Arsenic Resistance in Enterobacteria: its Transmission by Conjugation and by Phage

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A high proportion of strains of *Escherichia coli* (599 of 716 strains), *Shigella* (15 of 18), *Proteus* (32 of 33) and *Klebsiella pneumoniae* (42 of 61), but not of *Salmonella* (15 of 209), were resistant to sodium arsenite; the incidence of arsenite resistance was higher in animal than in human strains of *E. coli*. Transmissible arsenite resistance, which was mainly plasmid-borne, was common in resistant strains of *K. pneumoniae* (22 of 34 tested) and *Salmonella* (11 of 12), but not in resistant strains of *E. coli* (10 of 98), *Proteus* (0 of 32) or *Shigella* (0 of 6). In three *K. pneumoniae* strains from which the arsenite resistance genes could not be transferred by direct methods, they were mobilized by implanting the conjugative plasmids F or I into them. In two *S. typhimurium* strains with transmissible arsenite resistance, the resistance genes were located in the genome of the phage with which they were lysogenized. In several of the enterobacterial strains the plasmid-borne arsenite resistance was not associated with antibiotic resistance or any other character known to be transmissible. In many of the *K. pneumoniae* strains, though, it was often associated with transmissible antibiotic, mercury and tellurite resistance and especially with transmissible lactose utilization. The available evidence strongly suggested that the arsenite resistance genes were located on the Lac plasmid. The conjugative plasmids in several of the *K. pneumoniae* strains were temperature-sensitive. Many of the arsenite-sensitive *K. pneumoniae* strains grew on culture media containing mixtures of sodium arsenite and sodium nalidixate at concentrations of each which by itself would not permit their growth. Arsenite-resistant *K. pneumoniae* cultures did not grow under such conditions but any arsenite-sensitive mutants they contained did.

INTRODUCTION

Transmissible arsenic resistance was first demonstrated by Hedges & Baumberg (1973) in a strain of *Escherichia coli* in which it was associated with antibiotic resistance. Summers & Jacoby (1977) found it in 'stock' antibiotic resistance plasmids, and Smith *et al.* (1978) found it in temperature-sensitive antibiotic resistance plasmids obtained from a large collection of enterobacterial strains and from sewage and polluted river water. Nakahara *et al.* (1977) also found a high incidence in mercury resistance-bearing isolates from hospital in-patients. Because of these findings, it was decided to survey unselected enterobacterial strains for transmissible arsenic resistance paying special attention to its relationship to other transmissible characters, especially antibiotic resistance, and to the effect of temperature on its transmission rate. The results are reported in this paper. Sodium arsenite was used throughout this study because it is much more active against enterobacteria than is sodium arsenate; the latter was only used in a confirmatory sense. Unexpectedly, all the strains of *E. coli* K12 maintained in this laboratory were found to be resistant to arsenite. So, as it was desirable to use this organism as a potential recipient in the transmission

experiments, an arsenite-sensitive mutant was first obtained from one of the $\kappa 12$ strains by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment.

METHODS

Bacterial strains. All the *Escherichia coli*, *Salmonella*, *Shigella*, *Klebsiella pneumoniae* and *Proteus* strains surveyed for arsenite resistance were believed to be epidemiologically unrelated. They had been maintained in this laboratory for at least one year on Dorset egg medium at 5 °C. A smooth plasmid-free *Salmonella typhimurium* strain of phage type 36 and an arsenite-sensitive mutant of a *lac pro his trp phe E. coli* $\kappa 12$ strain were used as potential recipients of transmissible arsenite resistance. The mutant was obtained by testing isolates of the arsenite-resistant (*Asi*^r) parent strain that had been treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine according to the method of Glover (1968). It is referred to throughout as the *E. coli* $\kappa 12$ strain. Both potential recipient strains were spontaneous chromosomal mutants resistant to sodium nalidixate (*Nal*^r) and rifampicin (*Rif*^r); mutants resistant to ampicillin, streptomycin and spectinomycin were also available for transferring characters from these strains.

Cultural conditions. Unless stated otherwise, all bacterial strains were grown in 10 ml nutrient broth or on solid media (Oxoid, no. 2) for 24 h at 37 °C.

