Role of the Arginine Deiminase System in Protecting Oral Bacteria and an Enzymatic Basis for Acid Tolerance

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The arginine deiminase system was found to function in protecting bacterial cells against the damaging effects of acid environments. For example, as little as 2.9 mM arginine added to acidified suspensions of *Streptococcus sanguis* at a pH of 4.0 resulted in ammonia production and protection against killing. The arginine deiminase system was found to have unusual acid tolerance in a variety of lactic acid bacteria. For example, for *Streptococcus rattus* FA-1, the pH at which arginolysis was reduced to 10% of the maximum was between 2.1 and 2.6, or more than 1 full pH unit below the minimum for glycolysis (pH 3.7), and more than 2 units below the minimum for growth in complex medium (pH 4.7). The acid tolerance of the arginine deiminase system appeared to be primarily molecular and to depend on the tolerance of individual enzymes rather than on the membrane physiology of the bacteria; pH profiles for the activities of arginine deiminase, ornithine carbamoyltransferase, and carbamate kinase in permeabilized cells showed that the enzymes were active at pHs of 3.1 or somewhat lower. Overall, it appeared that ammonia could be produced from arginine at low pH values, even by cells with damaged membranes, and that the ammonia could then protect the cells against acid damage until the environmental pH value rose sufficiently to allow for the reestablishment of a difference in pH (ΔpH) across the cell membrane.

The arginine deiminase system occurs in a variety of bacteria, including mycoplasmas, halobacteria, *Pseudomonas* sp., *Bacillus* sp., and lactic acid bacteria (5). The system has three main enzymes: arginine deiminase (EC 3.5.3.6), ornithine carbamoyltransferase (EC 2.1.3.3), and carbamate kinase (EC 2.7.2.2). In addition, there are regulatory elements (5) and, at least in some bacteria, an inducible transport system (7) which may involve ornithine-arginine antiport across the cell membrane (6). ATP can be synthesized in the reaction catalyzed by carbamate kinase and can be used for the growth of some organisms. In fact, when arginine is present, halobacteria or *Pseudomonas aeruginosa*, ordinarily considered to be strict aerobes, can grow anaerobically with ATP derived from arginolysis (9, 23). For organisms such as *Streptococcus faecalis*, ATP production from arginolysis is sufficient to permit slow growth, but for the closely related *Streptococcus faecium*, arginolysis cannot support growth without some other source of ATP. Still, the arginine deiminase system is retained even by *S. faecium* and so must serve some function. Recently, we have suggested (16) that a major function of the system is in the acid-base physiology of the bacteria.

In the communities of bacteria in dental plaque, the arginine deiminase system appears to be involved in the return of the pH value to neutrality or somewhat above neutrality, after acidification caused by the catabolism of ingested sugars (11). The sources of arginine for plaque bacteria include the diet and saliva, which contains the so-called pH-rise peptide siian (H₂N-Gly-Gly-Lys-Arg-COÖH). Plaque bacteria with the arginine deiminase system include *Streptococcus sanguis*, *Streptococcus milleri*, *Streptococcus rattus*, and *Lactobacillus fermentum*. In addition, *S. faecalis* and *S. faecium* have been isolated from the oral flora of many people (8). The arginine deiminase system is regulated by catabolite repression and appears to be inducible by arginine (5, 7, 19, 21). It seems that the system may play important roles in oral ecology, possibly in protecting less acid-tolerant organisms, such as *S. sanguis*, from damage during the drop in the plaque pH to 4.0 or slightly below caused by glycolysis by more acid-tolerant bacteria, such as *Streptococcus mutans* and *Lactobacillus casei*. In this report we present data on the protective role of the arginine deiminase system and on the enzymatic basis for its relative acid tolerance.

