GENETIC CHARACTERIZATION OF AN *ESCHERICHIA COLI* PLASMID ASSOCIATED WITH HYDROGEN SULFIDE PRODUCTION AND DRUG RESISTANCE

by

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CHAPTER I

INTRODUCTION

During the past two decades, H_2S producing variants of Escherichia coli have been detected worldwide (6, 11, 19, 20, 23, 27).

The production of hydrogen sulfide (H_2S) has been a commonly used biochemical trait in the taxonomic classification of the Enterobacteriaceae (13). H_2S can be produced either through the reduction of an inorganic sulfur source such as thiosulfate or tetrathionate or of an organic sulfur source such as cysteine (7, 19). Standard media used to detect H_2S production include kliger iron agar (KIA), peptone iron agar (PIA), sulfide indole motility (SIM) and triple sugar iron (TSI). These media contain thiosulfate as a primary sulfur source and a salt of a heavy metal e.g.: ferric ammonium citrate or ferrous ammonium sulfate. H_2S produced from the reduction of thiosulfate reacts with the metal salts to produce an insoluble black precipitate of ferrous sulfide which becomes macroscopically visible after 18-24 h. incubation. Most Proteus, Salmonella, Citrobacter, and Edwardsiella produce H_2S from thiosulfate while Escherichia and Shigella species apparently lack this trait (13).

In 1971, Layne et al. (20) described two clinical isolates of Escherichia coli which produced H_2S on SIM agar. Extrachromosomal DNA was demonstrated in these strains and

it was suggested that the trait for H_2S production might be plasmid mediated. Attempts to cure the strains of this extrachromosomal DNA with ethidium bromide and acridine orange and to demonstrate conjugal transfer of the H_2S trait were unsuccessful.

Lautrop et al. (19) isolated twenty-six H_2S producing strains of E. coli which included thirteen different serotypes and eleven different fermentation types. It was demonstrated that one of the H_2S producing E. coli was able to transfer its H_2S trait to E. coli K12.

In 1972, Stoleru et al. (26) described a transmissible plasmid coding for H_2S production and resistance to tetracycline (Tc-H_2S). Subsequent studies by Bouanchaud et al. (4) showed that the Tc-H_2S plasmid was a covalently closed DNA molecule with a base ratio of 50% GC, a contour length of 20 ± 2 μm (40×10^6 daltons) and was present in a ratio of 1-2 copies per host chromosome.

In 1973 Ørskov and Ørskov (27) reported the transmissibility of the H_2S trait in eleven H_2S positive E. coli. Resistance to tetracycline and the ability to ferment raffinose were co-transferred in some cases. Three strains carrying the H_2S, raffinose and tetracycline resistance characters were further studied. It was suggested that these strains contained both fi^+ and fi^- transfer factors carrying the H_2S, raffinose and Tc markers.

In this study, a hydrogen sulfide producing variant of

E. coli is characterized and genetic evidence is presented that the gene(s) for the production of H_2S from thiosulfate are associated with a transmissible plasmid complex which also possesses resistance determinants for tetracycline (Tc), ampicillin (Ap), sulfonamide (Su) and streptomycin (Sm).

CHAPTER II

MATERIALS AND METHODS

Bacterial strains.

Bacterial strains used in this investigation are listed in Table 1. Stock cultures of these strains were maintained on penassay agar slants at 4°C.

Media and material.

The complex media used were nutrient agar (Difco), L broth and L agar (22), and penassay agar (Difco).

The formulas for the minimal media used in these experiments have been described (9). A carbon source, usually glucose at 0.5% final concentration, and desired nutritional supplements were aseptically added to the minimal medium after autoclaving. Nutritional supplements were purchased from Calbiochem (Los Angeles, Calif.), and were used in the following concentrations (μg/ml): L-arginine HCl, 22; L-histidine HCl, 22; L-threonine, 40; adenine, 40; uracil, 40; thiamine HCl, 2; L-leucine, 20; and L-methionine, 10.

The diluent used was buffered saline gelatin (BSG) containing NaCl 0.85%, KH_2PO_4 0.03%, Na_2HPO_4 0.06% and gelatin 100 μg/ml (9).