Sensitivity tests with antibiotics and inorganic compounds. Antibiotics were tested by the disc diffusion method and inorganic compounds were tested by the strip diffusion method (Smith *et al.*, 1978) using strips that had been soaked in aqueous solutions of sodium arsenite (1%, w/v), sodium arsenate (10%, w/v), mercuric chloride (0.1%, w/v) or potassium tellurite (0.01%, w/v). Minimum inhibitory concentrations were determined by the method of Smith *et al.* (1978).

Identification of enterobacterial strains possessing transmissible arsenite resistance. Nutrient broth was inoculated with 0.02 ml each of broth cultures of an *Asi*^r enterobacterial strain and either the *E. coli* $\kappa 12$ strain or the *S. typhimurium* 36 strain. After incubation, the mixed culture was left at room temperature for 24 h (in case the conjugative mechanism of arsenite resistance transfer was temperature-sensitive) and then 0.1 ml was inoculated on to a plate of MacConkey agar containing sodium nalidixate (20 µg ml⁻¹) and sodium arsenite (40 µg ml⁻¹). Colonies that grew on this medium were picked, purified by re-plating and their identity and arsenite resistance was confirmed. When necessary, the proportion of the *E. coli* $\kappa 12$ or *S. typhimurium* 36 organisms that had acquired arsenite resistance was estimated from viable counts of mixed cultures (Miles & Misra, 1938) on MacConkey agar containing sodium nalidixate alone or sodium nalidixate and arsenite. The above procedure was modified when *Asi*^r *K. pneumoniae* strains were being examined by using rifampicin (40 µg ml⁻¹) instead of sodium nalidixate, because sodium nalidixate-resistant mutants are common in most *K. pneumoniae* cultures (Smith, 1972).

Identification of lysogenic strains. The supernates of centrifuged 6 and 24 h broth cultures were spot-inoculated on to plates of tryptose agar (Difco) containing 0.02 M-CaCl₂ and sodium nalidixate (20 µg ml⁻¹) and which had previously been inoculated with a 'lawn' of the *E. coli* $\kappa 12$ or the *S. typhimurium* 36 strain. After incubation, the plates were examined for phage plaques. In other phage studies in which it was essential that the supernates should contain no live bacteria, they were either filtered through a membrane filter of average pore diameter 450 nm or held at 58 °C for 30 min; such preparations were always tested for bacteriological sterility.

Plasmid removal. This was attempted by serial passage of strains in broth at 44 °C or in broth containing sodium lauryl sulphate (Tomoeda *et al.*, 1968) or acridine orange (Hirota, 1960).

Lactose and sucrose utilization. The ability of enterobacterial strains to utilize lactose or sucrose as their sole source of energy was determined by the method of Smith *et al.* (1978).

RESULTS

Susceptibility of enterobacterial strains to sodium arsenite

The 1037 enterobacterial strains examined were classified according to their susceptibility to sodium arsenite in strip diffusion tests (Table 1). The markedly resistant strains grew right up to the edge of the strips; the zone of bacterial inhibition in the case of the moderately resistant strains was 3 to 7 mm and in the case of the sensitive strains, 11 to 20 mm. Examination of 90 strains revealed that the minimum inhibitory concentration (m.i.c.) was 500 to 2000 µg ml⁻¹ for the markedly resistant strains, 125 to 250 µg ml⁻¹ for the moderately resistant strains and 8 to 32 µg ml⁻¹ for the sensitive strains. The m.i.c. for the *S. typhimurium* 36 recipient was 16 µg ml⁻¹ and for the *Asi*^s *E. coli* $\kappa 12$ recipient was 32 µg

Table 1. *Susceptibility of enterobacterial strains to sodium arsenite: the incidence of transmissible arsenite resistance*

Strains	No. of sensitive/resistant strains			No. examined for transmissible resistance	No. whose resistance was transmitted to:	
	Sensitive	Moderately resistant	Markedly resistant		<i>E. coli</i> κ 12	<i>S. typhimurium</i> 36
<i>Salmonella</i> spp.	194	2	13	12	9	10
Human invasive <i>E. coli</i>	33	20	1	98	10	4
Human enteropathogenic <i>E. coli</i>	28	54	2			
Human faecal <i>E. coli</i>	19	36	0			
Domestic animal <i>E. coli</i>	37	469	17			
<i>K. pneumoniae</i>	19	11	31	34	22	4
<i>Proteus</i> spp.	1	17	15	32	0	0
<i>Shigella</i> spp.	3	15	0	6	0	0

ml⁻¹; the m.i.c. for the Asi^r *E. coli* κ 12 strain, from which the recipient was derived, and for other *E. coli* κ 12 strains maintained in this laboratory was 250 μ g ml⁻¹.