**MATERIALS AND METHODS**

**Bacteria and growth media.** Table 1 describes the bacteria used in this study. Those without American Type Culture Collection (ATCC) (Rockville, Md.) or National Collection of Type Cultures (NCTC), Central Public Health Laboratory (London, England), designations were from our laboratory culture collection. They were grown routinely in tryptone-glucose-Marmite (TGM) broth (17) at 37°C in static culture, except for *L. fermentum*, which was grown in Rogosa SL broth (Difco Laboratories, Detroit, Mich.). The organisms were maintained over the long term in lyophiles or were maintained for regular use on agar medium, with weekly transfers.

**Acid tolerance assays.** For determinations of minimum pH values for growth, five tubes containing TGM or Rogosa broth were inoculated with a 10% volume of an overnight culture in the early stationary phase of growth. The cultures were then incubated at 37°C, and growth was assessed by determining the changes in the A₇₅₀. Changes in culture pHs were determined with a glass electrode. The pH at which each culture just entered the stationary phase was then estimated graphically.

For determinations of minimum pH values for glycolysis, cells were harvested from cultures in the late exponential phase of growth, washed once with a 5 mM MgCl₂ solution, and suspended to give dense suspensions with about 2 mg (dry weight) of cell per ml in 20 mM potassium phosphate buffer containing 1 mM MgCl₂. Glucose was added to the

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suspensions to give a final concentration of 55.6 mM, and the resulting drop in pH was determined with a glass electrode. The final pH was considered to be the minimum for glycolysis, because glucose was in excess and neutralization of the suspensions with base resulted in a new round of glycolysis.

For determinations of minimum pHs for arginolysis, derepressed cells were harvested from cultures in tryptone-Marmite medium with 0.25% glucose–1% arginine in the late exponential phase of growth, washed once with 5 mM MgCl₂ solution, and suspended to give suspensions with about 2 mg (dry weight) of cells per ml in 20 mM potassium phosphate buffer at the desired pH, 1 mM MgCl₂, and 1% (47.5 mM) arginine. The suspensions were incubated at 37°C, the pH was assessed with a glass electrode, and NH₃ production was monitored with an ammonia electrode as described previously (17).

Acid killing. Cells from cultures in the early stationary phase were harvested by centrifugation, washed, and suspended in sterile distilled water to give dense suspensions with about 10¹⁰ CFU/ml. Subsequent additions resulted in final suspensions with 21 mM potassium phosphate and 1 mM MgCl₂, after adjustment of the pH to the desired value with a concentrated HCl solution. Arginine was added to give the desired final concentrations. Samples were taken at intervals after final adjustment of the pH, diluted with 1% peptone broth (pH 7.0; Difco), and plated onto TGM agar by spreading the samples on the surface. The plates were incubated at 37°C for at least 48 h before final counts of the colonies were made.

Acid sensitivities of enzymes. For determination of the pH profiles for enzyme activities of the arginine deiminase system, cells were permeabilized by subjecting them to one freeze-thaw cycle in mercaptoethanol buffer (5 mM mercaptoethanol, 5 mM MgCl₂, and 50 mM potassium phosphate) and 0.025 ml of toluene per ml of cell suspension. Arginine deiminase activities of permeabilized cells were assayed in terms of production of citrulline from arginine, with chemical estimation of the citrulline by the method of Archibald (1). Ornithine carbamoyltransferase activities were assayed by assessing NH₃ production from citrulline (100 mM) added in arsenate buffer (500 mM) with the OCT kit from Sigma Chemical Co. (St. Louis, Mo.). Carbamate kinase was assayed in terms of NH₃ released from carbamyl phosphate, which was assessed with an NH₃ electrode.

Crystalline carbamate kinase and ornithine carbamoyl-transferase from S. faecalis were purchased from Sigma Chemical Co.

**Arginine permeability.** Permeabilities of cells to arginine at various pHs were assessed by means of the thick suspension or space technique (15). Cell pellets (wet weight, approximately 3.5 g) were mixed with equal volumes of solution containing 0.1 M arginine labeled uniformly with ¹³C (Amer sham International, Amersham, United Kingdom) at a specific activity of 990 μCi/mmol.

