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Inducible Plasmid-Determined Resistance to Arsenate, Arsenite, and Antimony(III) in *Escherichia coli* and *Staphylococcus aureus*

S. SILVER,[1,2*] K. BUDD,[1†] K. M. LEAHY,[1] W. V. SHAW,[3] D. HAMMOND,[3‡] R. P. NOVICK,[4] G. R. WILLSKY,[5§] M. H. MALAMY,[5] AND H. ROSENBERG[2]

Department of Biology, Washington University, St. Louis, Missouri 63130[1]; Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. 2601, Australia[2]; Department of Biochemistry, University of Leicester, Leicester LE1 7RH, U.K.[3]; The Public Health Research Institute of the City of New York, New York, New York 10016[4]; and Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111[5]

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Plasmids in both *Escherichia coli* and *Staphylococcus aureus* contain an "operon" that confers resistances to arsenate, arsenite, and antimony(III) salts. The systems were always inducible. All three salts, arsenate, arsenite, and antimony(III), were inducers. Mutants and a cloned deoxyribonucleic acid fragment from plasmid pI258 in *S. aureus* have lost arsenate resistance but retained resistances to arsenite and antimony, demonstrating that separate genes are involved. Arsenate-resistant arsenite-sensitive *S. aureus* plasmid mutants were also isolated. In *E. coli*, plasmid-determined arsenate resistance and reduced uptake were additive to that found with chromosomal arsenate resistance mutants. Arsenate resistance was due to reduced uptake of arsenate by the induced plasmid-containing cells. Under conditions of high arsenate, when some uptake could be demonstrated with the induced resistant cells, the arsenate was rapidly lost by the cells in the absence of extracellular phosphate. Sensitive cells retained arsenate under these conditions. When phosphate was added, phosphate-arsenate exchange occurred. High phosphate in the growth medium protected cells from arsenate, but not from arsenite or antimony(III) toxicity. We do not know the mechanisms of arsenite or antimony resistance. However, arsenite was not oxidized to less toxic arsenate. Since cell-free medium "conditioned" by prior growth of induced resistant cells with toxic levels of arsenite or antimony(III) retained the ability to inhibit the growth of sensitive cells, the mechanism of arsenite and antimony resistance does not involve conversion of AsO_2^- or SbO^+ to less toxic forms or binding by soluble thiols excreted by resistant cells.

Plasmid-determined resistances to arsenate, arsenite, and antimony(III) in *Staphylococcus aureus* have been known since 1968, when Novick and Roth (22) reported that these resistances (along with resistances to a variety of heavy metals, including Hg^{2+} and Cd^{2+}) were associated with three plasmids, now known as pI258, pII147, and pI524. Mutations were isolated that affected arsenate or arsenite resistance separately, suggesting that the genetic determinants were independent (22). Studies with deletion mutants indicated further that the genetic determinants for arsenate, arsenite, and antimony(III) resistance were "clustered" on the genetic maps of the plasmids (22). Arsenate resistance was shown to be inducible by prior exposure to arsenate (22). However, no evidence was obtained as to whether or not arsenite and antimony(III) resistances were inducible and, based on growth experiments, Hg^{2+} resistance was initially thought to be constitutive. That resistance was later shown to be inducible (35).

In subsequent years, recombinational, deletion, and cloning experiments (14, 20, 21) have refined the genetic maps of these three *S. aureus* plasmids, and evidence was obtained suggesting that the genes for arsenate and arsenite resistances were in the same operon (20, 21). The arsenate-arsenite-antimony resistance operon on plasmids of *S. aureus* is bounded by a region of inverted duplicated sequences of about 200 base pairs in length (26), suggestive of a trans-

† Present address: Department of Biology, Queen's University, Kingston, Ontario K7L 3N6, Canada.

‡ Present address: 5164 Imperial Drive, Richton Park, IL 60471.

§ Present address: Department of Biochemistry, School of Medicine, State University of New York, Buffalo, NY 14214.

poson structure. However, there is no evidence for transposition of these determinants at this time. The question of whether antimony resistance is due to the action of the same gene as is arsenite resistance or is due to a third gene is still not resolved.

Although arsenate, arsenite, and antimony(III) resistances were common determinants on *S. aureus* penicillinase plasmids (22), similar studies with plasmids of gram-negative bacteria awaited the 1973 report of Hedges and Baumberg (12) that a newly discovered plasmid (R773) conferred resistance to arsenate and arsenite [antimony(III) was not tested], as well as to tetracycline and streptomycin. At that time we screened a collection of about 50 mercuric resistance plasmids (25; originally obtained from the Hammersmith Hospital collection of N. Datta and R. W. Hedges) and found that about one out of five conferred arsenate resistance. These arsenate resistance plasmids included R828, one of the more thoroughly studied of the mercuric-mercurial resistance plasmids (25). Similarly, Summers et al. (30, 31) screened plasmids from the Hammersmith Hospital and other collections and found resistance to arsenate in plasmids of incompatability groups FII, N, and H_2. Arsenite resistance and antimony resistance were not reported. Smith (29) screened a wide range of enterobacteria and found many cases of transmissible arsenite resistance; most of these were due to plasmids, but sometimes arsenite resistance transducing phage occurred. In all 43 cases of transmissible arsenite resistance, arsenate resistance was also transferred (29). Antimony was not tested.

One complication in studies of arsenate resistance governed by plasmid genes is the existence of chromosomal mutations leading to arsenate resistance (27). These mutations result in loss of a phosphate transport system (Pit), which is relatively indiscriminate between phosphate and arsenate (5, 24, 37, 38). As will be shown below, the plasmid-determined arsenate resistance was additive with the chromosomally governed resistance.

During the last 10 years, there have been several unpublished studies on the mechanism of arsenate resistance governed by plasmid genes. These include work by W. V. Shaw and D. Hammond (Bacteriol. Proc., p. 45, 1971) showing that a newly isolated *S. aureus* plasmid, pPS85, confers inducible arsenate resistance due to a selective decrease in accumulation of arsenate at neutral pH and that the reduced uptake did not occur when chloramphenicol was present during the induction period. Willsky (Ph.D. thesis, Tufts University, Boston, Mass., 1977) studied arsenate resistance of *Escherichia coli* containing the plasmid R773 and reached the conclusion that the plasmid effected reduced uptake of arsenate by both of the two known phosphate transport systems. Rather than publish a series of preliminary reports of the system governing arsenate, arsenite, and antimony(III) resistances (which seem remarkably similar in both *S. aureus* and *E. coli*), we have decided to place our current understanding together in this single paper with conclusions from several studies widely spaced in time and geography.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used are listed in Table 1. Plasmids R773 and R828 were transferred into strains AN710 and AN1088 by conjugation at 37 (R773) or 22°C (R828), respectively, because plasmid R773 transferred poorly at 22°C and R828 transferred poorly at 37°C. Transconjugants were selected for tetracycline resistance (both plasmids) or Hg^{2+} resistance (R828) with nutritional counterselection against the donor strain. In different laboratories and experiments, a variety of media was used. Nutrient broth (Difco Laboratories) contains 3 g of beef extract and 5 g of peptone per liter of deionized water. Triethanolamine-buffered minimal medium (24) contained 50 mM triethanolamine, 10 mM $(NH_4)_2SO_4$, 15 mM KCl, and 1 mM $MgSO_4$, adjusted to pH 6.5 at 37°C. This was supplemented with K-phosphate buffer, generally to 1 mM phosphate, with 20 mM glucose unless otherwise stated, and with amino acid and vitamin requirements as needed. Occasionally, when comparisons were to be made between growth under differing conditions, cells were grown in a high-phosphate-buffered medium (medium 56; reference 24).

