Interconversion of Purine Mononucleotides in *Pasteurella pestis*

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Included among the five established determinants of virulence in *Pasteurella pestis* are the abilities of cells to accomplish the de novo biosynthesis of purines and to grow as dark pigmented (P+) colonies on a solid synthetic medium containing hemin. P+ isolates of *P. pestis* strain KIM-10 (mouse intraperitoneal LD50 < 10 cells) failed to convert exogenous guanine-8-14C to adenine residues of ribonucleic acid (RNA) when cultivated in a minimal medium which favored the pigmentation reaction. This conversion occurred in P+ cells grown in an enriched medium which did not support the pigmentation reaction and was observed in P- mutants cultivated in both types of media. Both P+ and P- isolates converted exogenous adenine-8-14C but not adenine-2-14C at a significant rate to guanosine residues of RNA when grown under a variety of conditions. This difference appeared to reflect a deficiency of adenine deaminase. The mouse intraperitoneal LD50 of purine-auxotrophs was about 10^5 cells when the metabolic block occurred prior to the de novo formation of inosine monophosphate (IMP). In contrast, the corresponding value for a mutant blocked between IMP and guanine monophosphatase was > 10^6 cells in mice and > 10^8 cells in guinea pigs.

Purine-dependent mutants of highly pathogenic bacteria such as *Pasteurella pestis*, *Bacillus anthracis*, *Klebsiella pneumoniae*, and certain species of *Salmonella* often exhibit dramatic decreases in virulence (9, 15, 16, 20). In the case of *B. anthracis*, auxotrophs blocked between inosine monophosphate (IMP) and adenosine monophosphate (AMP) were avirulent, whereas mutants blocked in the de novo synthesis of IMP or those unable to convert IMP to guanosine monophosphate (GMP) remained fully virulent (20, 21). However, guanine-auxotrophs of some of the gram-negative bacteria were of reduced virulence. The nature of the mutations promoting avirulence in *P. pestis* has not yet been clarified. One avirulent purine-auxotroph of this species (strain M1) exhibited an unusual requirement for hypoxanthine, xanthine, or guanine; adenine failed to support growth, suggesting that cells of strain M1 were unable to convert adenine to IMP. A second purine-auxotroph (strain T3) was able to grow on adenine but not hypoxanthine, xanthine, or guanine and thus resembled the avirulent mutants of *B. anthracis*. Unlike strain M1, strain T3 was isolated from an avirulent prototroph; thus no relationship between adenine dependence and avirulence in *P. pestis* could be established (9).

Cells of eight strains of *P. pestis* studied by Maysky (27) were unable to convert exogenous adenine-8-14C to guanine residues of nucleic acids. This observation indicates that the organisms were unable to convert adenine directly to IMP via hypoxanthine or indirectly via aminoimidazole-carboxamide ribotide (AICAR). An outline, modified from Magasanik (25), of pathways involving the de novo synthesis and interconversion of purine nucleotides as reported for enteric bacteria is shown in Fig. 1. However, a block in the latter pathway would necessarily prevent the biosynthesis of histidine (25), an amino acid which is not normally required by wild-type *P. pestis* (11, 18). All strains of *P. pestis* could convert guanine-8-14C to adenine residues of nucleic acids (27).

One purpose of this study was to resolve the evident discrepancy between the interconversion of purine ribotides and the nutritional requirements of *P. pestis*. A second objective was to determine which of the relevant enzymes are necessary for the expression of virulence.

**MATERIALS AND METHODS**

**Bacteria.** Mutants obtained from wild-type *P. pestis* strain KIM-10 were used in most experiments.
The prototroph is a typical virulent strain of the variety mediaevalis of Devignat (14).

**Media.** The composition of the minimal medium used for the selection and characterization of purine auxotrophs is shown in Table 1. An enriched medium, containing 1% yeast extract (Difco) and 2% Bacto Casitone (Difco) in place of the nitrogenous components of the minimal medium, was used for the isolation and routine cultivation of auxotrophs. The complex synthetic medium of Higuchi, Kupferberg, and Smith (19), modified as shown in Table 2, and the minimal medium described previously were used to prepare cells containing radioactive ribonucleic acid (RNA).

