Thermosensitive H1 Plasmids Determining Citrate Utilization

By H. WILLIAMS SMITH, Z. PARSELL AND P. GREEN

*Houghton Poultry Research Station, Houghton, Huntingdon,
Cambridgeshire PE17 2DA*

(Received 21 April 1978)

Twelve thermosensitive H1 plasmids from strains of *Salmonella typhi* that had caused outbreaks of chloramphenicol-resistant typhoid fever in Vietnam, Thailand and India mediated citrate utilization (Cit⁺) in a prototrophic *Escherichia coli* K12 strain but not in the *S. typhi* strains from which they were derived. Four H1 plasmids from a similar outbreak in Mexico differed from the Far Eastern plasmids in not mediating citrate utilization but in mediating mercury resistance. H1 plasmids resembling the Far Eastern and the Mexican plasmids in regard to citrate utilization and mercury resistance were found in sewage in Britain. Citrate utilization was transferred to eight pathogenic strains of *E. coli* and to one strain each of *Shigella flexneri* and *Shigella sonnei*. Cultures of Cit⁺ bacteria grew more rapidly in citrate media at 28 °C than at 37 °C. Plasmid mutants that were more efficient at utilizing citrate were present in all such cultures – they grew equally well or better at 37 °C than at 28 °C. None of 222 strains of *E. coli* or *Shigella* that contained a variety of different plasmids were able to utilize citrate. This property was not transferred to the prototrophic *E. coli* K12 strain from *Citrobacter* (3 strains), *Salmonella* (39 strains), *Proteus* (44 strains), *Klebsiella pneumoniae* (33 strains) or *Pseudomonas aeruginosa* (44 strains).

INTRODUCTION

Recently, enterobacterial strains and specimens of sewage and river water were searched for thermosensitive (ts) antibiotic resistance plasmids (Smith *et al.*, 1978). The 73 that were found were examined to see whether they belonged to compatibility groups H1 and H2 (Anderson, 1975) because plasmids of these groups are known to be thermosensitive (Smith, 1974; Anderson, 1975); 12 were of group H1 and 58 of group H2. These plasmids were then transferred to a prototrophic *Escherichia coli* K12 strain and the resulting R⁺ forms were examined to see whether they had acquired characters other than antibiotic resistance. Three of the 12 forms with H1 plasmids in them had acquired the ability to utilize citrate as sole energy source (Cit⁺). So, too, had a form of the *E. coli* K12 strain harbouring an H1 plasmid determining resistance to chloramphenicol, tetracyclines, streptomycin, spectinomycin and sulphonamides that had been obtained from a *Salmonella typhi* strain incriminated in an outbreak of typhoid fever in Vietnam (Butler *et al.*, 1973); this plasmid had been included in the investigation for comparison with the other ts plasmids. As a collection of H1 plasmids was available that had been obtained from *S. typhi* causing other outbreaks of typhoid fever in Vietnam and also in Thailand, India and Mexico, it was decided to obtain more information on transmissible citrate-utilizing ability; the results of the investigation are reported in this paper.

METHODS

Bacterial strains and plasmids. Of 16 strains of *S. typhi* possessing ts H1 plasmids determining resistance to chloramphenicol, tetracyclines, streptomycin, spectinomycin and sulphonamides that were studied, four were from cases of typhoid fever in Mexico (Center for Disease Control Weekly Report, 1972), two from cases in India (Paniker & Vimala, 1972), four from cases in Vietnam (Butler *et al.*, 1973) and six from cases in Thailand (Lampe *et al.*, 1974). For subsequent examination these plasmids, like the 12 H1 plasmids from the ts plasmid survey, were transferred to an antibiotic-sensitive *lac*⁺ prototrophic *E. coli* κ 12 strain, using a minimum salts agar medium containing lactose and tetracycline for selecting the R⁺ recipient organisms. The previous bacterial hosts of the three plasmids from the survey that were Cit⁺ were not known. They had been obtained from different specimens of untreated sewage in Britain: two determined resistance to neomycin and tetracyclines and one to tetracyclines only.

The other bacterial strains studied were stock cultures of *E. coli*, *Citrobacter*, *Salmonella*, *Proteus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* maintained in this laboratory on Dorset egg medium at 5 °C; they are believed to be epidemiologically unrelated.