Growth was monitored with a Klett-Summerson colorimeter with a no. 54 (green) filter for broth-grown cultures and with a no. 42 (blue) filter for minimal medium cultures. This difference was not important. Values obtained were approximately $\times 10^7$ cells/ml per turbidity unit under the different conditions used. For the over 100 separate conditions in the experiment in Fig. 1, 10-ml cultures in test tubes were aerated on a rotator drum at 37°C. For a smaller number of 10- to 25-ml cultures whose growth was followed periodically (e.g., see Fig. 2–4), 250-ml flasks fitted with "Klett side arms" were used with aeration by rapid shaking at 37°C.

Uptake and exchange experiments with *E. coli* strains AN710 and AN1088 were run in Canberra according to published methods (24) at 37°C in triethanolamine-buffered uptake medium with 50 μM ^{32}phosphate or 250 μM ^{74}arsenate, unless otherwise stated. *S. aureus* and *E. coli* uptake and exchange experiments were run in St. Louis in 50 mM Tris–50 mM maleic acid buffer adjusted to pH 7.0 with NaOH and supplemented with 0.2% glucose. Chloramphenicol (50 or 100 μg/ml) was present to prevent induction of the system during the assay, and 0.5 mM ^{74}arsenate was used unless otherwise specified.

RESULTS

Resistances to arsenate, arsenite, and antimony(III). Plasmid-governed resistances were

TABLE 1. *Bacterial strains and plasmids*

Strain	Genotype[a]	Reference
E. coli K-12		
J53	*pro met*	12, 25
J53(R773)	*pro met* plasmid R773 *asa*$^+$ *asi*$^+$ (*ant*$^+$) *tet*$^+$	12
J53(R828)	*pro met* plasmid R828 *asa*$^+$ *asi*$^+$ (*ant*$^+$) *mer*$^+$ *pma*$^+$ *tet*$^+$ *bla*$^+$ *kan*$^+$	25
AN710	*arg thi entA phoT101*	24
AN1088	*arg thi entA pit*	24
AN524	*arg thi entA phoT101 pit*	24
S. aureus		
RN1	Wild-type strain 8325	20, 22, 35
RN23	8325(pI258)	20, 22, 35
RN4	8325(pI524)	20, 22, 35
RN895	8325(pII147)	20, 22, 35
RN664	8325 with plasmid pRN1058 (*blaI1 asa-56* mutant of pI524)	R.P.N., unpublished data
RN1(pPS85)	8325 transduced with plasmid pPS85[b]	W.V.S., unpublished data
SL3	8325-4 with class III chimeric plasmid containing *Eco*RI fragments A and D of pI258	14

[a] Chromosomal gene mnemonics are according to Bachmann and Low (3); plasmid gene mnemonics are according to Novick et al. (19). *asi* (*ant*) indicates that it is not currently known whether antimony (*ant*) resistance results from a separate gene from that for arsenite (*asi*) resistance.

[b] From strain PS85, which is the propagating strain for phage 85 of the staphylococcal typing series. Plasmid pPS85 *bla*$^+$ *mer*$^+$ *cad*$^+$ *asa*$^+$ (*pma*$^+$ *asi*$^+$ and *ant*$^+$ were never tested).

determined by measuring the zones of inhibition surrounding disks containing arsenic or antimony salts placed on the surface of petri dishes (data not shown; 35) and by following turbidity during growth in liquid cultures (Fig. 1). All of the wild-type plasmids considered in this paper conferred resistances to arsenate, arsenite, and antimony(III). There were some quantitative differences between *E. coli* and *S. aureus* strains without plasmids. For example, *E. coli* strain J53 was somewhat more resistant to arsenate and arsenite (Fig. 1A and B) than was *S. aureus* strain RN1 (Fig. 1D and E). There were also differences in plasmid-determined resistances. For example, the ratio of 50% inhibitory concentrations for the plasmid-containing strain and the strain without a plasmid was greater for *S. aureus* with arsenate and greater for *E. coli* with arsenite. Increased phosphate buffer was totally without effect on the toxicity of arsenite salts (Fig. 1B) or antimony(III) (data not shown), but did affect arsenate resistance. Nutrient broth contains 2.85 mM inorganic phosphate (measured by the colorimetric phosphomolybdate method; data not shown). Addition of 5.25 mM phosphate had a protective effect on the growth of both the sensitive plasmidless strain and the resistant strain J53(R773) in the presence of increasing arsenate concentrations. Analogous experiments showed that inorganic phosphate protected both the sensitive *S. aureus* strain RN1 and the plasmid-containing resistant strains RN4(pI524) and RN1(pPS85) from arsenate toxicity. The induction of arsenate resistance in RN1(pPS85) was prevented by the presence of high levels of inorganic phosphate (data not shown).

In addition to the interaction of phosphate levels and arsenate resistance, other differences in media affected resistance levels for *E. coli* both with and without plasmids. For example, *E. coli* strain J53 was somewhat more sensitive to arsenite inhibition and more resistant to antimony inhibition in triethanolamine medium than in nutrient broth (data not shown). Both strains J53 and J53(R773) were exceedingly resistant to antimony(III) salts in medium M56. The bases for these media effects were not pursued.

Resistance to arsenate was genetically separable from resistance to arsenite. Novick has a collection (unpublished) of over 30 mutants of plasmids pI258, pI524, and pII147 affected for arsenate or arsenite resistance or both. Although deletion mutations caused simultaneous loss of all three resistances [arsenate, arsenite, and antimony(III)], point mutants were found that were arsenate sensitive but arsenite resistant or arsenate resistant and arsenite sensitive. One such mutant derived from plasmid pI524 (in strain RN664) was shown in the experiment in Fig. 1D–F to be arsenate sensitive but substantially or completely as resistant to arsenite and antimony as was strain RN4, which carries the parental plasmid. Löfdahl et al. (14) cloned fragments from plasmid pI258 into small vector plasmids. Two of their chimeric plasmid classes expressed arsenite resistance but not arsenate re-

