THIOPHENE METABOLISM BY *E. COLI*

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Technical Progress Report

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* David P. Clark was on sabbatical leave from January 1, 1991, through July 15, 1991. This report covers the whole of this period.

EXECUTIVE SUMMARY

The manuscript by Kiswar Y. Alam and David P. Clark "Cloning and Sequencing of Genes Involved in Thiophene Oxidation by *E. Coli*" has been accepted by the Journal of Bacteriology and is due to appear in the October 1991 issue.

Using the polymerase chain reaction, we have synthesized a DNA segment carrying the regulatory region of the *thdF* gene and have inserted this segment in front of the *lacZYA* structural genes carried on the plasmid pRS415. The expression of β-galactosidase (product of the *lacZ* gene) under the control of the *thdF* regulatory region has been investigated. The *thdF-lacZ* fusion is expressed at a high level in late exponential and early stationary phase, in agreement with our previous observations that the thiophene oxidation system works optimally under these conditions. No induction was found with either tryptophan or indole (the substrate and product, respectively, of tryptophanase which is encoded by *tnaA*, the gene next to *thdF*).

When a *thdF*::Kan insertion mutation was transduced into the thiophene oxidizing strains NAR11 and NAR30, both derivative strains continued to show a positive response on indicator plates with thiophene carboxylate as substrate. As postulated earlier, the *thdF* gene is not the only gene involved in thiophene oxidation and, although involved, is not essential.

BACKGROUND

The objective of this project is to investigate the mechanism of degradation of sulfur-containing heterocyclic molecules by mutant strains of *Escherichia coli* K-12. We have previously isolated multiple mutants of *E. coli* which had gained the capacity to oxidize thiophene compounds and their furan analogs. We have focussed on the *thdA* mutation in our subsequent research, as this appears to be in a regulatory gene central to the thiophene/furan oxidation system. The *thdF* gene appears to be more directly involved in the oxidation reactions, whereas *thdC* and *thdD* are apparently required for increased protection against the toxic effects of thiophene and furan compounds.

PUBLICATIONS

The paper by Alam and Clark concerning the cloning and sequencing of *thdF* has been revised. The revision has been accepted by the Journal of Bacteriology and should be published in October 1991. Reprints will be forwarded with our next report.

RESULTS

The *thdF* Regulatory Region

The regulatory region in front of the *thdF* gene contains a large stem and loop structure and a possible promoter region. This region runs from coordinates 785 to 1027 of the published sequence of the *thdF* region (see manuscript by Alam and Clark that accompanied our previous report).

We made two PCR primers:

> Primer #387 (Coordinates 785-809) with an artificial EcoRI site, and
> Primer #383 (Coordinates 1001-1027) with an artificial BamHI site.

These were synthesized on our department's Applied Biosystems DNA synthesizer.

Using DNA of plasmid pKA10 which carries the *thdF* gene as template, we used the PCR reaction to generate the *thdF* regulatory region with an artificially introduced restriction site at either end. The PCR product was purified by electrophoresis on an agarose gel followed by the Gene-Clean procedure. The purified PCR product was digested with EcoRI and BamHI, as was DNA of the fusion vector pRS415. The Eco/Bam *thdF* regulatory fragment was ligated into the Eco/Bam cut pRS415 vector.

The ligated products were transformed into strain JM103 and the transformation mixture was plated onto LB agar with ampicillin to select for pRS415 and Xgal to monitor β-galactosidase production.

Several colonies containing plasmids were picked in the hope that they contained the *thdF* regulatory region successfully cloned in front of the *lacZYA* genes of pRS415. Analysis of the suspects by agarose gel electrophoresis and restriction enzyme analysis proved ambiguous. The problem is that pRS415 is 10.8kb whereas the insert is only 250 bp and tends to get lost among the RNA at the bottom of the gel. Therefore, plasmid minipreps were made of several suspects, and double stranded DNA sequencing was performed using Primer #387 as the starting point. Sufficient sequence was obtained to decide whether the *thdF* regulatory region had been inserted or not. Suspects pKB 33, 34, 36, and 37 proved to contain inserts, whereas one, pKB35, was negative.