**Fig. 1. Schema illustrating the enzymatic pathways (broken lines) or individual reactions (solid lines) of the de novo biosynthesis and interconversions of purine nucleotides as reported for enteric bacteria. Abbreviations are defined in the text; numerals refer to auxotrophic strains possessing corresponding metabolic blocks.**

**Table 1. Composition of minimal synthetic medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc (mm)</th>
<th>Component</th>
<th>Conc (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt solutions&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>Amino acids&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>25</td>
<td>L-Isoleucine</td>
<td>0.5</td>
</tr>
<tr>
<td>Citric acid</td>
<td>10</td>
<td>L-Methionine</td>
<td>0.5</td>
</tr>
<tr>
<td>Ammonium lactate</td>
<td>10</td>
<td>L-Phenylalanine</td>
<td>0.5</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2.5</td>
<td>L-Valine</td>
<td>0.5</td>
</tr>
<tr>
<td>FeCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.1</td>
<td>Glycine</td>
<td>10</td>
</tr>
<tr>
<td>MnCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.01</td>
<td>L-Threonine</td>
<td>0.5</td>
</tr>
<tr>
<td>Miscellaneous solutions</td>
<td></td>
<td>L-Tyrosine</td>
<td>0.5</td>
</tr>
<tr>
<td>d-Glucose&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10</td>
<td>L-Arginine</td>
<td>0.5</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.5</td>
<td>L-Aspartic acid</td>
<td>10</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Neutralized with 5 N NaOH.

<sup>b</sup> Prepared as X10 stock; sterilized by autoclaving.

<sup>c</sup> Prepared as X100 stock; sterilized by filtration.

<sup>d</sup> Prepared as X100 stock; sterilized by autoclaving.

<sup>e</sup> Sterilized by autoclaving.

**Isolation of purine auxotrophs.** Mutations were induced in *P. pestis* by irradiation with ultraviolet light (UV), and auxotrophs were selected by a modification of the penicillin technique (24); all operations were performed at 26 C. After incubation on slopes of enriched medium for 24 hr, the cells were removed and diluted in sterile 0.033 M potassium phosphate buffer, pH 7.2 (phosphate buffer), to yield a concentration of 5 × 10<sup>7</sup> viable organisms per ml. About 6 ml of this suspension received sufficient irradiation with UV to reduce viability by 50%. To 5 ml of liquid enriched medium (prepared at double strength) in a screw-cap tube (20 by 125 mm) was added 5 ml of irradiated cells. The tube was then slanted and, after incubation for 24 hr, the cells were collected by centrifugation at 10,000 × g for 15 min, washed twice with phosphate buffer, and transferred in 5 ml of phosphate buffer to a sterile 50-ml Erlenmeyer flask fitted with a cotton stopper. The flask was aerated on a shaker for 2 hr, and the organisms were centrifuged and resuspended in phosphate buffer at a concentration of 5 × 10<sup>8</sup> cells per ml. To 4.5 ml of minimal medium in a screw-cap tube (20 by 125 mm) was added 0.5 ml of cell suspension. The tube was slanted and, after incubation for 8 hr, 0.05 ml of a fresh sterile 0.7% solution of potassium penicillin G (E. R. Squibb and Sons, New York, N.Y.) was added aseptically to yield a final concentration of approximately 100 units per ml. After further incubation for 2 to 3 days, samples of 0.1 ml were plated directly onto the surface of solid enriched medium. Colonies arising after incubation for 2 days were replicated onto solid minimal medium. The latter step, however, was not essential, because about 50% of the surviving cells were auxotrophs and the majority of these exhibited a nutritional requirement for hypoxanthine.

**Characterization of purine auxotrophs.** The nutritional response to purines was determined by layering 5 ml of minimal agar, inoculated with 10<sup>6</sup> cells per ml, onto plates of solid minimal medium. After overnight incubation at 26 C, drops of 0.001 M or saturated solu-
tions of purines or their derivatives were spotted on the agar surface; zones of growth were recorded after further incubation for 3 to 4 days.