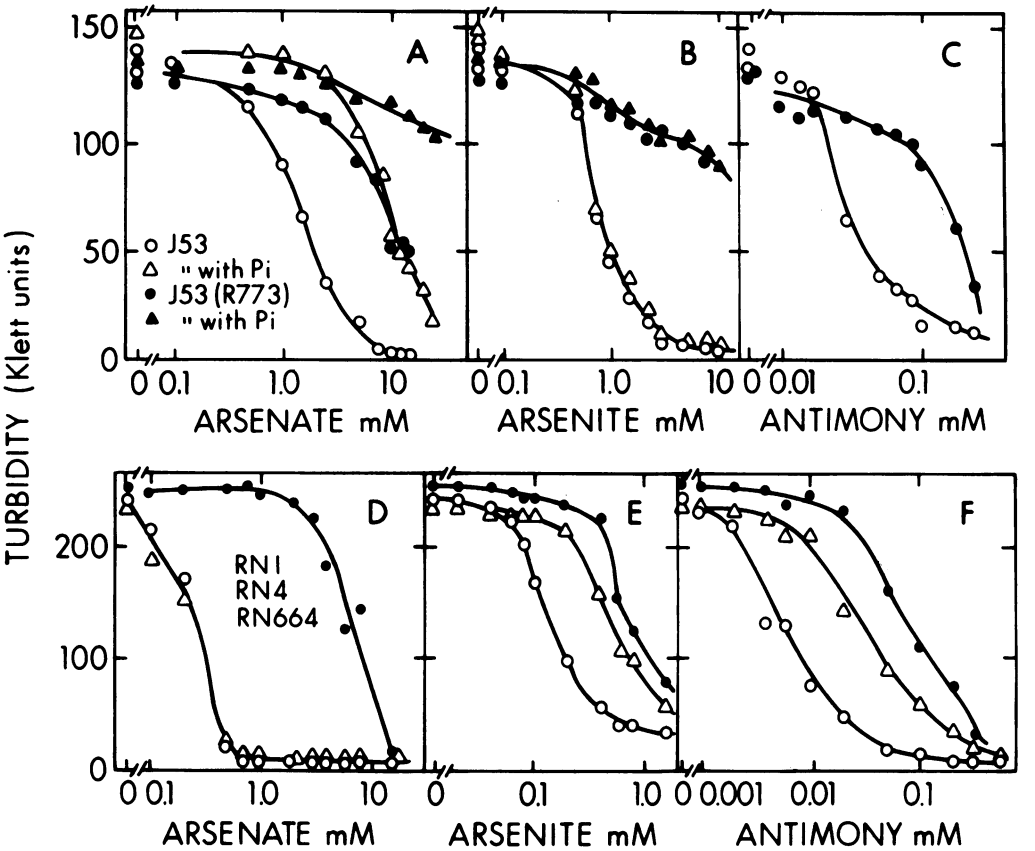

FIG. 1. *Resistance to arsenate, arsenite, and antimony in E. coli and S. aureus. Overnight cultures of E. coli strains J53, J53 with plasmid R773, S. aureus strains RN1 (without a plasmid, ○), RN4 (with plasmid pI524, ●), or RN664 (with a mutant variant of plasmid pI524, △) were diluted 100 times into fresh nutrient broth (to which 5.25 mM phosphate [P_i] buffer was also added where indicated). Na_2HAsO_4 (arsenate), $NaAsO_2$ (arsenite), or $SbKOC_4H_6O_6$ [antimony(III)] was added to 10-ml samples as indicated. After incubation with aeration on a "roller drum" for 6 h at 37°C, growth was determined as turbidity in a Klett-Summerson colorimeter with a no. 54 (green) filter. A turbidity of 100 Klett units corresponds to 0.22 mg (dry weight) per ml for S. aureus and to 0.25 mg (dry weight) per ml for E. coli.*

sistance (14). A class III cloned chimeric plasmid (14) showed arsenate sensitivity and arsenite resistance in our experiments similar to that of strain R664 in Fig. 1D and E. This class III chimeric plasmid also conferred resistance to antimony in both liquid growth experiments, as in Fig. 1F, and disk inhibition zone experiments (data not shown).

In disk zone inhibition studies, the strain with chimeric plasmid class III (14) was somewhat hypersensitive to arsenate, that is, somewhat more sensitive than the plasmidless strain RN1. This strain was also somewhat hypersensitive to inorganic Hg^{2+} and to fluoresceinmercuric acetate. Plasmid pI258 confers resistance toward both compounds. We do not have an explanation for these results.

With *E. coli* K-12 strains, there are two genetically defined phosphate transport systems governed by chromosomal genes (24, 37). Strain AN710 has the inorganic phosphate transport (Pit) system with a K_m of 25 μM phosphate and a V_{max} of 60 nmol/min per mg of dry weight (24) but is lacking the phosphate-specific transport (Pst) system with a K_m of less than 0.5 μM phosphate and a V_{max} that increases severalfold during phosphate starvation (24) or after arsenate addition (38) from 15 to 70 nmol of phosphate per min per mg of dry weight. The K_i for arsenate as a competitive inhibitor of both phosphate transport systems is about the same (25 μM arsenate; reference 24). Therefore, the Pit system with equal K_m and K_i is relatively less specific than the Pst system with its K_m for phosphate two orders of magnitude lower than the K_i for arsenate. Pit^+ Pst^- strains such as

AN710 are relatively arsenate sensitive (as sensitive as those with both the Pit and the Pst systems), whereas Pit$^-$ Pst$^+$ strains such as AN1088 are relatively arsenate resistant.

Plasmid-mediated arsenate resistance was shown to be additive to chromosomal resistance (i.e., Pit$^-$ Pst$^+$) in *E. coli* growth experiments using the series of strains GR2131 (Pit$^+$ Pst$^+$), GR5178 (Pit$^+$ Pst$^-$), and U7 (Pit$^-$ Pst$^+$) in Boston with plasmid R773 (data not shown), and in Canberra with strains AN710(R773), AN710(R828), AN1088(R773), and AN1088-(R828) (Fig. 2 and data not shown). Note that after a delay as shown in Fig. 2, AN1088 did grow in the presence of 10 mM arsenate (and 1 mM phosphate), whereas strain AN710 did not (data not shown). AN1088 grew also with 25 mM or 50 mM arsenate (but the delay with 50 mM arsenate was over 9 h). At all three concentrations (10 mM, 25 mM, and 50 mM arsenate), AN1088(R773) grew more rapidly and with less delay than did the plasmidless AN1088 strain, demonstrating that the plasmid-determined resistance occurred in both Pit$^+$ Pst$^-$ and Pit$^-$ Pst$^+$ strains and was additive with the chromosomally determined resistance of Pit$^-$ strains.

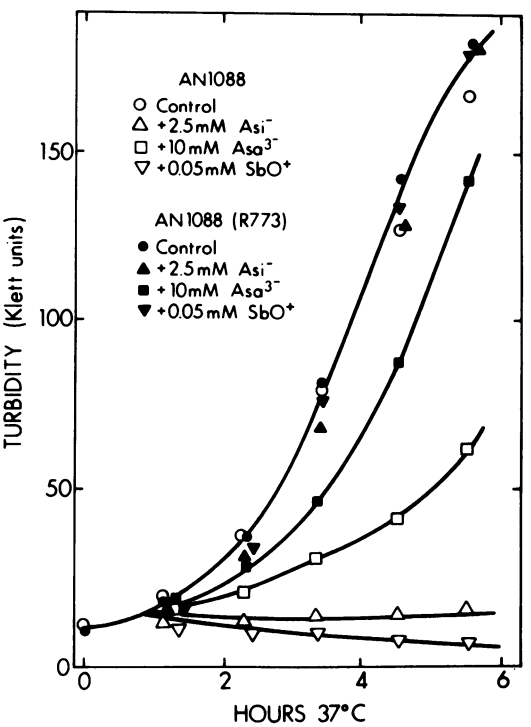

FIG. 2. *Resistance to arsenate, arsenite, and antimony with E. coli strain AN1088 with chromosomal resistance to arsenate. Overnight cultures of strains AN1088 and AN1088(R773) in triethanolamine-buffered minimal medium with 1 mM P_i (and 20 mM glucose as carbon source) were diluted into fresh medium, grown 2.5 h into log phase, and diluted again, and 2.5 mM arsenite (Asi$^-$), 10 mM arsenate (Asa^{3-}), or 0.05 mM antimony(III) (SbO$^+$) was added. Growth was monitored by turbidity (Klett units with a no. 42 blue filter) during aeration by shaking in a 37°C bath.*

The level of plasmid-mediated resistance depended upon the carbon source used for growth. With glucose-grown cells, 10 mM arsenate with 1 mM phosphate gave a clear distinction between the plasmid-containing and the plasmid-free cells (Fig. 2 and additional experiments with Pit$^+$ cells). With glycerol as carbon source (which induces synthesis of an additional transport system capable of inorganic phosphate transport), both plasmid-containing and plasmid-free cells were inhibited by 10 mM arsenate with 1 mM phosphate (data not shown). Lower concentrations (5 mM arsenate and 1 mM phosphate) were needed to distinguish between the resistant and the sensitive cells (data not shown). We do not know whether this difference is due to the differing intermediate metabolism of glucose versus glycerol or to the induction of the additional phosphate (and possibly arsenate) transport system.