### RESULTS

**Protection against acid death.** Typical data on the protection afforded by arginine against acid killing are presented in Fig. 1 for S. rattus FA-1. At pH 3.0, cells in suspension were killed rapidly within the first hour after acidification, and 47.5 mM arginine only slightly protected the population. However, at pH 3.5 and 4.0, arginine totally protected the bacteria, at least over a 6-h period. In these experiments, 47.5 mM arginine was used initially for protection, to provide an excess of the amino acid. However, as shown by the data presented in Fig. 2, 4.8 or 2.9 mM arginine was fully as protective as the higher concentration. Figure 2 shows the results for S. sanguis NCTC 10904 at an initial pH of 4.0. In Fig. 1 and 2 only sample data from a large number of experiments with a variety of plaque bacteria and with P. aeruginosa are presented. The data indicate the similar protective effects of arginine against acid death. It appeared that arginolysis and ammonia production were required for the protective effect because arginine did not protect the arginine deiminase-negative bacterium S. mutans GS-5.

**Acid tolerance of growth, glycolysis, and arginolysis.** To obtain a comparative view of the acid tolerance of arginolysis by intact cells relative to that to other cell functions, we determined the pH values at which growth stopped in complex media, at which glycolysis stopped in suspensions of nongrowing cells given excess glucose, and at which the rate of arginolysis was reduced to 10% of its maximum value. In some respects, the values obtained for the different functions were not strictly comparable because acid tolerance was affected by changes in environmental conditions and especially by differences in the levels of potassium and magnesium (18). However, as shown by the data presented in Table 1, for most of the test organisms the differences in the acid tolerances of the tested functions were major. In general, arginolysis was more acid tolerant than was glycolysis.

### Table 1. Acid tolerance of growth, glycolysis, and arginolysis

<table>
<thead>
<tr>
<th>Organism</th>
<th>pH at which growth stopped in complex medium</th>
<th>Minimum pH for glycolyzing suspensions</th>
<th>pH at which arginolysis rate was reduced by 90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. rattus FA-1</td>
<td>4.65 ± 0.01 (4.3)</td>
<td>3.74 ± 0.01</td>
<td>2.1–2.6</td>
</tr>
<tr>
<td>L. fermentum ATCC 14932</td>
<td>5.00 ± 0.00 (4.4)</td>
<td>3.63 ± 0.06</td>
<td>2.2–2.7</td>
</tr>
<tr>
<td>S. faecium ATCC 9790</td>
<td>4.73 ± 0.02 (4.7)</td>
<td>3.70 ± 0.02</td>
<td>3.2–3.8</td>
</tr>
<tr>
<td>S. lactis ATCC 19435</td>
<td>5.68 ± 0.12 (4.2)</td>
<td>3.94 ± 0.10</td>
<td>3.1–3.7</td>
</tr>
<tr>
<td>S. sanguis Challis</td>
<td>5.15 ± 0.02 (4.8)</td>
<td>3.93 ± 0.01</td>
<td>3.6</td>
</tr>
<tr>
<td>S. milleri’ ATCC 9895</td>
<td>4.75 ± 0.12 (4.3)</td>
<td>4.35 ± 0.10</td>
<td>4.2–4.4</td>
</tr>
<tr>
<td>S. rattus BHT</td>
<td>4.96 ± 0.08 (4.3)</td>
<td>4.03 ± 0.01</td>
<td>4.1</td>
</tr>
<tr>
<td>S. sanguis ATCC 10556</td>
<td>5.14 ± 0.08 (4.7)</td>
<td>4.40 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>S. sanguis NCTC 10904</td>
<td>5.04 ± 0.01 (4.8)</td>
<td>4.11 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>S. mutans GS-5</td>
<td>4.73 ± 0.02 (4.2)</td>
<td>3.67 ± 0.03</td>
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</tr>
</tbody>
</table>

*a Values in parentheses are minimum pHs in culture after extended incubation.

*b pHs were estimated from plots of the arginolytic rate versus pH. In each case, the lower number was determined experimentally, while the higher number was obtained by extrapolation. When only one number is shown, the pH for 90% inhibition was estimated from only experimental values without extrapolation.