SIM medium (Baltimore Biological Laboratory) and KIA (BBL) were initially used to test for H_2S production. To facilitate the detection of H_2S production by colonies on

TABLE 1.

BACTERIAL STRAINS[a]

Strain Number	Relevant phenotype	Source
142(PU1)[b]	Prototroph, H_2S, Tc, Ap, Sm, Su	A. Balows
χ656[c]	F^-, thr^-, $purE^-$, $pyrC^-$, his^-, xyl^-	R. Curtiss III
χ656Nal^r[c]	F^-, thr^-, $purE^-$, $pyrC^-$, his^-, xyl^-, Nal^r	χ656
χ1025[c]	F^-, thr^-, leu^-, thi^-, lac^-	C. Berg
χ289[c]	F^-, prototroph	R. Curtiss III
χ402[c]	F^-, met^-	R. Curtiss III
CSH70[c]	Hfr, met^-, arg^-, thi^-	J. Miller

[a]Symbols and definitions of phenotype

Superscript $^-$: Not utilized or not synthesized.

Sm, Su, Tc, Ap, Nal^r: Refer to resistance to streptomycin, sulfonamide, tetracycline, ampicillin and nalidixic acid respectively.

H_2S: Refers to production of hydrogen sulfide from thiosulfate

[b](PU1): Refers to the plasmid complex carried by strain 142 which confers the ability to produce H_2S from thiosulfate and also confers resistance to streptomycin, sulfonamide, tetracycline and ampicillin.

[c]Derivatives of E. coli K12.

the surface of agar plates, a modified KIA medium was developed with the following composition in g per liter: polypeptone peptone (BBL), 20; sodium thiosulfate, 8.5; ferric ammonium citrate, 1; glucose, 1; sodium chloride, 5; agar, 18. The medium was adjusted with 1N NaOH to pH 7. H_2S producing clones were detected as black colonies on the surface of plates incubated aerobically at 37°C.

Tetrathionate reduction was tested on the media described by Le Minor et al. (21).

Ethidium bromide and acridine orange were purchased from Calbiochem (Los Angeles, Calif.). Potassium tetrathionate was a gift from E. Merck (Darmstadt, Germany). Oxytetracycline and streptomycin sulfate were obtained from Pfizer Laboratories (New York, N.Y.). Ampicillin trihydrate was from Bristol Laboratories (East Syracuse, N.Y.). Sulfadiazine was from Beecham Laboratories (Bristol, Tenn.).

Biochemical and antibiotic sensitivity tests.

The biochemical tests were as described by Edwards & Ewing (13). Antibiotic sensitivity tests were performed on pennassay agar containing 100 µg Tc, 100 µg Sm and 100 µg Ap unless otherwise indicated. Mueller-Hinton agar with 5% defibrinated horse blood was used to test for sensitivity to sulfonamide at a concentration of 100 µg/ml.

Mating procedures.

The donor and recipient strains were grown separately

in L broth without aeration to a titer of approximately $2x10^8$ bacteria /ml. The cultures were mixed to achieve a donor to recipient ratio of 1:1 and then incubated at 37°C without aeration for 120 minutes. The mating mixture was then diluted, and samples were plated on selective media for the isolation of transconjugant clones. The phenotypes of the transconjugants were determined after purification on the medium used to select the transconjugant class. Unselected donor markers were scored by cross-streaking or replica plating to appropriate selective media.

Transduction.

The phage P1L4 used in our transduction experiments was supplied by Dr. Roy Curtiss III. Transduction was performed as described by Miller (24). Transducing particles were prepared by propagation on strain χ1025 carrying the PU1 plasmid. Transduction assays were carried out at a multiplicity of infection (MOI) of 1 on strain χ656.

Curing.

Curing experiments were performed on the original strain 142(PU1) and on E. coli K12 recipient strains carrying the PU1 plasmid. The procedures for acridine orange and ethidium bromide curing have been described by Hirota (18) and Bouanchaud (5) respectively.

Mobilization of non-transmissible transconjugants and transductants.

Non-transmissible transconjugants and transductants were mobilized by a modification of a resistance mobilization test described by Guerry et al. (14). Log phase cultures of intermediate strains carrying non-transmissible resistance determinants were mixed (1:1) with a log phase culture of the F^+ donor strain χ402. After 2 h. incubation, a final recipient (χ289) was added and the three strains were incubated overnight. The mating mixture was then diluted and plated on minimal medium containing 100 μg/ml of streptomycin or tetracycline. Only final recipients receiving resistance determinants from the intermediate strains could grow on this medium.