Citrate utilization. The standard method of determining ability to use citrate as sole energy source was to inoculate approximately 10⁸ viable organisms from washed broth cultures in duplicate on to plates of Simmons citrate agar (Difco B91). One set of plates was incubated at 28 °C and one at 37 °C. The plates were examined daily for 4 d and the sizes of the colonies that grew on them were noted. When growth factor-requiring organisms were tested, the medium was supplemented with the necessary factor at 30 µg ml⁻¹, e.g. for *Salmonella typhi* it was supplemented with tryptophan, and for *Shigella sonnei* and *Proteus* spp. it was supplemented with nicotinic acid. The Oxoid formulation (CM155) of Simmons citrate agar was also tested but its routine use was abandoned because at 28 °C Cit⁺ colonies grew more poorly on it than on the medium of Difco formulation. The growth of some strains was also studied in a liquid medium, Koser citrate medium (Difco B15). In routine tests, the tubes of media were inoculated with approximately 10⁴ viable organisms from washed broth cultures, incubated at 37 °C and at 28 °C and examined daily for visual evidence of bacterial growth. In experiments with the prototrophic *E. coli* κ 12 strain, the inocula employed were a 10-fold decreasing series from 10⁸ to 10² viable organisms.

Sensitivity tests. Antibiotic sensitivity was tested by the disc diffusion method; sensitivity to inorganic compounds was tested by the strip diffusion method (Smith *et al.*, 1978). For detecting resistance to sodium arsenite, plasmids were first implanted in a *Salmonella typhimurium* strain because *E. coli* κ 12 is moderately resistant to this compound (Smith, 1978).

Identification of bacterial strains possessing transmissible citrate-utilizing ability and antibiotic resistance. Nutrient broth was inoculated with 0.2 ml each of broth cultures of the strain to be tested and of a spontaneous chromosomal mutant of the *E. coli* κ 12 strain that was resistant to sodium nalidixate (Nal^r) and rifampicin (Rif^r). After incubation at 28 °C for 24 h, the mixed culture was inoculated on to plates of culture media containing sodium nalidixate (20 µg ml⁻¹), if the prospective donor strain was *E. coli*, *Proteus* or *Salmonella*, or rifampicin (80 µg ml⁻¹), if it was *Klebsiella pneumoniae* or *Pseudomonas aeruginosa*. For detecting transfer of antibiotic resistance, the medium was MacConkey agar containing an additional antibiotic (20 µg ml⁻¹) to which the donor, but not the recipient, was resistant. For detecting transfer of ability to utilize citrate, the medium was citrate agar. The plates of MacConkey agar were incubated at 37 °C for 24 h; those of citrate agar were incubated at 28 °C and at 37 °C for 4 d. This procedure was also used to detect strains that could receive plasmids determining antibiotic resistance and citrate-utilizing ability; the prospective recipient strains were Nal^r mutants and the donor strain was the prototrophic *E. coli* κ 12 strain. To ascertain whether both antibiotic resistance and citrate-utilizing ability had been acquired by recipient organisms, either colonies that had grown on one of the two kinds of selection media were tested to see whether they would grow on the other kind, or the mating cultures were inoculated on to plates of MacConkey medium containing the antibiotic to which the recipient strain was resistant and these plates, after incubation, were replica-plated (Lederberg & Lederberg, 1952) on to the two kinds of selection media.

Identification of plasmids that were thermosensitive and that belonged to compatibility group H1. This was achieved by the methods previously described (Smith *et al.*, 1978).

Attempts to obtain citrate-utilizing mutants of the prototrophic *E. coli* κ 12 strain. Approximately 10¹¹ viable organisms from washed broth cultures were inoculated on to two sets of plates of citrate agar and into two sets of tubes of liquid citrate medium. One set of each was incubated at 37 °C and the other at 28 °C for 4 d. The plates were then examined for the presence of colonies and the tubes were subcultured lightly into tubes of fresh liquid citrate medium and examined, after incubation, for visual evidence of bacterial growth.