*c Organism was grown in Rogosa medium with 5% glucose.
colysis, which was more acid tolerant than was growth in complex medium. Typically, there was nearly a full pH unit difference in acid tolerance between these functions. For example, the pH at which growth of *S. rattus* FA-1 stopped in TGM broth at 37°C was 4.65 ± 0.01. (The standard deviation refers to values for the five replicate cultures used for each organism.) For most of the organisms tested, the pH continued to fall after growth had stopped, and for *S. rattus* FA-1 the final culture pH was 4.3. The minimum pH value for glycolysis by cells in suspension with 20 mM potassium phosphate buffer–1 mM MgCl₂ was 3.74 ± 0.01. This value was lower than the final pH of the cultures, even though glucose was present in excess in both situations. The pH at which arginolysis by cells in suspension with 20 mM potassium phosphate buffer–1 mM MgCl₂ was reduced to 10% of its maximum value was significantly lower (between 2.1 and 2.6). Plots of the data for *S. rattus* FA-1 used to obtain the values presented in Table 1 are presented in Fig. 3. The variance in estimation of the pH for 90% reduction in arginolysis was related to the extremes of the possible extrapolations to zero activity shown in Fig. 3C. The zero value at pH 2.1 was assessed experimentally; that at pH 2.6 was obtained by extrapolation. Similar plots were used to obtain the data presented for the other organisms.

There was a clear hierarchy for acid tolerance among the test organisms. In establishing this hierarchy, it was important that the same experimental conditions were used for all organisms. The only exception was *L. fermentum*, which did not grow well in TGM medium.

For *S. rattus* FA-1, the minimum pH value for glycolyzing suspensions was some 0.9 units below that at which growth stopped in complex medium. The pH values at which arginolysis was 90% inhibited were some 1.1 to 1.6 units lower than that in the glycolytic minimum. Similar differences were found for *S. faecium* ATCC 9790, *S. sanguis* Challis, *S. lactis* ATCC 19435, *S. milleri* ATCC 9895, and *S. sanguis* ATCC 10556. Growth of *L. fermentum* was relatively sensitive to acidification, at least in Rogosa medium, but glycolysis and arginolysis were both relatively acid tolerant. Arginolysis by *S. rattus* BHT or *S. sanguis* NCTC 10904 was relatively acid sensitive.

**Acid sensitivities of the enzymes of the arginine deiminase system.** The enzymes of the arginine deiminase system appeared to have inherent acid tolerances, as indicated by the data presented in Fig. 4 and Table 2. The tolerance of the enzymes was assessed with permeabilized cells in an effort to maintain the enzymes under conditions as close to those of the normal cytoplasm as possible. The enzymes were assayed for activity in the direction of catabolism and ATP synthesis. The assay conditions were such that the substrates were saturating. The data in Fig. 4 indicate the activities of arginine deiminase, ornithine carbamoyltransferase, and carbamate kinase of *S. rattus* BHT over a range of pHs, mainly on the acid side of neutrality. Table 2 presents pH values for 90% inhibition of the enzymes of *S. rattus* BHT, *S. rattus* FA-1, *S. sanguis* ATCC 10904, and *L. fermentum* ATCC 14932. Carbamate kinase was highly resistant to acid damage. Arginine deiminase and ornithine carbamoyltransferase were both more acid sensitive but were still active at relatively low pHs (about 3.0). Thus, the acid resistance of the system, even for the most sensitive organisms, does not appear to depend on the maintenance of a pH gradient across the cell membrane, as does the acid resistance of the glycolytic system (3).

The acid tolerances of commercially available carbamate kinase and ornithine carbamoyltransferase isolated from *S. faecalis* were also assessed, and as shown by the data presented in Fig. 5 and 6, the isolated enzymes were

**FIG. 1.** Acid killing of *S. rattus* FA-1. Cells were suspended in 21 mM potassium phosphate buffer with 1 mM MgCl₂ and 0.0 mM arginine (open symbols) or 47.5 mM arginine (closed symbols). Then, the pHs of the suspensions were adjusted with HCl solution, as indicated, and samples were taken at the indicated times after pH adjustment for dilution and plating.