Fertility inhibition.

Phenocopies of the Hfr strain CSH70 were obtained as described by Curtiss et al. (10). The phenocopies were then mated with a donor strain carrying the PU1 plasmid. Selection was made for strain CSH70 clones which had received the PU1 plasmid. Following purification, CSH70 clones carrying the PU1 plasmid were tested for sensitivity to the donor specific phages f_1 and f_2.

CHAPTER III

RESULTS

Origin and properties of E. coli strain 142(PU1).

Strain 142(PU1) was first described by Layne et al. (20) as a H_2S producing variant of E. coli. Aside from the ability to produce H_2S from thiosulfate, the strain exhibited the biochemical properties characteristic of E. coli (Table 2). This strain was not typable with H, O, or K E. coli antisera nor with Salmonella or Arizona antisera and was non motile (Albert Balows - personal communication). Antibiotic sensitivity tests showed that 142(PU1) was resistant to high levels of tetracycline (Tc), ampicillin (Ap), sulfonamide (Su) and streptomycin (Sm). (Table 3). Further study revealed that strain 142(PU1) carries a transmissible plasmid complex (designated PU1) which confers resistance to Tc, Ap, Sm and Su and also the ability to produce H_2S from thiosulfate. The PU1 plasmid complex was conjugally transferred from strain 142 to E. coli K12 recipients at a frequency of approximately 10^{-5} per donor cell. Most transconjugants received all the drug resistance markers as well as the trait for H_2S production.

Spontaneous segregation of the H_2S, Tc, Ap, Sm and Su traits was checked in strains 142(PU1) and χ656(PU1) carrying the PU1 plasmid complex. The H_2S trait was lost at a frequency of 2.5 $\times 10^{-3}$ but there was no segregation of any

TABLE 2.

BIOCHEMICAL CHARACTERIZATION OF STRAINS
142(PU1), χ656 AND χ656(PU1)

Substrate of test	E. coli 142(PU1)	χ656	χ656(PU1)
Indole	+	+	+
Methyl red	+	+	+
Acetoin (VP)	-	-	-
Citrate	-	-	-
Tryptophan deaminase	-	-	-
Urease	-	-	-
Gelatin	-	-	-
Arginine dihydrolase	-	-	-
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	-	-
Glucose	+	+	+
ONPG	+	+	+
Sucrose	-	-	-
Mannitol	+	+	+
Inositol	-	-	-
Sorbitol	+	+	+
Arabinose	+	+	+
Rhamnose	+	+	+
Reduction of tetrathionate	-	-	-
Raffinose	-	-	-
H_2S from thiosulfate	+	-	+

TABLE 3.

LEVELS OF DRUG RESISTANCE OF STRAINS 142(PU1) χ656 AND χ656(PU1).[a]

Strain	Minimal Inhibitory Concentration (μg/ml)			
	Tc	Ap	Sm	Su
142(PU1)	>500	>500	>500	>500
χ656	<5	<5	<5	<10
χ656(PU1)	>500	>500	200	> 500

[a]Drug resistance levels for tetracycline, ampicillin, and streptomycin were determined on penassay agar. Sulfonamide resistance was determined on Mueller-Hinton agar containing 5 percent defibrinated horse blood.

antibiotic marker among more than a thousand clones tested. Neither ethidium bromide nor acridine orange affected the rate of segregation of the drug resistance markers or of the H_2S trait.

Transconjugant classes observed following transfer of the PU1 plasmid to E. coli K12.

The frequency of transfer of the PU1 plasmid to E. coli K12 was approximately 10^{-5} per donor cell. Following transfer of the PU1 plasmid complex to strain χ656 Nal^r twelve transconjugant classes were identified based on an analysis of unselected drug resistance markers and ability to produce H_2S from thiosulfate (Table 4).

Transconjugant classes 1,2,3,4,5,6 and 12 were transmissible. A common feature of the transmissible classes is the presence of the ampicillin resistance determinant. The remaining classes i.e. 7,8,9,10 and 11 were non-transmissible and none contained the ampicillin resistance determinant. All non-transmissible classes could be mobilized by the transfer factor F (Table 4).