Induction of resistance. When uninduced *S. aureus* cells with plasmid pI258 were exposed to 3 or 6 mM arsenate in nutrient broth, there was a delay of 5 to 7 h before growth commenced (Fig. 3A). Prior exposure to 0.1 mM arsenate, arsenite, or antimony salts allowed immediate growth on up to 6 mM arsenate (Fig. 3B–D). Note that 0.1 mM antimony itself was toxic under these conditions and, therefore, although growth commenced without a delay, the eventual growth yield was reduced (Fig. 3D). Experiments with 0.02 or 0.05 mM Sb(III) as inducer gave less inhibition in nutrient broth with *S. aureus* strains RN23(pI258) and RN4(pI524) and with *E. coli* strains J53(R773) and J53(R828) (data not shown). We were able to show the arsenate, arsenite, and antimony were all inducers of arsenate resistance with all plasmids tested. Whereas arsenate resistance was clearly inducible in *S. aureus*, the onset of inhibition of growth by arsenite and antimony(III) was so gradual in nutrient broth (see below) that we were unable to demonstrate inducibility of arsenite or antimony resistance in *S. aureus*. In triethanolamine-buffered minimal medium with *E. coli*, the onset of growth inhibition by arsenite and antimony(III) was more abrupt, and we readily saw that arsenite resistance was inducible by prior exposure to arsenate (Fig. 4), arsenite, or antimony (data not shown). In this triethanolamine-buffered medium with *E. coli* strain J53(R773), all nine combinations of in-

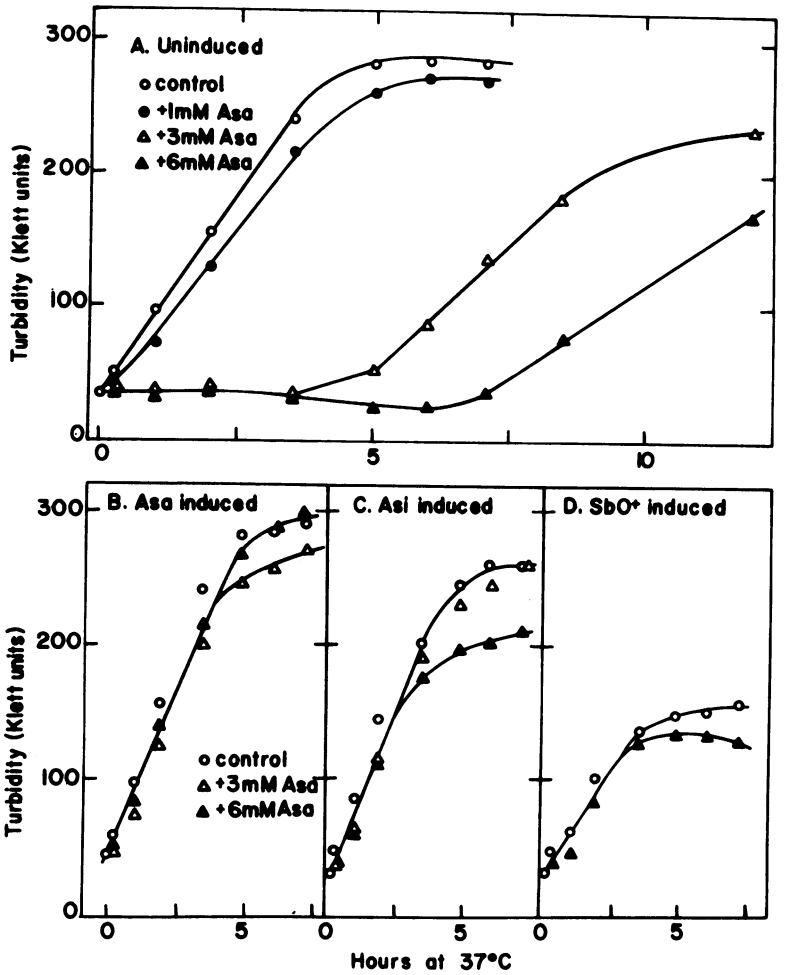

FIG. 3. *Induction of arsenate resistance by arsenate, arsenite, and antimony. S. aureus strain RN23 (with plasmid pI258) was grown overnight in nutrient broth, diluted 1:10 in fresh nutrient broth [without (A) or with 0.1 mM arsenate (B), arsenite (C), or antimony (D)]. After 2 h of growth and induction with aeration at 37°C, the cultures were diluted 1:5 with fresh nutrient broth, in the absence or in the presence of 1, 3, or 6 mM arsenate. Turbidity (no. 54 filter) was monitored during growth at 37°C.*

ducers and resistances were demonstrated; arsenate, arsenite, and antimony(III) induced resistance toward inhibitory concentrations of arsenate, arsenite, and antimony(III) (Fig. 4 and data not shown).

Toxicity of arsenate, arsenite, and antimony(III). Growth over many hours is often the least incisive means of monitoring plasmid-determined resistances. As a step toward a biochemical characterization, *S. aureus* strains were grown, induced by addition of subtoxic levels of arsenate, and then exposed to [^{14}C]-uracil for 10-min pulses after addition of concentrations of arsenate, arsenite, and antimony that best distinguished the resistant from the sensitive strains. Within 10 min of exposure, 3 or 6 mM arsenate reduced the incorporation of [^{14}C]uracil more than 95% with the sensitive strain, but not measurably with the resistant strain RN23(pI258) (data not shown). The inductive effects of arsenite and antimony(III) (which inhibited the sensitive strain RN1 significantly more than the resistant strain) were more gradual, continuing over the 2 h of the experiment.

Altered uptake of arsenate with plasmid-containing strains. Induced plasmid-containing *E. coli* strain AN710 accumulated negligible ^{74}arsenate (Fig. 5) under conditions where the plasmidless strain accumulated approximately comparable amounts of ^{32}phosphate and ^{74}arsenate. This result was reproducible in many

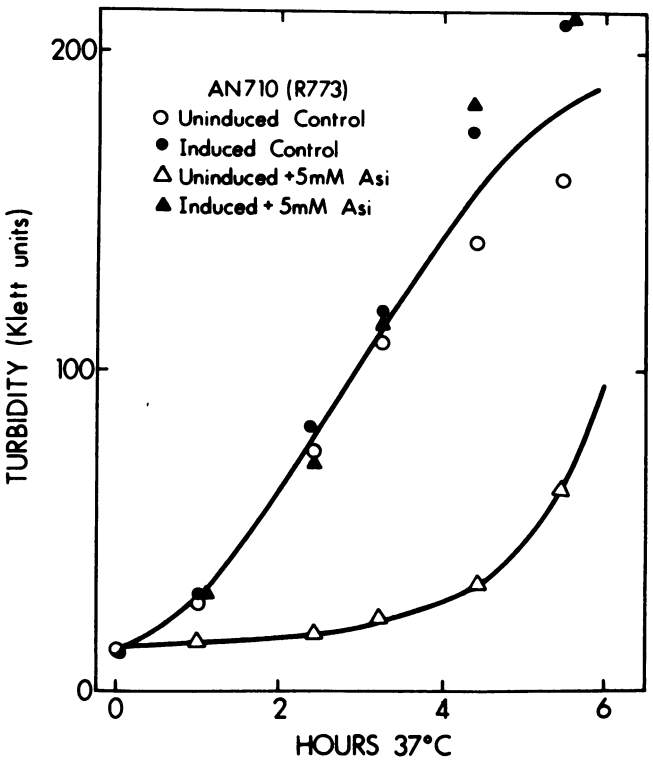

FIG. 4. *Induction of arsenite resistance by arsenate. E. coli strain AN710(R773) was grown overnight in triethanolamine medium with 1 mM P_i and 20 mM glucose. The cells were diluted into fresh medium, and 0.1 mM arsenate was added to the induced culture. After 2 h at 37°C, the cells were diluted again, and 5 mM arsenite was added as indicated.*