Attempts to obtain stable citrate-utilizing strains from unstable strains. Organisms picked from colonies on plates of citrate agar were submitted to five serial passages, each of 24 h, in liquid citrate medium at 28 °C and at 37 °C. The final passaged culture was then re-inoculated on to citrate agar, a colony was picked after incubation and the process was repeated.

Table 1. Citrate-utilizing ability and mercury resistance of forms of the prototrophic *E. coli* K12 strain possessing H1 antibiotic resistance plasmids from different sources

No. of forms	Source*	Plasmids possessed		
		Antibiotic resistance mediated†	Citrate utilization	Mercury resistance
6	<i>Salmonella typhi</i> from Thailand	CmTcSmSpcSu	+	—
4	<i>S. typhi</i> from Vietnam	CmTcSmSpcSu	+	—
2	<i>S. typhi</i> from India	CmTcSmSpcSu	+	—
4	<i>S. typhi</i> from Mexico	CmTcSmSpcSu	—	+
3	Sewage	TcNm or Tc	+	—
1	River water	CmSu	—	+
5	River water	CmApSu or Tc	—	—
1	Sewage	Tc	—	—
2	Pig <i>Escherichia coli</i>	Tc	—	—

* Except where stated, all the sources were in Britain.

† Cm, Chloramphenicol; Tc, tetracycline; Sm, streptomycin; Spc, spectinomycin; Ap, ampicillin; Su, sulphonamides; Nm, neomycin.

Plasmid removal. This was achieved by submitting strains to three serial passages in nutrient broth at 44 °C. After the final passage, the cultures were replica-plated on to MacConkey agar containing chloramphenicol or tetracycline (each 20 µg ml⁻¹) and on to citrate agar.

RESULTS

Citrate utilization and other characteristics mediated by H1 plasmids

The citrate-utilizing ability and mercury resistance of the forms of the prototrophic *E. coli* K12 strain to which the 16 H1 antibiotic resistance plasmids of the *S. typhi* strains and the 12 from the ts plasmid survey had been transferred are shown in Table 1; none of them were resistant to sodium arsenite or potassium tellurite. The forms possessing the plasmids of the *S. typhi* strains isolated in the Far Eastern countries (Thailand, Vietnam and India) differed from those possessing the plasmids of the Mexican *S. typhi* strains in that they utilized citrate but were not mercury resistant; three forms possessing plasmids from the ts plasmid survey resembled those possessing the Far Eastern *S. typhi* plasmids and one resembled those possessing the Mexican *S. typhi* plasmids.

Transfer of citrate-utilizing ability from the 15 Cit⁺ *E. coli* K12 forms, like that of their antibiotic resistance determinants, was thermosensitive, and the genetic elements responsible for its transfer belonged to compatibility group H1.

Growth of citrate-utilizing E. coli K12 in culture media containing citrate

On citrate agar. No difference was detected in cultural characteristics between the forms of the *E. coli* K12 strain harbouring the 15 plasmids that mediated citrate utilization. Growth on plates of citrate agar at 28 °C was clearly visible after 2 d incubation by which time the colour of the medium had changed from green to blue; after 4 d incubation well-separated colonies were 1 to 1.5 mm in diameter. The 15 Cit⁺ forms grew much more poorly at 37 °C; after 4 d incubation most of the colonies were just visible to the naked eye. However, within 2 d incubation, 50 to 200 large mutant colonies (1.5 to 2 mm diam.) were present on the plates inoculated with all 15 Cit⁺ forms, the colonies being even larger after 4 d incubation (Fig. 1); for convenience, these mutants are referred to as Cit⁺⁺. They were also present on plates incubated at 28 °C but were more difficult to discern because of the heavy surrounding growth of Cit⁺ organisms. Subcultures of Cit⁺⁺ organisms grew more rapidly at 37 °C than at 28 °C. They were, however, unstable in that they usually yielded a majority

Fig. 1. Two plates of citrate agar inoculated in a similar manner with a prototrophic *Escherichia coli* $\kappa 12$ strain harbouring a plasmid determining citrate utilization obtained from a strain of *Salmonella typhi*. The bacterial growth on the left plate, which had been incubated at 28 °C for 3 d, consists of very many citrate-utilizing colonies (Cit^+) and a small number of more efficient citrate-utilizing mutants (Cit^{++}). Only the Cit^{++} mutants are discernible on the right plate, which had been incubated for 3 d at 37 °C.