Arsenite sensitivity was most common amongst the *Salmonella* strains, the degree of sensitivity tending to be higher in serotypes of groups B and D than in serotypes of groups C and E. Of the human *E. coli* strains examined, a higher proportion of sensitive strains was found amongst the invasive strains, i.e. those that had been isolated from generalized infections, than amongst the enteropathogenic strains or the strains isolated from the faeces of healthy human beings. A very much lower proportion of sensitive strains was found amongst strains isolated from domestic animals despite the fact that they had been isolated from similar situations. One reason for this was that arsenite sensitivity, to a certain extent, was serotype-dependent and arsenite-sensitive serotypes were more common amongst the human strains. For example, many of the invasive strains from human beings belonged to O-groups O4, O6 and O18 which were usually arsenite-sensitive, whereas most of the invasive strains from animals belonged to serotype O78:K80, an arsenite-resistant serotype.

Arsenite sensitivity was fairly common amongst the *Klebsiella pneumoniae* strains examined but less so amongst the *Proteus* strains which were mainly *P. mirabilis*.

Incidence of transmissible arsenite resistance

The resistance in 43 of 182 Asi^r enterobacterial strains examined was transmissible (Table 1). The highest incidence was amongst the resistant *Salmonella* strains, but if all the strains in each group of organisms are considered, irrespective of whether they were sensitive or resistant, then the highest incidence was amongst the *K. pneumoniae* strains. Transmissible resistance was found in only a low proportion of the resistant *E. coli* strains and not at all in the resistant *Proteus* and *Shigella* strains. The m.i.c. of sodium arsenite for the *E. coli* κ 12 and *S. typhimurium* 36 strains that had acquired arsenite resistance in these experiments was approximately the same as that for the strains that had donated it.

Of the 11 *Salmonella* strains whose resistance was transmissible, five were *S. typhimurium*, three were *S. senftenberg* and one each were *S. thompson*, *S. agona* and *S. worthington*. Of the 10 *E. coli* strains with transmissible resistance, three had been isolated from human beings, four from pigs, two from calves and one from a chicken.

Apart from two *S. typhimurium* strains, nos 345 and 348, all the enterobacterial strains whose resistance was transmitted to the *S. typhimurium* 36 recipient also transmitted their resistance to the *E. coli* κ 12 recipient. The latter, in general, was the better recipient, especially from the *K. pneumoniae* donor strains. The rate of transfer of the arsenite resistance genes of the different enterobacterial strains varied greatly. When some of them were transferred between organisms of the *E. coli* κ 12 strain and between organisms of

Table 2. *Characters associated with the transfer of arsenite resistance from different enterobacterial strains*

Character transferred	No. of strains tested . . .	No. of strains from which transfer of arsenite resistance was associated with transfer of another character		
		<i>Salmonella</i> spp. 11	<i>E. coli</i> 10	<i>K. pneumoniae</i> 22
Arsenate resistance		11	10	22
Mercury resistance		1	2	12
Tellurite resistance		0	0	11
Antibiotic resistance		3	6	14
Lactose utilization		0	1	21
Sucrose utilization		1	0	0
Colicin production		0	2	0

S. typhimurium 36, the proportion of recipient organisms that acquired them was as high as 10^{-1} or as low as 10^{-6} to 10^{-7} . The transfer of those from eight of the 22 *K. pneumoniae* strains and from one of the 10 *E. coli* strains was temperature-sensitive in that they were transferred at much lower rates at 37 °C than at 28 °C and 22 °C.