similar experiments with short time courses (10 to 30 s). In other experiments with longer time courses, measurable arsenate was accumulated by the resistant cells, but the uptake by induced resistant cells was always strikingly less than that found with sensitive or uninduced resistant cells. Similar results were obtained with strain AN1088 with and without plasmid R773 (Table 2). In these experiments with no added phosphate in the incubation medium, the chromosomal resistance mutant strain AN1088 accumulated less arsenate than did the sensitive strain AN710. The introduction of plasmid R773 reduced the arsenate uptake still further (Table 2 and additional data).

The reduced arsenate uptake required induction by arsenate, arsenite, or antimony, just as did resistance of growth to arsenate. In the uptake experiment in Fig. 6, *E. coli* strains AN710 and AN710(R773) were grown into log phase and "induced" or not in the presence of 0.1 mM arsenate for 80 min. Half the cultures contained chloramphenicol to prevent protein synthesis. After centrifugation and washing, the cells were suspended, and the uptake of 250 μM arsenate was followed (Fig. 6). The sensitive strain AN710 was unaffected by induction. In some experiments, there was a general inhibition of cellular metabolism by this regimen, including an inhibition of phosphate as well as of arsenate uptake. With the resistant strain AN710(R773), reduced arsenate uptake was found only with the cells induced in the absence of chloramphenicol. Similar results were obtained with resistant *S. aureus* cells (data not shown).

In broths and in Tris-buffered media, both *E. coli* (data not shown) and *S. aureus* (Fig. 7) strains with resistance plasmids took up reduced but measurable levels of ^{74}arsenate. The relative uptake by resistant strains when compared with sensitive strains depended importantly upon time of incubation, concentration of arsenate and phosphate present, and pH of the incubation medium (Fig. 7). Short-term assays at lower arsenate concentrations (Fig. 7A) showed essentially complete elimination of arsenate uptake with the resistant cells. Longer incubations with higher concentrations of arsenate allowed some uptake of arsenate by the resistant cells, especially at acid pH (Fig. 7B). Net phosphate ac-

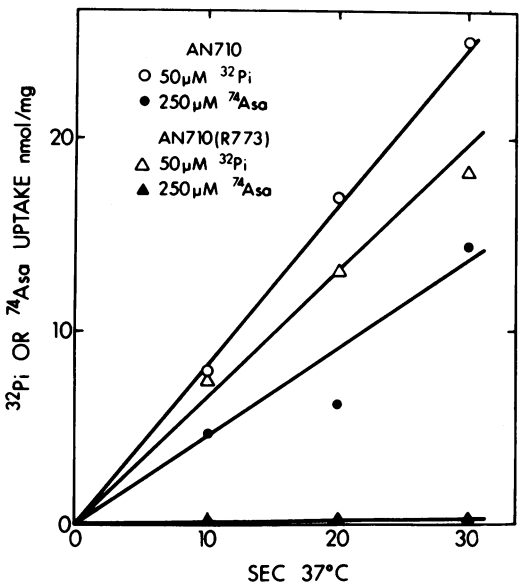

FIG. 5. *Absence of arsenate uptake by the induced plasmid-containing strain. E. coli strains AN710 and AN710(R773) were grown overnight on limited (1 mM) glucose in medium 56 (high phosphate). Glucose (20 mM) was added, and after 1.5 h (one cell doubling), 0.1 mM arsenite was added as inducer. After an additional cell doubling, the cells were centrifuged, washed three times in triethanolamine medium, and suspended in triethanolamine uptake medium as described by Rosenberg et al. (24). $^{32}P_i$ (50 μM) or ^{74}Asa (250 μM) was added, and samples were filtered, washed, and counted.*

cumulation was maximum around pH 6 in a 3-min incubation with low (10 μM) phosphate (Fig. 7C), and with longer incubation at higher concentrations no specific peak was found (Fig. 7D). Arsenate uptake, however, showed two pH optima of 5.5 and 7 with 10 μM arsenate and the sensitive cells. With the resistant cells at low concentrations and short incubation times, significant uptake occurred only below pH 5 (Fig. 7A); with longer incubations and higher concentrations, the maximal uptake occurred at a pH of about 5.5. At higher pH, the difference in arsenate uptake between the sensitive and the induced resistant *S. aureus* was greater (Fig. 7B). These results can be compared with the pK_a for $H_2PO_4^-/HPO_4^{2-}$ of 7.12 and for $H_2AsO_4^-/HAsO_4^{2-}$ of 6.77, but one cannot deduce the charged form of the accumulated phosphate and arsenate from such preliminary results. Harold and co-workers (9, 10) defined two phosphate-arsenate transport systems in *Streptococcus faecalis* by differences in pH profiles and use of a mutant strain lacking the major (optimum above pH 7) system. Both systems could accumulate arsenate, as well as phosphate, albeit with differing efficiencies depending upon conditions. Comparable pH experiments with only one or the other phosphate transport system do not appear to have been done with the *E. coli* mutants. Harold and Spitz (10) concluded from experiments with inhibitors that phosphate and arsenate entered *S. faecalis* in an electroneutral form ($H^+H_2PO_4^-$ or $H^+H_2AsO_4^-$) and that the uptake systems were directly dependent upon energy from ATP and not from the membrane electrical or pH gradient. We do not know whether the reduced uptake by the induced resistant cells results from a direct blockage of arsenate influx or from an accelerated efflux of accumulated arsenate. The pH profile reflects the sum of both processes.

The interaction between phosphate and arsenate uptake. Since arsenate is known to be a competitive inhibitor of phosphate uptake, one effect of the arsenate resistance plasmid might be to alter the kinetic constants (K_m for phosphate, K_i for arsenate, K_m for arsenate, V_{max} for phosphate, V_{max} for arsenate) of the apparently shared membrane transport systems. The presence of the plasmid pI258 in strain RN23 of *S. aureus* did not affect the K_m (about 70 μM phosphate) or V_{max} (about 25 nmol of phosphate/min per mg of dry weight) of phosphate transport by this strain; nor did it affect the K_i (about 300 μM arsenate) of arsenate as a competitive inhibitor of phosphate uptake (Fig. 8). With strains *S. aureus* RN1 and RN1(pPS85), similar results were found. There was no difference between the sensitive and resistant strains with regard to V_{max} for ^{32}phosphate uptake. In a Tris-glucose-saline medium, the sensitive cell showed a K_m of 47 μM ^{32}phosphate and a K_m of 850 μM ^{74}arsenate. The absence of an effect of the arsenate resistance plasmid on the kinetic parameters of phosphate transport was surprising, but equivalent results were obtained with *E. coli* strains AN710 and AN1088 with plasmid

TABLE 2. *Net arsenate accumulation by E. coli strains*

Strain	^{74}Arsenate uptake (nmol/mg of dry weight)[a]
AN710	10.2 ± 1.4
AN710(R773)	1.65 ± 0.4
AN1088	3.5 ± 1.0
AN1088(R773)	0.67 ± 0.27

[a] Log phase cells growing in triethanolamine medium with 1 mM phosphate were induced by the addition of 0.1 mM arsenate 1 h before harvesting and washing. Then 250 μM ^{74}arsenate was added, and samples were filtered and washed at 1, 2, 4, and 6 min of incubation. The values of the averages ± standard deviation for these four samples are given, although equilibrium had not been reached by 1 min.