The resulting response facilitated the identification by enzymatic assay of blocks in the de novo pathway of purine biosynthesis. Mutant cells were incubated overnight at 26°C on a model R25 shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) in 100 ml of liquid enriched medium per 1-liter flask, washed twice in phosphate buffer, and then aerated under the same cultural conditions for 24 hr in liquid minimal medium. The organisms were then washed in phosphate buffer, suspended at a concentration of about 10^11 cells per ml of cold 0.1 M tris(hydroxymethyl)-ammoniummethane-hydrochloride (pH 7.4) plus 0.001 M mercaptoethanol, and disrupted by treatment for 1 min with an ultrasonic probe (Instrumentation Associates, New York, N.Y.). After removal of cellular debris by centrifugation, quantitative determinations of enzymes were performed by methods outlined by McElroy (28) and Magasanik (26); qualitative procedures are described under Results. In some cases the supernatant fluids from the cultures were assayed by the Braun-Marshall test (15).

Virulence determinants. The ability of cells to absorb hemin and thus grow as dark or pigmented (P^+^) colonies was determined with the hemin agar of Jackson and Burrows (22). The modified magnesium oxalate agar described previously (4) was used as an assay for calcium dependence. The expression of this property closely correlates with the ability to produce the virulence or V and W antigens (VW^+^) (7, 8). Remaining determinants of virulence and the LD_50_ were assayed by methods described previously (2, 7).

Known avirulent P^- and VW^- mutants were isolated for use in experiments requiring large numbers of cells. The interrelationship between these isolates is shown in Fig. 2.

### Purine nucleotide interconversions

A modification of the Schmidt-Thannhauser technique (30) was used to isolate mononucleotides from cells cultivated with radioactive purines. To 50 ml of liquid minimal or complex synthetic medium per 500-ml flask was added 5 X 10^-4 M radioactive purine (specific activity of 0.13 μC/μ mole). After inoculation with 10^7 cells per ml, the cultures were aerated at 26°C for 24 to 36 hr. The cells were harvested by centrifugation, washed twice with phosphate buffer, and extracted for 90 min with 10 ml of cold 5% HClO_4_. The resulting precipitate was again centrifuged and washed with 10 ml of cold 5% HClO_4_ prior to extraction in a closed vessel for 15 min at 50°C with 10 ml of ether:ethanol (1:1, v/v). The suspension was centrifuged and the precipitate was dissolved in 10 ml of 0.5 M KOH; after hydrolysis for 14 hr at 37°C, the solution was chilled to 0°C and incubated for 1 hr with 10 ml of added cold 20% HClO_4_. The resulting precipitate was removed by centrifugation and re-extracted with 1 ml of cold 20% HClO_4_, and the combined supernatant fluids were neutralized with 5 N KOH. After KClO_4_ was removed by centrifugation, the sample was brought to dryness on a model 3-2100 flash evaporator (Buchler Instruments, Inc., Ft. Lee, N.J.), reconstituted with distilled water to 10 ml and centrifuged, and the supernatant fraction was again brought to dryness. The sample was dissolved in 5 ml of distilled water and refrigerated for 1 hr to permit precipitation of remaining KClO_4_. The clear solution was then decanted, dried, and dissolved in 2 ml of distilled water.

Samples of 20 to 50 μl were subjected to electrophoretic analysis on Whatman no. 1 paper in 0.05 M ammonium acetate buffer (pH 3.5) at 65 v per cm for

### Table 2. Composition of complex synthetic medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt solution^b</td>
<td></td>
</tr>
<tr>
<td>K_2HPO_4</td>
<td>25</td>
</tr>
<tr>
<td>Citric acid</td>
<td>10</td>
</tr>
<tr>
<td>Potassium gluconate</td>
<td>10</td>
</tr>
<tr>
<td>Ammonium acetate</td>
<td>10</td>
</tr>
<tr>
<td>MgCl_2</td>
<td>2.5</td>
</tr>
<tr>
<td>FeCl_3</td>
<td>0.1</td>
</tr>
<tr>
<td>MnCl_2</td>
<td>0.01</td>
</tr>
<tr>
<td>Vitamin solution^c</td>
<td></td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.01</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>0.01</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.005</td>
</tr>
<tr>
<td>Miscellaneous solutions</td>
<td></td>
</tr>
<tr>
<td>D-Xylose</td>
<td>61</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0.1</td>
</tr>
</tbody>
</table>

^a Neutralized with 5 N NaOH.
^b Prepared as ×10 stock; sterilized by autoclaving.
^c Prepared as ×10 stock; sterilized by filtration.
^d Prepared as ×100 stock; sterilized by filtration.
^e Sterilized by autoclaving.