Classes 2,7,10,11 and 12 were resistant to high levels of streptomycin; the minimal inhibitory concentration (mic) was greater than 500 µg/ml. Each of these classes was also resistant to sulfonamide. Class 6 was resistant to intermediate levels of streptomycin, mic 200 µg/ml and also was resistant to sulfonamide. Classes 1,3,8 and 9 were

TABLE 4.

TRANSCONJUGANT CLASSES OBSERVED FOLLOWING CONJUGATION OF (χ656 PU1, χ656 PU1 H_2S^-) AND χ656 Nal^r

Transconjugant classes	Frequencies of each class Cross A[a] Selected Markers			Cross B[b] Selected Marker	Transmissibility
	Tc	Ap	Sm	Tc	
1. H_2S,Tc,Ap, Sm[d]	-	.50	-	-	+
2. Tc,Ap,Su,Sm[f]	-	.06	.16	.18	+
3. Tc,Ap, Sm[d]	-	.28	-	.64	+
4. Ap	-	.02	-	-	+
5. H_2S, Ap	-	.02	-	-	+
6. H_2S,Tc,Ap,Su,Sm[e]	.96	.12	.18	-	+
7. Tc, Su,Sm[f]	-	-	-	.10	-[c]
8. Tc, Sm[d]	-	-	-	.08	-[c]
9. H_2S,Tc, Sm[d]	.04	-	-	-	-[c]
10. Su,Sm[f]	-	-	.54	-	-[c]
11. H_2S Su,Sm[f]	-	-	.10	-	-
12. Ap,Su,Sm[f]	-	-	.02	-	+

[a] From the mating of χ656 PU1 and χ656 Nal^r.

[b] From the mating of χ656 PU1, H_2S^- and χ656 Nal^r.

[c] The non-transmissible classes 7,8,9,10 and 11 were all mobilized by the transfer factor F.

[d] Resistant to low level of streptomycin i.e. minimal inhibitory concentration (mic) 50 µg/ml.

[e] Resistant to intermediate level of streptomycin i.e. mic 200 µg/ml.

[f] Resistant to high level of streptomycin i.e. mic greater than 500 µg/ml.

resistant to low levels of streptomycin, mic 50 μg/ml and were sensitive to sulfonamide.

Transductant classes observed following transduction of the PU1 plasmid with phage P1L4.

Five transductant classes were obtained by transduction using P1L4 grown on χ1025 carrying the PU1 plasmid (Table 5). Like the transconjugants, the transductant classes carrying the ampicillin resistance marker (classes 1,3 and 4) were transmissible; however, some representatives of classes 1 and 4 were non-transmissible and non-mobilizable. All representatives of classes 2 and 5 were sensitive to ampicillin, non-transmissible and non-mobilizable.

Classes 1,2 and 5 were resistant to low levels of streptomycin, mic 50 μg/ml. No high or intermediate levels of streptomycin resistance nor resistance to sulfonamide were detected in any transductant classes.

Evidence that the PU1 plasmid possesses an fi^- transfer factor.

The entire PU1 plasmid complex was transferred to phenocopies of Hfr strain CSH70. The PU1 plasmid was stably maintained in the Hfr strain which remained sensitive to the donor specific phages f_1 and f_2. We take this as evidence that this plasmid complex possessed an fi^- transfer factor(s) unable to inhibit F function.

TABLE 5.

TRANSDUCTANT CLASSES OBSERVED FOLLOWING TRANSDUCTION OF PU1 PLASMID WITH PHAGE P1L4[a]

Transductant class	Phenotype	Relative frequency of each class[b] Selected Marker Tc	Ap	Transmissibility
1	H_2S,Tc,Ap,Sm[d]	.58	.76	+[e]
2	H_2S,Tc Sm[d]	.33	-	-[c]
3	Ap	-	.17	+
4	Tc,Ap	.04	.06	+[e]
5	Tc Sm[d]	.04	-	-[c]

[a]Transduction frequency ~5 x 10^{-6} for all classes.

[b]Based on an analysis of 48 transductants selected for resistance to Tc and 47 transductants selected for resistance to Ap.