Using a tetracycline resistance determinant as marker, the conjugative plasmids F and I were implanted in three of the *K. pneumoniae* strains whose arsenite resistance had not been transmitted. The arsenite resistance genes in all three strains were mobilized by both F and I, their subsequent transfer rates to the *E. coli* K12 recipient strain being 10^{-2} to 10^{-4} . It was also possible to transmit the natural arsenite resistance from an *E. coli* K12 strain in which F was chromosomally located, but not from one in which it was extrachromosomally located, to the *E. coli* K12 recipient strain, suggesting that the location of the arsenite resistance genes in *E. coli* K12 is chromosomal.

Characters transferred with arsenite resistance

The characters that the two recipient strains acquired with arsenite resistance from the 43 enterobacterial donor strains are shown in Table 2. All the Asi^r forms of the recipient strains were resistant to sodium arsenate. The m.i.c. of this compound for the *E. coli* K12 strain was 800 µg ml⁻¹ and for the *S. typhimurium* 36 strain was 200 µg ml⁻¹; the m.i.c. for the Asi^r forms of both strains was 6400 µg ml⁻¹ or greater. Many of the Asi^r forms of both recipients had also acquired resistance to antibiotics, mercuric chloride and potassium tellurite and the ability to utilize lactose as energy source; this was particularly so in the case of the forms that had acquired their arsenite resistance from the *K. pneumoniae* donor strains. Three of the *E. coli* donors and seven of the *Salmonella* donors did not transfer any other identifiable character with arsenite resistance. These seven *Salmonella* donors included the two, 345 and 348, that transmitted arsenite resistance to *S. typhimurium* 36 but not to *E. coli* K12.

The examination of multiple isolates of recipient organisms from matings with 14 of the *K. pneumoniae* strains that transferred arsenite and antibiotic resistance and lactose utilization, and from cultures of these strains subjected to procedures that promote plasmid loss, suggested that in six of them the genes for arsenite and antibiotic resistance and for lactose utilization were linked and that in another six the genes for arsenite resistance and for lactose utilization were linked; the arsenite resistance genes and lactose utilization genes also appeared to be linked in the three *K. pneumoniae* strains in which they were mobilized with F and I (not shown in Table 2).

Transfer of arsenite resistance by phage

Of all the forms of the *E. coli* $\kappa 12$ and *S. typhimurium* 36 recipient strains that had acquired arsenite resistance from the enterobacterial strains, only two were, as a consequence, lysogenic for the recipient strain from which they had been derived. These two were *S. typhimurium* 36 that had acquired the arsenite resistance of *S. typhimurium* 345 and 348, the only two enterobacterial strains that could transmit their arsenite resistance to *S. typhimurium* 36 but not to the *E. coli* $\kappa 12$ strain. The matings of strains 345 and 348 with *S. typhimurium* 36 were repeated and broth cultures were prepared from 40 Asi^r *S. typhimurium* 36 colonies from each mating; all 80 cultures were lysogenic for *S. typhimurium* 36. In addition, the two mating mixtures were inoculated on to plates of MacConkey medium containing only sodium nalidixate instead of this antibiotic and sodium arsenite. Of 40 *S. typhimurium* 36 colonies tested from the mating with strain 345, three were arsenite-resistant and lysogenic and 37 were arsenite-sensitive and non-lysogenic; the corresponding figures for the mating with strain 348 were one and 39. The mating mixtures were filtered through membrane filters and inoculated on to lawns of *S. typhimurium* 36 in a manner that yielded isolated plaques after incubation. The resistant growth within 16 plaques from each mating yielded Asi^r cultures of *S. typhimurium* 36; bacterial growth picked from all of six non-plaque regions of each lawn failed to grow on arsenite-containing media. That contact with strains 345 and 348 was not necessary for *S. typhimurium* 36 to acquire arsenite resistance was demonstrated by using filtrates of cultures of those organisms in the mating mixtures instead of the cultures themselves. Transfer of arsenite resistance also occurred from cultures in which the donor organisms had first been killed by chloroform or by holding at 58 °C for 30 min, procedures which did not kill the phage particles present. Cultures held at 62 °C (but not at 60 °C) for 30 min were devoid of infective phage and were unable to confer arsenite resistance on *S. typhimurium* 36. During these experiments with culture fluids of strains 345 and 348 that contained no living organisms, the resistant growth from plaques that these fluids produced on lawns of *S. typhimurium* 36 and bacterial growth from non-plaque regions of the same lawns were examined for Asi^r organisms of *S. typhimurium* 36. All of 200 plaques of the strain 345 phage and all of 165 plaques of the strain 348 phage yielded these organisms, but none of 150 non-plaque regions from the lawns containing strain 345 phage plaques or of 165 non-plaque regions from the lawns containing strain 348 phage plaques did. The phages from some of the 345 and 348 plaques were propagated on *S. typhimurium* 36 and plaques were re-examined for ability to transmit arsenite resistance; all of them did so. Cross-resistance tests (Bail, 1923) indicated that the 345 and 348 phages were identical.

