromatic compounds, although nitrogen heterocycles were not broken down.

We also isolated a fourth stage mutant, NAR41, which was unusual in showing decreased degradation of benzene and furan derivatives yet increased degradation towards thiophenes. Such strains should be especially valuable in coal desulfurization since the object is to degrade the sulfur compounds while leaving other ring structures intact.

The genetic analysis of our third generation *E. coli* mutant, NAR30, revealed three novel genes, required for thiophene catabolism. These have been designated *thdA*, at 12 min on the *E. coli* chromosome, *thdC* (92 min), and *thdD* (98 min). Details of the genetic analysis and growth properties of this mutant are given in our recent publication (*8*). The *thdE* mutation of NAR41 is still to be mapped. The optimization of growth conditions for coal desulfurization has been further advanced by the finding that the heterocyclic amino acid proline, together with the aromatic amino acids tyrosine and phenylalanine provide essentially full protection against thiophene toxicity.

We have mapped the *thdA* gene more accurately, relative to chromosomal genes by three point transductional crosses and are presently cloning the *thdA* region, making use of closely linked Tn*10* insertions.

In addition to the work with *E. coli* discussed in this presentation, our group has isolated a variety of natural bacterial isolates capable of degrading both mono- and polynuclear thiophenes and related sulfur compounds (*16*). Not surprisingly, most of the isolates are *Pseudomonas* species. The majority of such natural isolates had a very restricted substrate specificity (Ochman and Klubek, unpublished), and would therefore be unsuitable for coal desulfurization on their own. We intend to clone the thiophene degradative genes from the best of these natural isolates and introduce them into our *E. coli* thiophene degrader, in order to produce a superior coal desulfurizing organism. In particular, some of these Pseudomonads can degrade dibenzo–thiophene and other polynuclear ring compounds and would therefore nicely complement the abilities of the *E. coli* strains which have been developed to degrade mononuclear thiophene derivatives.

ACKNOWLEDGMENT

This work was supported by a contract from the US Department of Energy, Pittsburgh Energy Technology Center to the SIU Coal Technology Laboratory, Contract DE-FC01-83FE 60339.

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