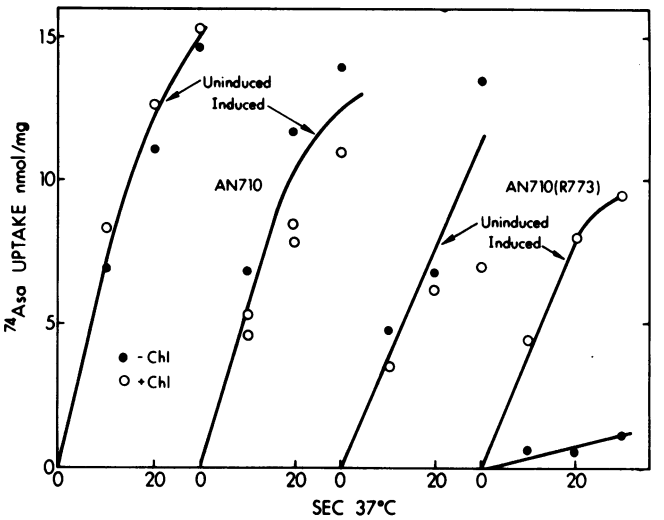

FIG. 6. *Induction of the block to arsenate uptake. E. coli strains AN710 and AN710(R773) were grown overnight in triethanolamine medium on limiting glucose. Glucose (20 mM) was added, and after 2.5 h (two cell doublings) the cultures were divided into four samples each. Arsenate (0.1 mM), 50 μg of chloramphenicol per ml, both, or neither were added, and the cultures were incubated for an additional 80 min. After centrifuging, washing, and suspension in uptake medium, uptake of 250 μM ^{74}arsenate was measured. "Uninduced" or "induced" refers to the absence or presence of the 0.1 mM arsenate during the 80-min incubation.*

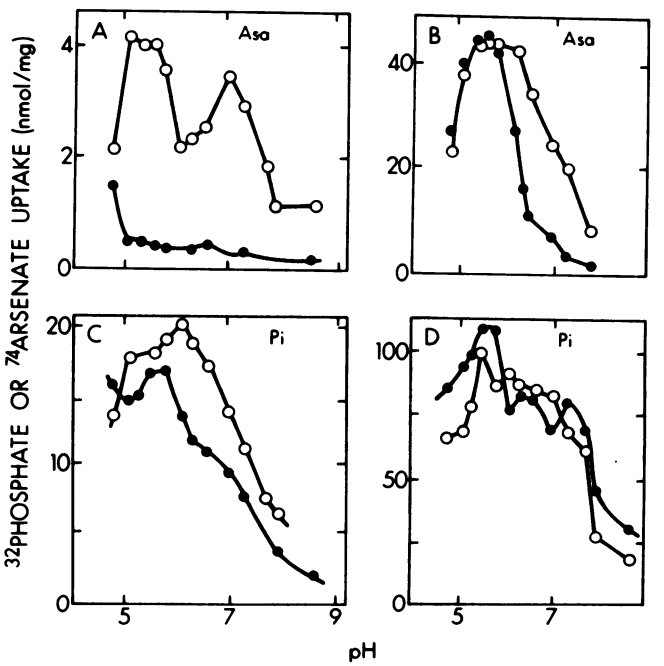

FIG. 7. *^{74}Arsenate and ^{32}phosphate uptake as a function of pH. S. aureus strains RN1 (sensitive, without a plasmid, ○) and RN1(pPS85) (resistant, ●) were grown into exponential growth phase, centrifuged, and suspended at a final density of 0.55 mg (dry weight) per ml in 50 mM Tris-maleate–50 mM glucose–100 mM NaCl medium at the different pH values indicated. (A and C) 10 μM ^{74}arsenate or ^{32}phosphate was added, duplicate 1-ml samples were filtered after 3 min at 37°C, and cell-bound radioactivity was determined. (B and D) 100 μM ^{74}arsenate or ^{32}phosphate was added, and samples were filtered and washed after 15 min.*

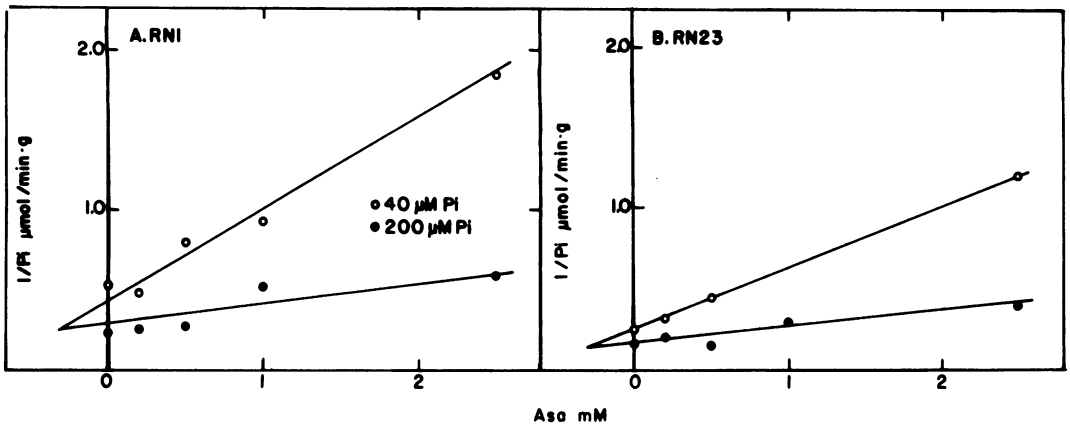

FIG. 8. *Dixon plot of arsenate as a competitive inhibitor of phosphate uptake in S. aureus strain RN1 (A, without plasmid) and RN23 (B, with plasmid pI258). Cells were grown into log phase in nutrient broth, induced for 1.5 h with 0.2 mM arsenate, centrifuged, washed, and suspended in Tris-maleic acid buffer with 10 mM glucose. The initial rate of uptake of 40 or 200 μM $^{32}P_i$ was measured as in Fig. 5 in the presence of 0, 0.25, 0.5, 1.0, or 2.5 mM arsenate. Samples were filtered and washed at 0.5 and 1 min.*

R773; the kinetic constants were consistent with those published (24) and were not affected by the presence of the plasmid (or induction by low arsenate during growth or both). Although arsenate was a competitive inhibitor of phosphate uptake in *E. coli*, with a K_i of 10 to 25 μM arsenate (in strains AN710 and AN1088), arsenate uptake itself generally did not show saturation kinetics at concentrations below 100 μM arsenate. Similarly, in *S. aureus*, where the K_i for arsenate as an inhibitor of phosphate transport was about 0.3 mM arsenate (Fig. 8), arsenate uptake did not saturate below 1 mM arsenate (data not shown). The absence of clear-cut kinetic results for ^{74}arsenate uptake, under conditions where reproducible results were obtained with ^{32}phosphate, placed serious limitations on our conclusions. Yet, it seemed as if the initial surface recognition site for arsenate as a competitive inhibitor of phosphate uptake was unaltered by the plasmids. Some subsequent step that did not saturate at the lower arsenate concentrations governed arsenate entrance into the cells.