Strains 345 and 348 were examined for ability to transfer arsenite resistance to 13 *Salmonella* strains belonging to eight serotypes. Transfer was achieved to nine of them, *S. typhimurium* (four strains), *S. heidelberg* (two strains), *S. agona*, *S. infantis* and *S. gallinarum*, the organisms that had acquired arsenite resistance also acquiring lysogenicity.

Using a tetracycline resistance determinant as marker, the conjugative plasmids F and I were implanted in strains 345 and 348. All four F⁺ or I⁺ forms failed to transfer arsenite resistance to the *E. coli* $\kappa 12$ recipient. Neither could filtrates of cultures of these forms or of spontaneous mutants of 345 and 348 resistant to streptomycin, spectinomycin or sodium nalidixate transfer their antibiotic resistance to the *S. typhimurium* 36 recipient. Loss of arsenite resistance or of lysogenicity was not demonstrated in isolates of cultures of strains 345 or 348 that had been subjected to acridine orange or sodium lauryl sulphate treatment or to serial passage in broth at 44 °C.

Fig. 1. Growth of *Asi*^r and *Asi*^s *K. pneumoniae* on Sensitest agar containing sodium nalidixate at 20 µg ml⁻¹ and sodium arsenite at (a) 80 µg ml⁻¹, (b) 40 µg ml⁻¹, (c) 20 µg ml⁻¹ and (d) 10 µg ml⁻¹. The four sections of each plate were inoculated with overnight broth cultures diluted 1/100 of an *Asi*^r strain (top left) and an *Asi*^s strain (bottom right) which both gave a positive nal/asi reaction and an *Asi*^r strain (top right) and an *Asi*^s strain (bottom left) which did not.

Effect on its virulence of implanting arsenite resistance genes in S. typhimurium 36

Groups of five 1 d-old chickens were each given orally approximately 10⁹ viable organisms of a form of the *S. typhimurium* 36 recipient strain in which had been implanted arsenite resistance genes from one of 18 of the enterobacterial donor strains. The mortality pattern in all 18 groups resembled that in uninfected control groups indicating that genes markedly affecting virulence had not been transferred with the arsenite resistance genes.

*Growth of *Asi*^s and *Asi*^r *K. pneumoniae* organisms in culture media containing sodium arsenite and sodium nalidixate*

Although rifampicin and sodium arsenite were used instead of sodium nalidixate and sodium arsenite for selecting organisms that had acquired arsenite resistance from the *K. pneumoniae* donor strains, because *Nal*^r mutants are common in cultures of *K. pneumoniae* (Smith, 1972), some of the mating mixtures were inoculated on to plates of culture media containing the latter combination of antibacterial agents. On plates inoculated with some, but not all, of the mating mixtures the few donor mutant colonies that grew were greatly outnumbered by colonies of the donor strains that consisted of organisms that were *Nal*^s and, surprisingly, *Asi*^s. Similar results were obtained when the plates were inoculated with broth cultures of the donor strains only or with cultures of some, but not all, sensitive strains of *K. pneumoniae*, the growth that occurred on plates inoculated with the latter always being heavier than that on plates inoculated with the former. This phenomenon