of colonies on citrate agar that were Cit^+ . For example, after four serial passages in nutrient broth at 37 °C, the ratios of Cit^{++} to Cit^+ organisms in cultures of four forms of the *E. coli* $\kappa 12$ strain harbouring different citrate-utilizing plasmids were approximately 1:5, 1:10, 1:100 and 1:1000. Three of these four plasmids were from *S. typhi* strains isolated in Vietnam, Thailand and India; the fourth was one of those found in the ts plasmid survey. (These are the four plasmids referred to in subsequent experiments.) It was only possible to obtain a stable Cit^{++} culture from one of the four forms.

The *S. typhi* strains from which 12 of the 15 Cit^+ plasmids had been obtained would not grow on citrate agar, even when tryptophan was added; they grew on the tryptophan-supplemented medium when the citrate was replaced by glucose.

In liquid medium. The tubes of liquid citrate medium inoculated with a 10-fold decreasing series from 10^8 to 10^2 viable organisms of the four forms of the *E. coli* $\kappa 12$ strain containing different citrate-utilizing plasmids were all turbid with bacterial growth after 6 d incubation at 28 °C; a slight degree of bacterial growth was observed after 3 d incubation in the tubes inoculated with 10^4 or more viable organisms. Tubes examined when the amount of bacterial growth in them was small contained only Cit^+ organisms; when the growth became heavy they contained mainly Cit^{++} organisms. After 6 d incubation at 37 °C, usually only the tubes that had received an inoculum of 10^4 or more organisms were turbid; there was little or no evidence of growth in any of the tubes after 3 d incubation. With continued incubation all the tubes became turbid, the principal organisms they contained being Cit^{++} .

It was not possible to obtain citrate-utilizing mutants of the *E. coli* $\kappa 12$ parent strain by cultivation on citrate agar or in the liquid citrate medium.

Fig. 2. Two plates of nalidixate-containing citrate agar inoculated with a mating culture of an *E. coli* $\kappa 12$ donor strain harbouring a plasmid determining citrate utilization and a Nal^r *E. coli* $\kappa 12$ recipient strain. The donor organisms on the right plate were normal citrate-utilizing organisms (Cit^+) and those on the left plate were the more efficient mutants (Cit^{++}). The plates were incubated at 37°C for 3 d. Many more recipient colonies of the Cit^{++} variety are present on the left than on the right plate.

Transfer of citrate-utilizing ability between E. coli $\kappa 12$ organisms: association between citrate-utilizing and antibiotic resistance genes

In matings between cultures prepared from Cit^+ organisms and from Cit^{++} organisms of the four forms of the *E. coli* $\kappa 12$ strain harbouring the different citrate-utilizing plasmids and a Nal^r mutant of this strain, approximately 10% of the organisms of the latter acquired the plasmids. The proportions of Cit^{++} to Cit^+ recipient organisms in the mating cultures in which the donor organisms were Cit^{++} were about 1:0, 1:10, 1:10 and 1:1000. The proportions in the four mating cultures in which the donors were Cit^+ , however, were similar to those in the donor cultures themselves, i.e. about 1:10⁶ (Fig. 2). Replica-plating of the mating cultures prepared in these experiments revealed that the organisms of the recipient strain were either Cit^+ (or Cit^{++}) R^+ or Cit^- R^- . In plasmid removal studies, 3 to 6% of the organisms of the donor strains used in these experiments lost both their citrate-utilizing genes and their antibiotic resistance genes; no organisms that had lost one or other were detected. In all other experiments in which the 15 citrate-utilizing antibiotic resistance H1 plasmids were transmitted between *E. coli* $\kappa 12$ strains, there was also no evidence of segregation of the citrate-utilizing or the antibiotic resistance genes.