Using pKB35 as negative control, we ran a series of β-galactosidase assays on the *thdF-lacZ* fusion constructs pKB 33, 34, and 36. Strains carrying these inserts were grown in rich broth for up to 48 hr and the β-galactosidase measured at different time points. As Table 1 shows, pKB 33, 34, and 36 show maximum expression at 24 to 31 hr in early stationary phase. Prolonged incubation to 48 hr results in a decline in activity from the maximum. The apparent induction in stationary phase is roughly 8-fold. However, cells at very early times were obtained by diluting an overnight culture into fresh medium, hence, they probably already

Table 1

β-Galactosidase vs. Growth Phase

Strain	β-Galactosidase (Specific Activity) at					
	2 hr	4 hr	6 hr	24 hr	31 hr	48 hr
pKB33	4,150	7,800	19,340	31,300	29,900	23,000
pKB34	4,850	8,790	20,600	35,900	30,800	24,000
pKB35	12	267	612	438	480	284
pKB36	3,674	8,230	17,600	32,500	30,200	17,600

carried some β-galactosidase due to stationary phase induction. To get around this, we grew overnight cultures, diluted them into fresh rich broth, grew them up to 100KU, and rediluted them before starting the growth curve. When this was done, the early values for β-galactosidase were much lower. Consequently, the induction ratios were substantially higher (average at 1 hr = 1,236 units, average at 48 hr = 23,320; induction = 19-fold).

We grew these cultures at 30° and 42° C instead of the normal 37° C. However, no difference due to temperature was observed (data not shown). We also incubated these cultures under anaerobic conditions. Three anaerobic cultures were tested. Rich broth alone does not allow anaerobic growth. Rich broth plus a fermentable sugar–either glucose or fructose–does allow growth in the absence of air. As Table 2 shows, non-growing (rich broth alone) cultures showed high values–they are in stationary phase. However, those anaerobic cultures which could grow showed the opposite–a massive reduction in β-galactosidase expression. Thus, *thdF-lacZ* is induced by the stationary phase, but is greatly repressed by anaerobic conditions. When cultures grown anaerobically were subcultured under aerobic conditions, they regained their typical high β-galactosidase levels, showing that no permanent change had occurred (data not shown).

Table 2

Effect of Anaerobic Conditions in Expression of *thdF-lacZ*

Strain	Growing (Fructose)		Growing (Glucose)		Non-growing
	24 hr	48 hr	24 hr	48 hr	24 hr
pKB33	4,620	613	2,260	370	23,000
pKB34	4,260	519	2,198	213	12,200
pKB35	0	272	0	144	400
pKB36	3,380	282	1,195	127	14,000

Finally, we grew one culture, pKB34, in air for up to 48 hr in various, different media (Table 3). All media contained M9 salts plus casamino acids (not rich broth) and we tested the effect of tryptophan, indole and glucose.

Table 3

Effect of Tryptophan, Indole, and Glucose on *thdF-lac* Expression

Medium Additives	β-Galactosidase (Specific Activity) at			
	2 hr	5 hr	24 hr	48 hr
Glycerol	3,930	15,700	17,900	27,100
Glycerol plus Tryptophan	3,780	15,600	16,000	25,500
Glycerol plus Indole	5,250	17,700	15,000	20,600
Glucose	1,901	7,330	3,310	13,970

pKB34 was grown in M9, casamino acids (0 1%) plus 0.4% of glycerol or glucose. Where indicated, 1mM tryptophan or indole was added.

Comparison of the data in Table 3 with the values for pKB34 grown in rich broth (Table 1) shows that growth in minimal glycerol medium had no significant effect relative to rich broth. Nor did the addition of tryptophan or indole affect β-galactosidase production. However, replacing glycerol with glucose resulted in moderate repression.

Overall, the *thdF-lacZ* fusions were induced by stationary phase, repressed by anaerobic growth, and subject to glucose repression.

Response to Indole

Tryptophanase, encoded by *tnaA*, the gene next to *thdF*, converts tryptophan to indole. Our theory is that the original *thdF* gene was involved in the further degradation of indole (a nitrogen heterocycle) and has been recruited for the degradation of sulfur heterocycles in our mutants.

We therefore tested indole for its effect on the regulation of *thdF-lacZ* (see above) but found nothing significant. We then tested indole for its toxic effect against a series of our *E. coli thd* mutants. Wild type *E. coli* are killed by 4mM indole, which is a moderately toxic compound (Table 4). NAR11, the *thdA* derivative of

Table 4

Toxicity of Indole Towards *thd* Mutants

Strain	Indole (mM)			
	1	2	4	8
DC 625	+ +	+ +	—	—
DC 625 *thdF*::Kan	+ +	+ +	—	—
NAR11	—	—	—	—
NAR11 *thdF*::Kan	+ +	—	—	—
NAR30	+ +	—	—	—
NAR30 *thdF*::Kan	+ +	+ +	—	—

Growth of the indicated strains was tested on tetrazolium indicator medium containing indole. + + = growth, — = no growth.

DC625, was much more sensitive to indole, whereas NAR30 *thdACD* was intermediate in response. Introduction of a disrupted *thdF* gene (i.e., *thdF*::Kan) into these strains resulted in partial protection of the sensitive *thd* strains, although it had no effect on the parent strain. This perhaps implies that *thdF* converts indole to

a more toxic derivative. In the parent, the *thd* system is inactive, hence indole sensitivity is much less. Furthermore, inactivation of *thdF* protects those strains whose *thd* system is active.

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