In the absence of exogenous arsenate or phosphate, the sensitive strain RN1 retained accumulated arsenate (Fig. 9), whereas the resistant strain lost accumulated arsenate, slowly from uninduced cells and more rapidly from induced cells (Fig. 9). We do not known whether this slow loss from the uninduced cells reflects a partially constitutive functioning of the arsenate resistance system. The difference was reproducible in other experiments. Rapid efflux of arsenate from preloaded induced *E. coli* J53(R773) was also studied (data not shown), and the energy dependence of arsenate efflux will be the subject of a subsequent communication (in preparation). Addition of 2 mM phosphate or arsenate (at 10 min in the experiment in Fig. 9) led to a rapid loss of radioactive arsenate by both sensitive and uninduced resistant cells (data not shown).

The possibility that arsenate might be detoxified by conversion to a less toxic form in the resistant cells was tested in two ways. (i) *E. coli* strain GR5158(R773) was grown in minimal medium containing 10 mM arsenate and 1 mM phosphate. After one cell doubling, the cells were removed by filtration and replaced by sensitive Pst$^-$ Pit$^+$ *E. coli* strain GR5200. The sensitive cells were inhibited, indicating that the arsenate had not been detoxified. (ii) ^{74}Arsenate extracted from resistant and sensitive cells of *S. aureus* and from medium exposed to resistant cells was subjected to thin-layer chromatography. No evidence for any form other than AsO_4^{3-} was found (data not shown).

Arsenite and antimony(III) resistances. We do not know the mechanism of arsenite or antimony resistances or whether these are biochemically different resistances. It seems useful to describe briefly our unsuccessful attempts in this direction. We had postulated that plasmid-determined arsenite resistance might be due to oxidation to arsenate (32). We can now eliminate that hypothesis. Using a colorimetric I_2-starch assay for arsenite, we could detect no measurable oxidation of arsenite to arsenate with induced cells of *E. coli* J53(R773) or *S. aureus* RN23(pI258). The detection level was less than 0.1 nmol/min per mg of cell dry weight. As a control for these experiments, arsenite oxidation was measured at a rate of 200 nmol/min per mg

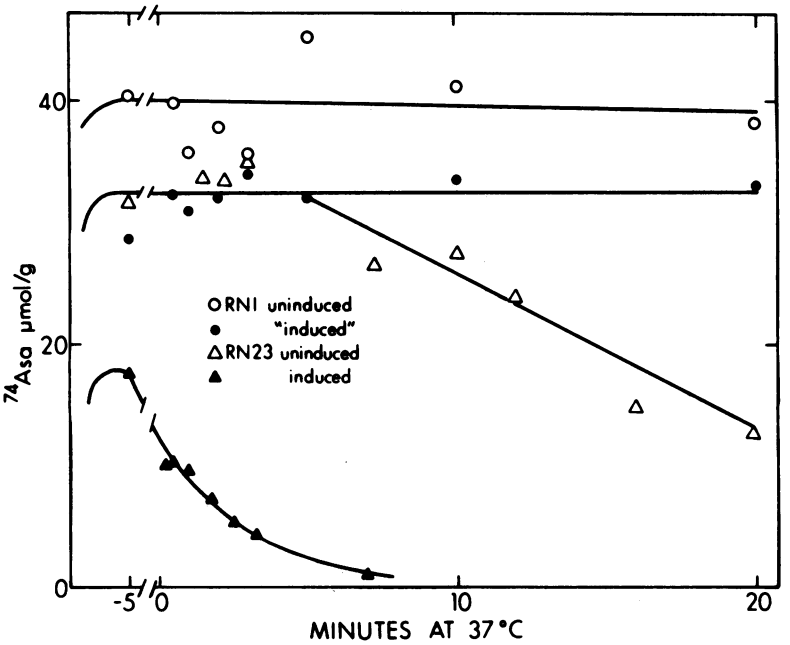

FIG. 9. *^{74}Arsenate retention by sensitive and by resistant S. aureus. Strains RN1 and RN23 were induced or not, centrifuged, washed, and suspended as in Fig. 8. ^{74}Arsenate (0.75 mM) was added; samples were filtered and washed after a 5-min uptake (−5 min); the remainder of the cultures were centrifuged and suspended in the same buffer, and samples were periodically filtered and washed.*

of dry weight by the induced cells of the soil *Alcaligenes* strain (23) kindly provided by H. L. Ehrlich. M. L. Taylor, T. P. Trezona, W. H. Taylor, and J. W. Myers (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, H44, p. 115) independently determined that plasmid R773 does not confer arsenite-oxidizing activity on *E. coli* cells.

A second hypothesis that might explain arsenite [and antimony(III)] resistance is that the plasmid-containing resistant cells excrete an effective chelating agent binding arsenite ions, thereby allowing cellular growth. The cellular excretion of a detoxifying dithiol compound (1, 7, 11) or other mechanisms of detoxification of extracellular arsenite was tested in an experiment in which sensitive *E. coli* J53 was added to medium "conditioned" by growth of induced resistant cells of strain J53(R773) in inhibitory concentrations of arsenite (Fig. 10). After three cell doublings of the resistant strain in the presence of 10 mM arsenite (end of log phase growth), the cells were centrifuged, and the residual bacterial cells were removed by filtration and replaced by plasmidless sensitive cells. The growth medium retained the ability to inhibit the growth of sensitive but not of resistant cells (Fig. 10), indicative of a lack of detoxification.

Uninduced cells of strain J53(R773) were also inhibited for several hours upon transfer to the culture filtrate of induced resistant cells in 10 mM arsenite (data not shown). This indicates that induction of resistance occurs within the cell and not by conditioning of the medium.

The resistant cells in Fig. 10 eventually grew to a yield of nearly 50% higher turbidity in the presence of 10 mM arsenite than in its absence. Resistant cells in arsenite have reproducibly shown this stimulation of growth. We do not have an explanation for this finding.

In an experiment analogous to that in Fig. 10, strain J53(R773) was shown not to detoxify antimony(III) added to triethanolamine growth medium (data not shown). As with the case of arsenite, we do not currently know the basis for antimony(III) resistance. ^{125}Sb(III) uptake experiments did not show a difference between the sensitive and the resistant cells (data not shown).