**FIG. 2.** Acid killing of *S. sanguis* NCTC 10904. The experimental conditions were the same as those described in the legend to Fig. 1, except that all suspensions were at pH 4.0; and 0.0 mM (Δ), 2.9 mM (○), or 4.8 or 47.5 mM (□) arginine was added to the suspensions. At each sampling time, except for 2 h, essentially the same counts were obtained for the three suspensions to which arginine was added.
somewhat more sensitive to acid than were the enzymes in permeabilized cells. Data are also presented showing that carbamyl phosphate was stable under the conditions of the assay. Even with the isolated enzymes, the pH values for activity extended far into the acid range. Carbamate kinase was acid tolerant without additions, but ornithine carbamoyltransferase was tolerant only in the presence of added protein, such as bovine serum albumin, or increased levels of the enzyme (Fig. 6).

Permeability to arginine. In a previous study (3) we found that cells of oral streptococci subjected to extreme acid conditions lost intracellular solutes, including Mg. This loss is indicative of the loss of the permeability function of the membrane, and the expectation is that cells under extreme acid conditions should be freely permeable to arginine. That cells of S. sanguis NCTC 10904 were permeable to arginine labeled with \(^{14}C\) at pHs 2.0, 3.0, and 4.0 was indicated by average space values of 4.6, 4.0, and 5.2, respectively, at the indicated pHs. Because the cells were between 70 and 80% water, corrected space or R values of about 0.7 or higher indicate that arginine had access to the cytoplasmic water of the cell (15). In fact, arginine appeared to be concentrated by the cells, possibly because of its positive charge.

DISCUSSION

Results of recent studies of the arginine deiminase system of bacteria have suggested that it serves a number of functions. First, the system can be a major or sole provider of ATP for the growth of a variety of organisms in a variety of situations. For example, arginine may be the main catabolite for nonfermenting bacteria such as Mycoplasma hominis (22), in which the system may exist in free cytoplasmic or membrane-bound forms (13). Arginolysis may supply ATP for the growth of fermentative streptococci such as S. faecalis when sugars are not available. For P. aeruginosa and Halobacterium organisms, arginine catabolism allows for anaerobic growth of otherwise strict aerobes. Second, in some organisms, the system may play biosynthetic roles. For example, the reaction catalyzed by carbamate kinase can work in the noncatabolic direction, especially at high pH values, to supply carbamyl phosphate for synthesis of citrulline or pyrimidines (20); however, most organisms have separate anabolic and catabolic enzymes. Carbamyl phosphate can also serve as a source of phosphate for the synthesis of glucose-1-phosphate in the pathway to glycogen (12).

The system is under tight regulatory control both from catabolite repression and from an induction circuit in most, but not all, organisms. In P. aeruginosa, the genes for the enzymes of the system are contiguous (arcA, arcB and arcC), presumably in an operon (14). In many organisms, the system includes a catabolism-specific transport system (6, 7, 19), and in S. mitis production of aminopeptidase I has been found (10) to be coordinately repressed and derepressed with the arginine deiminase system.

Results of our previous study (16) and those reported in here indicate that the arginine deiminase system can also function to protect bacteria against damage caused by acid environments. This protection depends on the pH rise associated with ammonia production and on the capacities of the bacteria to carry out arginolysis at low pH. The acid tolerance of the arginine deiminase system appears to be largely inherent and related to the molecular properties of the enzymes of the system. In this study we used permeabilized cells so that the enzymes being tested would at least be retained with the cell wall, possibly in association with other polymeric, cytoplasmic constituents. The enzymes, especially ornithine carbamoyltransferase, may have been protected against acid denaturation by association with other cytoplasmic elements but did not have the protection afforded by a working cell membrane. Cells subjected to potentially lethal acidification are probably similar to permeabilized cells, and we found previously that acidification

