Interaction of Arsenate with Phosphate-Transport Systems in Wild-Type and Mutant *Streptococcus faecalis*

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ABSTRACT

HAROLD, F. M. (National Jewish Hospital, Denver, Colo.), AND J. R. BAARDA. Interaction of arsenate with phosphate-transport systems in wild-type and mutant *Streptococcus faecalis*. J. Bacteriol. 91:2257-2262. 1966.—Arsenate competitively inhibits the growth of *Streptococcus faecalis*, primarily by competition with phosphate for a common transport system. Arsenate is itself accumulated by the cells; the uptake requires metabolic energy, and the intracellular arsenate level may reach 0.01 M. Cells loaded with arsenate have lost the capacity to take up radioactive glutamate, rubidium, phosphate, or arsenate itself, apparently by the uncoupling of adenosine triphosphate generation. The pH dependence of arsenate uptake is complex. At low concentrations of extracellular arsenate, uptake by the wild-type strain 9790 exhibits a single maximum about pH 8; mutant PT-1, previously shown to be defective in phosphate uptake, takes up essentially no arsenate. At high concentrations of arsenate, uptake by the wild type is bimodal with maxima at pH 5.5 and 9; the uptake curve for mutant PT-1 corresponds to the shoulder in the curve for the wild type. The apparent dissociation constant for arsenate uptake by the wild type is approximately 10^{-5} M from pH 5 to 9, whereas that for mutant PT-1 is about 5×10^{-5} M at pH 5 and rises rapidly with increasing pH. The results confirm the earlier conclusion that the lesion in mutant PT-1 resides in the transport of phosphate and arsenate. It is proposed that the wild type has two distinct transport systems, whereas the mutant has lost the one with alkaline pH optimum.

A previous paper from this laboratory described the isolation and characterization of a mutant of *Streptococcus faecalis* defective in phosphate uptake (4). This mutant differed from the parent strain in that phosphate uptake, and growth, were progressively inhibited as the extracellular pH was raised above 6. The defect was independent of the nature of the energy source, and did not involve other factors known to influence phosphate uptake, such as glycolysis, K⁺ transport, and phosphate efflux. It was therefore proposed that the lesion resides in an access system which mediates the passage of phosphate across the cytoplasmic membrane.

Uptake of inorganic phosphate of *S. faecalis* required a source of metabolic energy, and the phosphate was immediately incorporated into metabolic intermediates. Consequently, it was not possible to distinguish unambiguously between a lesion in phosphate transport and one in

phosphate metabolism. In many biochemical reactions, arsenate has been found to serve as an analogue of phosphate, and uptake of arsenate by microorganisms is mediated by the system which also transports phosphate (2, 5, 9, 10). However, arsenate is not usually incorporated into stable cellular metabolites. The present study of arsenate uptake in *S. faecalis* was undertaken to further clarify the nature of the metabolic lesion in mutant PT-1, defective in phosphate uptake.

MATERIALS AND METHODS

Organisms and growth media. *S. faecalis* ATCC 9790 and the mutant defective in phosphate uptake (strain PT-1) were grown at 37 C on the defined medium described previously (4). This medium, designated M, is a modification of that devised by Toennies and Shockman (12). Sodium maleate, 0.15 M, served as a buffer, and the subscript indicates the concentration of phosphate in micrograms of phos-

phorus per milliliter. Growth was followed by turbidimetry at 600 m μ .

Measurement of radioactive phosphate or arsenate uptake. Bacteria from an overnight culture in M₅₀₀ medium were diluted with fresh medium, incubated at 37 C, and harvested during the exponential or early stationary phase. They were washed with salts-maleate buffer (containing maleate and the inorganic salts present in the growth medium, except for phosphate), and resuspended in the same buffer at a density of 0.3 to 0.6 mg/ml (dry weight). In some experiments, tris (hydroxymethyl) aminomethane (Tris)-chloride (0.15 M) was added to extend the pH range. All incubations were carried out at 37 C with glucose (10 mg/ml) as energy source; growth of the bacteria could not occur because of the omission of amino acids and nucleic acid precursors.

The cell suspensions were incubated for 5 to 10 min, and P³²-inorganic phosphate or As⁷⁴-arsenate (henceforth abbreviated P³² and As⁷⁴) of known specific activity was then added. At intervals thereafter, samples of 1 to 3 ml were rapidly filtered through 0.45- μ Millipore filters; these were washed three times with cold salts-maleate buffer, glued to planchets, and counted. The washing procedure did not bring about loss of radioactivity from the cells. Uptake is expressed as micromoles of inorganic phosphate or arsenate per gram (dry weight) of cells, calculated from the known specific activity of the extracellular ion. This procedure is warranted by the finding (4) that P³² uptake is almost entirely net uptake, and does not involve exchange between extracellular and intracellular pools; the present results suggest that the same is true for arsenate.