<table>
<thead>
<tr>
<th>Component</th>
<th>Conc (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids^e</td>
<td></td>
</tr>
<tr>
<td>DL-Alanine</td>
<td>9.0</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>7.6</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>4.0</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>3.2</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>4.8</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>2.8</td>
</tr>
<tr>
<td>L-Valine</td>
<td>13.6</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>2.0</td>
</tr>
<tr>
<td>L-Proline</td>
<td>14.0</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>162</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>2.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.2</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>2.2</td>
</tr>
</tbody>
</table>
RESULTS

By use of the penicillin technique, 100 auxotrophs of strain KIM-10 (VW+, P+) were isolated which grew in the presence of hypoxanthine. These mutants were separated into three classes on the basis of their nutritional response to other purines and purine ribosides (Table 3). A representative isolate of each class was then preserved for determinations of virulence; VW- and P- mutants of these isolates were also selected (Fig. 2).

The first class of mutants, exemplified by isolate 46 (VW+, P+), exhibited a dual nutritional requirement for thiamine plus hypoxanthine, adenine, or aminimidazole carboxamide (AICA). Isolates of this phenotype were assumed to be blocked prior to the formation of aminimidazole ribotide (AIR), the last common intermediate of divergent pathways leading to IMP and the pyrimidine moiety of thiamine (29).

The nutritional response of mutant 61 (VW+, P+), an example of the second class of purine auxotrophs, was similar to that of isolate 46 (VW+, P+) except that a nutritional requirement for thiamine was not detected. The fact that cells of mutant 61 (VW+, P+) were thiamine-independent and able to grow in the presence of AICA suggested that the metabolic block was located before the biosynthesis of AICAR but after the formation of AIR. Subsequent study showed that cells of strain 61 (VW-, P+) excreted a compound yielding an orange color in the Bratton-Marshall test (εmax = 500 nm) which closely matched the spectrum of aminimidazole riboside. This accumulation is consistent with the absence of phosphoribosyl-aminimidazole carboxylase (EC 4.1.1.21), although it could also result from the loss of phosphoribosyl-aminimidazole-succinocarboxamide synthetase (EC 6.3.2.6), because the substrate of the latter spontaneously decarboxylates to AIR (30).

Mutants of the third class, such as isolate 45 (VW+ , P+) expressed a specific requirement for hypoxanthine (or inosine). Single-step mutants of this phenotype cannot be isolated from species of bacteria which possess the direct (deaminating) and indirect (histidine cycle) pathways leading from adenine to IMP (24). The fact that cells of strain 45 (VW+, P+) did not exhibit a nutritional requirement for histidine indicated that this mutant was capable of forming AICAR during the biosynthesis of imidazole-glycerol phosphate, a precursor of histidine (25). It seemed probable, therefore, that the induced metabolic block in strain 45 (VW+, P+) was located after the biosynthesis of AICAR but before the formation of...

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![Diagram](http://iai.asm.org/)  
**Fig. 2. Schema illustrating the origin of substrains used in this investigation.**  
A, induction by ultraviolet light and selection with penicillin; B, selection on magnesiu...
IMP. An excretion product from cells of isolate 45 (VW−, P+) was noted which yielded a purple color in the Bratton-Marshall test; the spectrum of this derivative closely matched that of AICA riboside (εmax = 540 nm). Attempts to demonstrate the conversion of IMP to AICAR in cell-free extracts of isolate 45 (VW−, P+) containing added tetrahydrofolic acid were not successful, whereas AICAR was demonstrated qualitatively in similar experiments utilizing strain KIM-10 (VW−, P+). These findings indicate that isolate 45 (VW+, P+) lacks aminoimidazole carboxamidribotide formyltransferase (EC 2.1.2.3).

Further study showed that adenine-8-14C was efficiently converted to GMP by strains KIM-10 (VW+, P−) and KIM-10 (VW−, P+) in minimal and complex synthetic media (Table 4). In contrast, results obtained in identical experiments with adenine-2-14C varied from essentially no conversion to values approaching 15% of those obtained with adenine-8-14C. This finding suggested that the primary mechanism for the conversion of adenine to GMP in the prototroph was via the histidine cycle rather than by deamination. Subsequent attempts to detect adenine deaminase (EC 3.5.4.2) in five derivatives of strain KIM-10 were not successful.