<table>
<thead>
<tr>
<th>Organism</th>
<th>pH for 90% inhibition of:</th>
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<tr>
<td></td>
<td>Arginolysis</td>
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<tr>
<td>L. fermentum</td>
<td>2.1</td>
</tr>
<tr>
<td>ATCC 14932</td>
<td></td>
</tr>
<tr>
<td>S. rattus FA-1</td>
<td>2.1</td>
</tr>
<tr>
<td>S. rattus BHT</td>
<td>3.6</td>
</tr>
<tr>
<td>S. sanguis NCTC</td>
<td>4.1</td>
</tr>
<tr>
<td>10904</td>
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can damage cell membranes sufficiently to allow for the loss of Mg from the cytoplasm. It seems, then, that the capacities of cells to carry out arginolysis at very low pHs, below the minimum for glycolysis, depend mainly on the inherent acid stabilities of the arginolytic enzymes rather than on the abilities of the cells to maintain a pH difference across the cell membrane in acidified environments. For some of the organisms tested, specifically the *S. sanguis* strains, the acid tolerance of the deiminase system in permeabilized cells appeared to be somewhat less than that of the individual enzymes of the system, which were assayed separately. Presumably, the differences had to do mainly with those of the assay conditions used. The arginolytic system is, therefore, very different from the glycolytic system, which contains enzymes such as enolase that are inhibited by pHs only slightly below 7.0 (4). Thus, the capacities of cells to carry out glycolysis at low pHs depends very much on the functioning of the cell membrane to develop a difference in pH (ΔpH) across the membrane and to maintain the cytoplasmic pH at a relatively alkaline level.

Although the capacities of cells to degrade arginine at pHs as low as 2.1 appeared to depend on the molecular stabilities of enzymes and not on the ΔpH, after the organisms produced sufficient ammonia to raise the local pH, the ΔpH could presumably be reestablished through the actions of proton-translocating ATPases. Once the selective barriers of the cell membrane became reestablished, the ammonium ion tended to be retained within the cell. Thus, each ammonia produced from arginine combined with a proton to yield an ammonium ion; this combination initially took place in the cytoplasm. Overall, it seems that the arginine deiminase system is well designed for a protective role, in that the inherent acid tolerance of the enzymes allows for ammonia production at pHs below those at which the cell membrane can work effectively, and the rise in pH associated with ammonia production should then allow the membrane to become functional again.

The organisms used in this investigation were mainly dental plaque bacteria, and so they came, at least originally,
from an environment where cycles of acidification and pH rise are thought to have major effects on the flora. The pH in plaque in vivo drops somewhat below 4.0 after sugar ingestion. Presumably, this low value reflects the minimum pH at which the more acid-tolerant members of the community, such as \textit{S. mutans} and \textit{S. sobrinus}, can carry out sugar degradation with acid production. The minimum value is similar to the lowest of the minimum values reported in Table 1 for glycolyzing suspensions. \textit{L. casei} from plaque can carry out glycolysis at pHs as low as about 3.5, but generally, it makes up only a small part of the plaque flora. The acid tolerances of plaque bacteria appear to be related primarily to proton-translocating, membrane ATPases, specifically to the amounts of the enzymes per cell and their pH optima for activity (2, 3). The arginine deiminase system therefore appears to be a secondary safety device. The less acid-tolerant organisms of plaque have this system. Organisms such as \textit{L. casei} and \textit{S. mutans} presumably do not need the system because they can function at the lower pH values in plaque. However, organisms such as \textit{S. sanguis} not only cannot function at these low pH values but they also are damaged by the acid conditions. Ammonia production from arginine or from peptides such as sialin in plaque may spare these sensitive organisms from irreversible damage. It seems that there must be still other ways to survive acidification. Thus, \textit{Actinomyces viscosus} is not very acid tolerant and also does not have the arginine deiminase system. Additional studies are required to define fully the peculiarities of acid-base physiology underlying the complex ecology of dental plaque and to distinguish population phenomena from single-organism phenomena.

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**LITERATURE CITED**