Radioactive arsenate was purchased from Abbott Laboratories, North Chicago, Ill., and its purity was checked by paper chromatography. Solvents used were picric acid-*t*-butanol-water (2:80:20, w/v/v) and isobutyric acid-water-25% ammonia-ethylenediaminetetraacetate (60:29:1:0.1, v/v/v/w) as described by Borst-Pauwels et al. (2), and also butanol saturated with either 20% acetic acid or 1.5 N NH₄OH (8). Solutions of As⁷⁴ were passed through a Millipore filter before use to remove any particulate radioactivity. The preparation of P³² solutions and the pretreatment of the filters have been described (4).

RESULTS

Inhibition of growth and phosphate uptake by arsenate. The addition of arsenate to *S. faecalis* 9790 growing exponentially resulted in competitive inhibition of growth. With extracellular inorganic phosphate between 10⁻⁴ and 10⁻² M, arsenate at one-half the phosphate level reduced the growth rate by approximately one-half, and the inhibition could be reversed by the addition of excess phosphate (Fig. 1). Incubation of the cells with arsenate, either in buffer or in various growth media, did not reduce the viable count.

The inhibition of growth is readily explained by the effect of arsenate on P³² uptake. Cells of strain 9790 were harvested and suspended in

buffer (pH 6) supplemented with glucose. After brief preincubation, samples of the suspension were pipetted into tubes containing various amounts of labeled inorganic phosphate and unlabeled arsenate; 3 min later, samples were filtered and counted, and the results were plotted according to the method of Lineweaver and Burk. As shown in Fig. 2, a series of straight lines were obtained which intercept the ordinate close together. The results suggest that the relationship between phosphate and arsenate is largely, but not entirely, a competitive one, presumably competition for the transport system. From the data presented in Fig. 2, the inhibition constant *K_i* was calculated to be 0.3 $\times$ 10⁻⁵ M. A similar pattern was obtained with mutant PT-1, but the *K_i* at pH 6 was far larger, 3 $\times$ 10⁻⁴ M. It should be stressed that this conclusion properly applies only to the initial rate of P³² uptake. Upon more prolonged incubation, both the rate and the total amount of phosphate uptake are reduced in the presence of arsenate. This phenomenon, which was also noted by Rothstein (10) in yeast, is presumably due to the uncoupling of adenosine triphosphate (ATP) generation as discussed below.

Uptake of As⁷⁴ by *S. faecalis* 9790. The finding that the phosphate-transport system has an affinity for arsenate comparable to, or even greater than, that for inorganic phosphate suggested that arsenate might itself be transported. The time course of As⁷⁴ uptake by strain 9790 is shown in Fig. 3. As had previously been found for phosphate, arsenate uptake was dependent upon the presence of an energy source. However, arsenate uptake ceased within a few minutes and the arsenate content then remained constant (for unknown reasons, uptake of As⁷⁴ from very low extracellular concentrations was transient). The total amount absorbed was less than one-tenth of the capacity for phosphate.

Arsenate taken up by the cells was tenaciously retained, even in the absence of an energy source. Upon prolonged incubation with phosphate and glucose, arsenate was slowly displaced (30 to 50% in 1 hr), but because of the profound effects of arsenate on energy metabolism this phenomenon was not further investigated.

Intracellular As⁷⁴ was completely (98%) removed by one extraction with cold 5% trichloroacetic acid (15 min at 0 C). The extract was shaken four times with ethyl ether saturated with water, neutralized, and subjected to two-dimensional paper chromatography (see Materials and Methods). Radioautography revealed but a single radioactive spot, whose location coincided with that of added carrier arsenate. It thus ap-

FIG. 1. Competitive inhibition of growth by arsenate. Cultures of *Streptococcus faecalis* 9790 were allowed to grow in M medium supplemented with 4×10^{-4} M or 4×10^{-3} M inorganic phosphate. When the exponential phase had been attained, various concentrations of arsenate were added. Two hours later, the inhibition of the first culture was reversed by addition of a 10-fold excess of phosphate.

pears that all the intracellular As^{74} is present as arsenate. With the assumption that the intracellular water space of *S. faecalis* 9790 is 2 g of water per g of dry weight (Zarlengo, *personal communication*) and that all the arsenate is present in free solution, the maximal intracellular arsenate concentration attained is of the order of 0.01 M.

Effect of pH on the uptake of As^{74} by strains 9790 and PT-1. Mutant PT-1 differs from the wild type, 9790, in that its capacity for phosphate uptake declines sharply at extracellular pH above 6. Since phosphate and arsenate appear to compete for a common transport system, the effect of pH upon the rate of As^{74} uptake was investigated.