Transfer of citrate-utilizing ability from E. coli $\kappa 12$ to other E. coli strains and to Shigella strains

The ability to utilize citrate was transferred from the four forms of the *E. coli* $\kappa 12$ strain possessing the different citrate-utilizing plasmids to Nal^r mutants of two *E. coli* strains that had caused generalized infections in human beings (O18:K1:H7 and O129:K?:H4), two human enteropathogenic strains (O78:H11 and O26:K60:H⁻), two piglet enteropathogenic strains (O141:K85,88 and O9:K103:H⁻), two calf enteropathogenic strains (O8:K85,99 and O9:K35,99), a *Shigella flexneri* strain and of a *Shigella sonnei* strain, the only strains to which transmission was attempted. All of 240 recipient isolates selected from the 12 matings (20 from each) employing the O18:K1:H7, the O9:K103:H⁻ and the O9:K35,99 strains were Cit^+ R^+ . So were most of the 96 isolates selected from these matings (eight from each)

because they were Cit⁺; however, seven from the matings with the O18:K1:H7 strain and one from each of the matings with the other two recipient strains were Cit⁺ R⁻ and possessed conjugative ability. All of 56 R⁺ recipient isolates selected from the 28 matings (two from each) in which the other seven strains were employed were Cit⁺ R⁺; so were 56 Cit⁺ isolates selected from these matings (two from each).

As for transfers between the *E. coli* K12 strains, a much higher proportion of the recipient organisms of the eight pathogenic *E. coli* strains and the two *Shigella* strains were of the Cit⁺⁺ mutant variety when the *E. coli* K12 donor culture was prepared from Cit⁺⁺ organisms rather than from Cit⁺ organisms. Cultures of Cit⁺ organisms of the 10 strains contained similar proportions of Cit⁺⁺ organisms as was found when *E. coli* K12 was the recipient, although, in general, the Cit⁺ organisms of these strains grew less well on citrate agar at 28 °C than did *E. coli* K12 Cit⁺ organisms but always better than they did at 37 °C. Neither Cit⁺ nor Cit⁺⁺ cultures grew as luxuriantly on citrate agar as *Salmonella typhimurium*, *Klebsiella*, *Proteus* or *Pseudomonas aeruginosa* strains. For example, at 37 °C an incubation period of 4 d was usually required before colonies of Cit⁺⁺ organisms became as large as colonies of *S. typhimurium* that had been incubated for 2 d. From an inoculum of 10⁴ viable organisms most of the Cit⁺ cultures produced visible growth in liquid citrate medium in 3 to 5 d at 28 °C and in 8 to 12 d at 37 °C; the Cit⁺⁺ cultures usually produced visible growth in 2 to 3 d at 28 °C and 37 °C.

A search for citrate-utilizing ability in plasmid-containing strains of E. coli and Shigella

None of 222 strains of *E. coli*, three strains of *Shigella dysenteriae* or two strains of *Shigella sonnei*, all of which harboured temperature-tolerant plasmids, grew on citrate agar incubated at 28 °C and 37 °C. About 100 of the plasmids provided the same pattern of antibiotic resistance as that provided by the plasmids of the *S. typhi* strains. Others mediated production of colicin, haemolysin, enterotoxin, K88, K99 and Vir antigens and lactose, sucrose and raffinose utilization.

Attempts to transmit citrate-utilizing ability from strains of bacterial genera possessing this property

No transfer of citrate-utilizing ability to the *E. coli* K12 strain was detected from *Citrobacter* (3 strains), *Salmonella* (39 strains), *Klebsiella pneumoniae* (33 strains), *Proteus* (44 strains) or *Pseudomonas aeruginosa* (44 strains).

DISCUSSION

The results suggest that plasmid-borne ability to utilize citrate is relatively rare and may be principally confined to H1 plasmids. Even so, the fact that plasmids possessing this characteristic were found in sewage in Britain as well as in *S. typhi* strains isolated in the Far Eastern countries (Vietnam, Thailand and India) reveal that it is widely distributed in nature.