which, for convenience, was referred to as the nal/asi reaction was also observed on plates of Sensitest Agar (Oxoid) but the relative proportions of sodium arsenite and sodium nalidixate in the culture medium were important for its demonstration (Fig. 1). Another method of demonstration was to lightly inoculate cultures over the surface of plates of Sensitest Agar containing sodium nalidixate ($20 \mu\text{g ml}^{-1}$) and to apply a disc containing $100 \mu\text{g}$ sodium arsenite to the centre of the inoculated area. After incubation, the discs on the plates that had been inoculated with Asi^r and Asi^s strains that gave the nal/asi reaction were surrounded by a band of bacterial growth several millimetres thick, sometimes commencing a few millimetres from the edge of the disc, and always consisting of Nal^s Asi^s organisms. Similar results could be obtained by using plates containing sodium arsenite and discs containing sodium nalidixate.

Of 19 Asi^s *K. pneumoniae* strains tested, 15 gave the nal/asi reaction. The corresponding figure for 42¹ Asi strains was 27, strains whose arsenite resistance was known to be transmissible being more common among those that reacted positively than among those that reacted negatively. The organisms that grew from all these 27 strains of *K. pneumoniae* were Asi^r and some of them were also deficient in other characters possessed by their parent; six, for example, were no longer antibiotic-resistant and 17 had reduced lactose utilizing ability.

Although none of the *E. coli*, *Salmonella*, *Proteus* and *Shigella* strains had been noted to give the nal/asi reaction when they were examined for transmissible antibiotic resistance, six *E. coli* strains and six *Salmonella* strains (three of each were arsenite-resistant) were examined by the additional methods referred to above; none of the 12 gave a positive reaction.

DISCUSSION

This study has revealed the existence of transmissible arsenite resistance in *Salmonella*, *E. coli* and *K. pneumoniae* but not in *Proteus* and *Shigella*, the incidence of this kind of resistance, and of arsenite resistance in general, being greatly different in each of these groups of enterobacteria. Furthermore, whereas in other reports (Hedges & Baumberg, 1973; Summers & Jacoby, 1977; Smith *et al.*, 1978) arsenite resistance was always associated with transmissible antibiotic resistance or transmissible mercury resistance (Nakahara *et al.*, 1977), in the present investigation it appeared to exist in several strains in the absence of such resistance or of any other characteristic known to be transmissible. All the evidence, like that provided by the previous studies, indicated that most of the transmissible arsenite resistance was plasmid-borne. In many of the *K. pneumoniae* strains, the genes for arsenite resistance were closely associated with other plasmid-borne genes such as those for lactose utilization and for resistance to mercury, tellurite and a wide range of antibiotics. The arsenite resistance genes and the lactose utilization genes, at least, were undoubtedly located on the same plasmid and this was very probably the non-conjugative Lac plasmid of Reeve & Braithwaite (1973). If this is always the case, then the *K. pneumoniae* strains from which we transferred arsenite resistance and lactose utilization must also possess conjugative plasmids that promote their transfer. It is noteworthy that we were able to mobilize the lactose utilization and arsenite resistance genes in three *K. pneumoniae* strains by means of the conjugative plasmids F and I.

The Asi^r *S. typhimurium* strains 345 and 348 differed from all the other strains with transmissible arsenite resistance in that their arsenite resistance genes were incorporated in the genome of a phage with which they were lysogenized. They therefore provide another naturally occurring example of resistance genes so located; the first to be reported was ϕAmp which contained genes for ampicillin resistance (Smith, 1972). In lysogenic organisms, ϕAmp was extrachromosomally sited. We could find no evidence that the phages of strains 345 and 348 were similarly sited – they were not eliminated from organisms of these strains by ‘plasmid-removing’ procedures nor was the arsenite resistance of these strains trans-

mitted to *E. coli* k12 after F and I were implanted in them. The available evidence, including that of the cross-resistance tests, indicated that the two phages were identical. However, no epidemiological relationship could be established between strains 345 and 348; 345 had been isolated in 1966 and 348 in 1967.

The basis of the nal/asi reaction remains a mystery. It is not difficult to appreciate that when two compounds are added to culture media in certain proportions their antibacterial effects may be diminished. But it is not known why most Asi^s but not Asi^r *K. pneumoniae* organisms grew on media containing sodium arsenite and nalidixate or why organisms of the other enterobacterial strains examined did not show this effect.

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