At low concentrations of extracellular As^{74} , such as 10^{-5} M, uptake by the wild type exhibited a single maximum at pH 8; the mutant took up virtually no As^{74} at any pH (Fig. 4A). However, at the high concentration of 10^{-4} M As^{74} , the pH dependence of As^{74} uptake by the wild type became bimodal, with a maximum at about pH 9 and a shoulder at about pH 5.5. At this concentration, there was also considerable uptake by mutant PT-1 but only below pH 6, corresponding to the shoulder in the curve for strain 9790 (Fig. 4B). These results suggest that the wild type has two distinct but overlapping transport systems for arsenate, one of which has been deleted in the mutant.

Direct measurements of the apparent dissocia-

tion constant, K_m , for As^{74} uptake by the two strains are consistent with this interpretation. Cells were incubated with various concentrations of As^{74} in buffer for 2 min and then filtered; the results were analyzed by the Lineweaver-Burk method. Even though the rate of arsenate uptake begins to decline after 2 min, linear plots were obtained, and the pattern was similar to that previously described for phosphate uptake: in strain PT-1 the K_m was 5×10^{-5} M at pH 5 and rose to 5×10^{-4} M at pH 6; at pH 7 the slow rate of uptake was proportional to the concentration of As^{74} , and at pH 8 uptake was no longer detectable. By contrast, in strain 9790 the K_m ranged from 0.5 to 1×10^{-5} M at pH 5, 6, 7, and 8. At high concentrations of As^{74} , the points deviated from the linear Lineweaver-Burk plot, but the presence of a second and higher K_m in the curve for strain 9790 could not be unambiguously demonstrated.

Inhibition of ATP generation by arsenate. It was previously shown by Rothstein (10) that yeast cells loaded with arsenate have lost the capacity for phosphate uptake; the findings were attributed to inactivation of the phosphate-transport system by arsenate. Similar observations were made with *S. faecalis*. Cells of strain 9790 were harvested during the exponential phase, washed, and resuspended in salts-maleate buffer at pH 7. The suspension was divided; 2×10^{-4} M arsenate was added to one part, and both were supplemented

FIG. 2. Competitive inhibition of radioactive phosphate uptake by arsenate. Cells of *Streptococcus faecalis* 9790 were incubated briefly in salts-maleate buffer plus glucose at pH 6. Samples were pipetted into tubes containing various amounts of phosphate and arsenate, and the cells were filtered out 3 min later. The reciprocal of the rate of uptake is plotted as a function of the reciprocal of the phosphate concentration.

FIG. 3. Time course of As^{74} uptake. Cells of *Streptococcus faecalis* 9790 were incubated in salts-maleate buffer (pH 7) with various concentrations of As^{74} . Glucose was present in all except the last tube.

with glucose and incubated for 30 min. The cells were then recovered by filtration, washed, resuspended in fresh buffer in the absence of arsenate, and examined for their capacity to take up radioactive phosphate. As shown in Fig. 5A, phosphate uptake was severely inhibited. However, this inhibition was not restricted to phosphate uptake, as cells loaded with arsenate were equally deficient in the uptake of Rb^{86} and of

glutamate- $1-C^{14}$ (Fig. 5B and C). Since all three uptake processes are dependent upon glucose metabolism, it seems probable that inhibition by arsenate is due to interference with ATP generation. This is also a likely explanation for the rapid cessation of arsenate uptake itself (Fig. 3).

Other metabolic phenomena were also subject to inhibition by arsenate. *S. faecalis* subjected to phosphate starvation in M_0 medium was capable of limited nucleic acid synthesis at the expense of intracellular phosphate esters of low molecular weight; this conversion was inhibited by 10^{-4} M arsenate, presumably by interference with ATP production. It is noteworthy, however, that addition of arsenate (2×10^{-4} M) to exponential-phase cells under the conditions of the present experiments did not reduce the rate of conversion of glucose to lactic acid.

DISCUSSION

The essential conclusions drawn from the present study concern the interaction of arsenate with the transport system for phosphate. In agreement with earlier experiments with *Staphylococcus aureus* by Mitchell (9) and with yeast by Rothstein (10) and by Borst-Pauwels et al. (2), arsenate competes with phosphate for the transport system, and is itself accumulated by *S. faecalis*. Arsenate uptake is energy-dependent, and can occur against an apparent concentration gradient of 1:100 or more. Interpretation of the present experiments is simplified by the finding that all the intracellular arsenate could be recovered as such. No evidence was obtained for significant reduction to arsenite (2) or incorporation into cellular metabolites (6). With the reservation that transient formation of an unstable intermediate (11) has not been excluded, it follows that metabolic transformation of arsenate and of phosphate is not essential for transport. In other words, the transport system for phosphate and arsenate meets the essential criteria employed to define a permease (7).