Although it seemed probable at one time that the same H1 plasmid was responsible for the chloramphenicol resistance of the *S. typhi* strains causing the outbreaks of typhoid fever in the Far Eastern countries and in Mexico, it is now clear that this is not so; not only were the Mexican plasmids we examined Cit⁻ but they also mediated mercury resistance, a property not shared by the Far Eastern plasmids. The Far Eastern plasmids had been found in several different phage types of *S. typhi* whereas the Mexican ones were in the same phage type (Anderson, 1975). It is of interest that H1 plasmids resembling both the Far Eastern and the Mexican ones in regard to citrate utilization and mercury resistance were present in the collection obtained from British sewage, but they differed in regard to the kinds of antibiotic resistance they provided. However, before one can be certain on all these aspects of plasmid relationship, DNA studies would be required to establish that the antibiotic resistance genes and the citrate-utilizing genes, and the mercury resistance genes for that matter,

are located in the same plasmid. Most of the evidence presented in this paper strongly suggests that the antibiotic resistance genes and the citrate-utilizing genes are so located but it must be borne in mind that in some of the experiments in which the plasmids were transferred to wild strains of *E. coli*, a small number of recipient organisms were isolated that possessed the citrate-utilizing genes but not the antibiotic resistance genes; there can, of course, be explanations for this other than that the genes are located on separate plasmids.

Because the forms of the *E. coli* K12 strain containing the 15 Cit plasmids that were identified grew in a similar and highly characteristic manner on citrate agar, it seems likely that the genes determining citrate utilization in all 15 were identical. Although neither they nor the pathogenic *E. coli* or *Shigella* strains in which they had been implanted grew as luxuriantly as *Salmonella*, *Proteus*, *Klebsiella* and *Pseudomonas*, they utilized citrate sufficiently to be classified as citrate-positive in routine tests. In the *S. typhi* strains from which 12 of the 15 had been obtained, though, the plasmids failed to express citrate utilization. Presumably, this is due to some metabolic difference between the *S. typhi* strains and the *E. coli* and *Shigella* strains. Host differences of this nature were also observed in regard to two plasmids determining substrate utilization studied by Smith *et al.* (1978) – a *lac*⁺ prototrophic *E. coli* K12 strain possessing these plasmids utilized sucrose but not raffinose whereas a *S. typhimurium* strain possessing them utilized sucrose and raffinose.

The unstable mutants found in all cultures harbouring plasmids mediating citrate utilization and referred to, for convenience, as Cit⁺⁺ differed from their parent organisms in at least two respects – in utilizing citrate more efficiently and in doing it equally well or better at 37 °C than at 28 °C. Nevertheless, because cultures of recipient organisms from matings with Cit⁺⁺ donors contained many more Cit⁺⁺ organisms than similar cultures from matings in which the donors were Cit⁺, it is apparent that the mutation to Cit⁺⁺ is solely a function of the plasmid, with no chromosomal involvement.

We are grateful to Dr P. M. Biggs, Mr R. Sampson, Miss Susan Shaw and Professor J. T. Smith for assistance in a variety of ways.

REFERENCES

- ANDERSON, E. S. (1975). The problem and implications of chloramphenicol resistance in the typhoid bacillus. *Journal of Hygiene* **74**, 289–299.
- BUTLER, T., LINH, N. N., ARNOLD, K. & POLLACK, M. (1973). Chloramphenicol-resistant typhoid fever in Vietnam associated with R factor. *Lancet* *ii*, 983–985.
- CENTER FOR DISEASE CONTROL WEEKLY REPORT (1972). Typhoid fever – Mexico. *Morbidity and Mortality* **21**, 177–178.
- LAMPE, R. M., MANSUWAN, P. & DUANGMANI, C. (1974). Chloramphenicol-resistant typhoid. *Lancet* *i*, 623–624.
- LEDERBERG, J. & LEDERBERG, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *Journal of Bacteriology* **63**, 399–406.
- PANIKER, C. K. J. & VIMALA, K. N. (1972). Transferable chloramphenicol resistance in *Salmonella typhi*. *Nature, London* **239**, 109–110.
- SMITH, H. WILLIAMS (1974). Thermosensitive transfer factors in chloramphenicol-resistant strains of *S. typhi*. *Lancet* *ii*, 281–282.
- SMITH, H. WILLIAMS (1978). Arsenic resistance in enterobacteria: its transmission by conjugation and by phage. *Journal of General Microbiology* **109**, 49–56.
- SMITH, H. WILLIAMS, PARSELL, Z. & GREEN, P. (1978). Thermosensitive antibiotic resistance plasmids in enterobacteria. *Journal of General Microbiology* **109**, 37–47.