[c]Non-transmissible classes 2 and 5 not mobilized by the transfer factor F.

[d]Classes 1,2 and 5 resistant to low levels of streptomycin; mic, 50 μg/ml.

[e]Some representatives of classes 1 and 4 were non-transmissible and non-mobilizable.

CHAPTER IV

DISCUSSION

Unlike some other plasmids coding for H_2S production, PU1 does not carry the genes for tetrathionate reduction or raffinose fermentation (19, 27). H_2S production has been shown to be linked to a single resistance determinant i.e. tetracycline, in a plasmid designated Tc-H_2S (4, 26). In addition to Tc, the PU1 plasmid complex confers resistance to Ap, Sm and Su. Furthermore, unlike the previously described Tc-H_2S plasmid, PU1 was not cured by ethidium bromide. PU1 thus differs in several important properties from the H_2S plasmids previously described.

All transconjugant classes carrying the Ap resistance marker were transmissible while all classes lacking this trait were non-transmissible but mobilizable (Table 4). This suggests that the gene(s) coding for Ap resistance is closely linked to the genes coding for transfer function i.e. RTF. Anderson and Natkin (1) reported such a closely linked element, the Ap-ΔR factor, transduced by P1kc as a single unit and transferable after transduction.

With the exception of class 6, all transconjugant classes resistant to Su (classes 2,7,10,11,12) were also resistant to high levels of Sm (mic >500 μg/ml). Transconjugant classes sensitive to Su (classes 1,3,8, and 9) were resistant only to low levels of Sm (mic 50 μg/ml). The reason

for the intermediate level of Sm resistance (class 6) is unexplained at the present time.

In the conjugation experiments, PU1 segregated at high frequencies into 2 different components (H_2S-Tc-Ap-Sm and Sm-Su) different in transmissibility properties (see Table 4). Furthermore, the H_2S-Tc-Ap-Sm component was transduced by P1L4 as a single unit but no transduction of the Su-Sm component was observed (Table 5).

Based on the above data, we predict that 2 physically distinct plasmids comprise the PU1 plasmid complex: first, a transmissible plasmid with resistance determinants for Tc, Sm (low level), Ap (linked to RTF) and the gene(s) for H_2S production from thiosulfate; and secondly, a non-transmissibile plasmid conferring resistance to Sm (high level) and Su.

Transductional data supports the notion that we are dealing with 2 different plasmids in the PU1 plasmid complex (see Table 5). The majority of transductants selected for Tc and Ap contained the H_2S, Tc, Ap and Sm markers and were transmissible, indicating that the entire hypothesized plasmid (H_2S-Tc-Ap-RTF-Sm) was transduced. The other transductant classes most likely resulted from the incorporation of different portions of the H_2S-Tc-Ap-RTF-Sm plasmid in the transducing phage head. Transductant classes carrying the Ap resistance marker (classes 1,3 and 4) were transmissible supporting the transconjugant data suggesting the close

linkage of Ap and RTF. Classes 2 and 5 were non-transmissible and non-mobilizable by the F factor. These classes may lack genes essential for transferability and mobilization or may be associated with the host chromosome (12, 15, 25). Further experiments will be required to distinguish between these possibilities.

The hypothesized Sm-Su plasmid apparently is not transducible by P1L4. Sm resistance in the transductant classes was always of low level (mic 50 μg/ml) and all classes were sensitive to Su. Non-transmissible Sm-Su plasmids previously described have been a very homologous group of plasmids of contour length of about 5.7 μm (3) which should be easily transduced by phage P1L4. Our inability to detect Sm-Su transductants is presently unexplained. The trait for H_2S production may be associated with Sm-Su non-transmissible plasmid (class 11 Table 4). Physical studies on representatives of the transconjugant and transductant classes are presently being conducted to determine the actual number of plasmid species present in the PU1 plasmid complex. If the H_2S gene(s) are shown to be physically linked to more than one plasmid species and if they can be transferred to unrelated plasmids (i.e. R64, R100 . . .) on a rec A background, we will attempt to assess what role, if any, translocating genetic elements play in the movement of the H_2S gene(s) between different plasmids (2,8,16,17). Such studies may help to explain the rather rapid worldwide appearance of

these genes in a genus i.e. Escherichia not previously known to possess them.

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