Previous studies on phosphate uptake in *S. faecalis* 9790 and mutant PT-1 (4) revealed a striking difference in the pH dependence of uptake: whereas in 9790 the rate of uptake was relatively independent of pH, in PT-1 uptake was maximal at pH 6 and declined rapidly at higher pH, becoming negligible above pH 7. These and other findings were interpreted to mean that mutant PT-1 is defective in phosphate transport. However, because of the rapid incorporation of phosphate into metabolic pathways, an unambiguous distinction between transport and metabolism could not be made. Moreover, the results were consistent with either of two alternative hypotheses. (i) The parent strain, 9790, has a single transport system for phosphate, desig-

FIG. 4. Effect of pH on the uptake of As^{74} by *Streptococcus faecalis* strains 9790 and PT-1. Cells were suspended in Tris-maleate buffer of various pH, supplemented with glucose. After brief preincubation, As^{74} was added, and the cells were recovered by filtration 5 min later, washed, and counted. (A) 10^{-5} M As^{74} ; (B) 10^{-4} M As^{74} .

FIG. 5. Effect of preincubation with arsenate on subsequent uptake of metabolites. Cells of *Streptococcus faecalis* 9790 were incubated for 30 min in salts-maleate buffer (pH 7) with either glucose alone or glucose plus 2×10^{-4} M arsenate. The cells were then filtered, washed, resuspended in buffer, and incubated with and without glucose and the following metabolites: (A) 0.5×10^{-3} M P^{32} , (B) 0.5×10^{-3} M Rb^{86} , (C) 3×10^{-5} M glutamate-1- C^{14} .

nated α ; the mutation results in the formation of an altered system, designated β , which differs from the normal one with respect to its pH dependence. (ii) Both the α and the β system are present in the parent strain; the mutation results in the loss of the more efficient α system, exposing the pH-dependent β system.

The present results bear upon the resolution of both issues. Mutant PT-1 has a pH-dependent defect in arsenate uptake, entirely analogous to

the defect in phosphate uptake. Since the arsenate undergoes no metabolic transformation, this observation strongly supports the localization of the genetic lesion in the transport step. Moreover, studies on the pH dependence of arsenate uptake provided evidence for the presence in the wild type of two distinct transport systems for arsenate: the α system has an optimum at pH 8 or 9 (depending on the extracellular arsenate concentration) and a K_m for arsenate of the

order of 10^{-5} M over the entire range from pH 5 to 9. The species transported has not been defined. However, it will be noted that at high pH (e.g., pH 9 in Fig. 4) arsenate uptake is strongly stimulated by raising the extracellular As^{74} concentration; this suggests that the singly charged H_2AsO_4^- ion, whose concentration will become limiting at high pH, is the ion transported by the α system. The β system has an optimum at pH 5.5, and a K_m which rises rapidly with increasing pH. In mutant PT-1, the β transport system alone is present, the mutation consisting in the deletion of the α system for phosphate and arsenate transport. In this connection, it should be pointed out that the pH dependence of phosphate uptake is not bimodal. Two factors may obscure the participation of the β system in phosphate transport by the wild type. At pH 5, the affinities of the two systems for inorganic phosphate are nearly identical (4); moreover, the rate of phosphate uptake (unlike that of arsenate) is controlled by the intracellular concentration of phosphorylated compounds (4), and therefore the contributions of two independent transport systems need not be additive.

In addition to its interaction with the phosphate-transport system, arsenate has long been recognized as a competitive inhibitor of phosphate-dependent enzymes such as phosphorylases (3) and oxidative phosphorylation. In the present study, evidence was obtained suggesting that intracellular arsenate interferes profoundly with ATP generation. Arsenate is known to uncouple glyceraldehyde-3-phosphate dehydrogenase (13), a key enzyme in energy metabolism of *S. faecalis* 9790 which derives ATP entirely from the conversion of glucose to lactic acid (1). Indeed, in extracts, 0.01 M arsenate (the intracellular level attained during arsenate uptake) was more than sufficient to render this reaction independent of inorganic phosphate. It is therefore reasonable to attribute the rapid cessation of arsenate uptake, as well as the inhibition of other transport processes (Fig. 5), to the uncoupling of this reaction. The observation of Rothstein (10) that yeast cells pretreated with arsenate have lost the capacity for phosphate uptake may also be in part explained in this way, though it is clear that the situation in yeast is very complex (5).

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