DISCUSSION

Plasmid-determined resistances to arsenic and antimony were among the earlier known resistances governed by plasmid systems (22). These resistances have been found with both gram-negative and gram-positive bacteria (12, 16–18, 22, 29) and occur in clinical isolates at frequencies sometimes exceeding 50% (16–18). Nevertheless, this is the first published report deline-

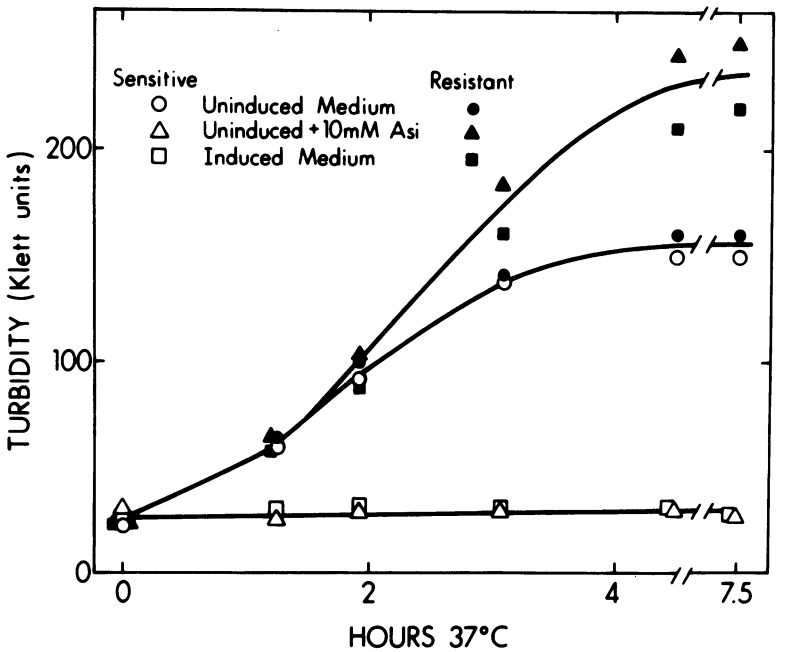

FIG. 10. *Absence of detoxification of arsenite by arsenite-resistant E. coli. Strains J53 and J53(R773) were grown into log phase in triethanolamine-buffered minimal medium. A portion of the resistant J53(R773) culture was induced by addition of 0.3 mM arsenite, followed 30 min later by arsenite to a total of 10 mM. After a further 3.5 h at 37°C (three cell doublings), all three cultures (the sensitive strain and the induced and uninduced cultures of the resistant strain) were centrifuged (10 min, 6,000 rpm); the supernatant fluids from the resistant cell cultures were filtered (0.45-μm pore size membrane filters) to remove residual cells; and the suspended bacterial cells were inoculated in the culture filtrates at about 25 Klett units turbidity. Arsenite (10 mM) was added where indicated, and subsequent growth followed during aeration at 37°C. The sensitive strain was suspended in culture filtrate from uninduced resistant cells, with 10 mM arsenite added, or in filtrate from induced resistant cells. Induced resistant cells were suspended in culture filtrate from uninduced resistant cells, with 10 mM arsenite added, or filtrate from induced resistant cells.*

ating specific properties of the system, its regulation, and the mechanism of arsenate resistance. Although some questions have now been resolved, others remain unanswered, especially those of the mechanisms of arsenite and antimony resistances.

The most striking conclusion is that the properties of the arsenic-antimony system seem essentially the same in *E. coli* and in *S. aureus*. Plasmids in both organisms govern an inducible resistance to arsenate, arsenite, and antimony(III). Although arsenate resistance has been distinguished from arsenite resistance both by mutation (*S. aureus*) and by competitive protection against arsenate toxicity by phosphate (both organisms), we do not know whether arsenite and antimony(III) resistances are specified by a single genetic locus or two. Both arsenite and antimony(III) are toxic by virtue of their interactions with cysteine residues of proteins (1, 7, 11). Protection against toxicity by these agents can be afforded in animal systems by adding dimercaptols (e.g., 33) with higher affinities for arsenite and antimony than the reactive thiols of target enzymes (e.g., 1). The medium exchange experiment in Fig. 10 [and similar experiments with antimony(III)] suggests that excretion of mono- or dimercaptols by the resistant bacterial cells is not likely to be the mechanism of plasmid-determined resistance. Since arsenite and antimony(III) in these media retained their toxicity, the mechanism of resistance must lie at the cell surface or within. Two possibilities remain: (i) the resistant strains might block normal accumulation of arsenite and antimony(III) or have systems for excretion of these toxic ions; or (ii) the intracellular target enzymes might be altered by the plasmid system, or entirely new and relatively resistant enzymes might be coded for by one or more plasmid genes.

Toxic materials are generally thought to enter cells by the same transport systems as used for structurally related nutrients (8, 27). Less than 5% of the toxic levels of arsenite (measured by the iodometric color test) or antimony(III) (fol-

lowed with ^{125}Sb) was accumulated by the cells, and we found no significant difference between sensitive and resistant strains with regard to uptake or binding or both (data not shown). If arsenite and antimony(III) enter bacterial cells by transport systems functioning for normal nutrients (as arsenate enters via phosphate transport systems), then we do not know which transport systems might be functioning in these cases.

The alternative hypothesis of an altered intracellular target enzyme (8) has the virtue of precedent in that plasmid-mediated trimethoprim resistance is due to a drug-resistant plasmid-coded dihydrofolate reductase (2) and plasmid-mediated sulfonamide resistance is due to a similarly plasmid-encoded resistant dihydropteroate synthase (28, 39). The analogy with trimethoprim and sulfonamides breaks down, however, in that the targets in these cases are single sensitive enzymes and a drug-resistant homologous enzyme specified by the plasmid will suffice. No such straightforward substitution mechanism seems likely for arsenite and antimony resistances, since neither is sufficiently target specific.

The mechanism of arsenate resistance seems more clear. As might be expected, arsenate functions (9, 10, 24, 27, 37) as an analog for the phosphate transport systems, for which there appear to be two (certainly in *E. coli* and probably in *S. aureus*). The inducible plasmid-determined resistance is additive to the chromosomally determined resistance associated with mutations eliminating the less selective (for phosphate over arsenate) of these two systems (Fig. 2; 24, 37, 38). Although the inducible plasmid system did not affect the kinetic parameters for phosphate uptake via either system (e.g., Fig. 8) and did not affect the K_i for arsenate as an inhibitor of phosphate uptake (essentially the same 25 μM arsenate for both systems in *E. coli* with or without a plasmid), the inducible plasmid-determined resistance eliminated (Fig. 5) or grossly reduced (Fig. 6 and 7) arsenate uptake by the cells. In addition, under those conditions where arsenate uptake could be demonstrated with the resistant cells, the accumulated arsenate was rapidly excreted (Fig. 9). Accumulated arsenate was retained by the sensitive cells unless external phosphate was added, and an arsenate-phosphate exchange occurred. With the existence of a rapid efflux system, one can ask whether a direct block on uptake need be invoked at all. It is sufficient, in principle, to attribute the absence of net uptake to an efflux system acting to eject the arsenate ions from the cells as fast as they are accumulated. The complete absence of arsenate uptake was measured during the first few seconds of exposure (Fig. 5), whereas the experiments during which the resistant cells accumulated some (although reduced) arsenate occupied several minutes, and our measurements of efflux generally showed cellular residence times of a few minutes (data not shown). It seems plausible that both effects (reduced uptake and accelerated efflux) occur additively. This would then make the plasmid-determined arsenate resistance system similar to two extensively studied plasmid systems. Plasmid-mediated tetracycline resistance is the prototype of a resistance system involving efflux of the toxic substrate. The basic mechanism of tetracycline resistance is a block on net uptake and retention (6, 13). It is unclear how many membrane proteins are involved (13) or how they interact. Tetracycline resistance appears, however, to involve accelerated efflux (4, 13, 15). Another heavy metal resistance system existing on the same *S. aureus* plasmids (21, 22, 26) with which we have studied arsenate, arsenite, and antimony resistances is that to Cd^{2+} ions (34, 36). Again, there is a relationship with a "natural" ion transport system, since Cd^{2+} appears to be accumulated through the cellular Mn^{2+} transport system (36). As with the arsenate-phosphate system, Cd^{2+} resistance involves reduced uptake with accelerated efflux (Z. Tynecka, Z. Goś, and J. Zając, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K130, p. 159; J. Bacteriol., in press).

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