Attention was next directed towards determining why mutants blocked in the de novo synthesis of IMP were unable to grow in the presence of exogenous guanine, guanosine, or xanthine. In preliminary experiments, a large number of apparent revertants of strain 45 (VW−, P+) were observed to form colonies on solid minimal medium supplemented with guanine. The fact that these organisms failed to grow in the absence of guanine indicated that they had not recovered the ability to convert AICAR to IMP but rather had acquired the ability to convert guanine to IMP. Further study showed that all of 50 suppressor mutants of this type obtained from strain 45 (VW−, P+) had undergone the mutation from P+ to P−. A similar relationship between expression of the P− phenotype and ability to grow in the presence of guanine was also noted in the case of strains 46 (VW−, P−) and 61 (VW−, P−). In addition, all of 25 P− clones obtained from strain 45 (VW−, P+), after selection on hemin agar plus hypoxanthine, were able to grow in the presence of guanine.

Addition of 0.01% hemin to the minimal agar described in Table 1 resulted in a medium which supported an intense pigmentation reaction similar to that of the hemin agar of Jackson and Burrows (22). In contrast, the pigmentation reaction was not expressed by cells of the P+ phenotype on complex synthetic medium (Table 2) supplemented with the same concentration of hemin. It was of interest, therefore, to determine whether P− cells blocked in the de novo synthesis of IMP could grow in complex synthetic medium supplemented with guanine. P− cells of strain 45 grew almost as rapidly on complex synthetic medium containing added 0.0005 M guanine as did cells of the P− phenotype (Table 5).

An additional attempt was made to characterize the metabolic block between exogenous guanine and IMP in P+ cells grown in minimal medium. Ribotides were isolated from cells of strains KIM-10 (VW−, P+) and KIM-10 (VW+, P+) after cultivation in minimal and complex synthetic media containing guanine-8-14C. The specific activity obtained in the recovery of GMP was essentially identical to that of the exogenous guanine regardless of the medium or the strain employed (Table 6). In contrast, no radioactivity was detected in AMP recovered from P+ cells after growth in the minimal medium although both P+

### Table 4. Conversion of adenine-8-14C to adenine residues (isolated as adenosine monophosphate) and guanine residues (isolated as guanosine monophosphate) of ribonucleic acid by growing cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Specific activity of isolated mononucleotides</th>
<th>AMP</th>
<th>GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIM-10 (VW+, P−)</td>
<td>Minimal synthetic</td>
<td>0.14</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>KIM-10 (VW−, P+)</td>
<td>Minimal synthetic</td>
<td>0.12</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>KIM-10 (VW+, P−)</td>
<td>Complex synthetic</td>
<td>0.11</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>KIM-10 (VW+, P−)</td>
<td>Complex synthetic</td>
<td>0.10</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

a Specific activity of exogenous adenine-8-14C was 0.13 μC/μm.

### Table 5. Doubling time of P+ and P− cells of strain 45 in minimal and complex synthetic medium supplemented with purine

<table>
<thead>
<tr>
<th>Strain</th>
<th>Added purine (0.0005 M)</th>
<th>Doubling time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Minimal synthetic medium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45 (VW+, P+)</td>
<td>None</td>
<td>&gt;24 hr</td>
</tr>
<tr>
<td>45 (VW+, P+)</td>
<td>Hypoxanthine</td>
<td>4 hr 10 min</td>
</tr>
<tr>
<td>45 (VW−, P+)</td>
<td>Guanine</td>
<td>&gt;24 hr</td>
</tr>
<tr>
<td>45 (VW+−, P−)</td>
<td>None</td>
<td>&gt;24 hr</td>
</tr>
<tr>
<td>45 (VW−, P−)</td>
<td>Hypoxanthine</td>
<td>2 hr 45 min</td>
</tr>
<tr>
<td>45 (VW−, P−)</td>
<td>Guanine</td>
<td>2 hr 40 min</td>
</tr>
<tr>
<td><strong>Complex synthetic medium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45 (VW+, P+)</td>
<td>None</td>
<td>&gt;24 hr</td>
</tr>
<tr>
<td>45 (VW+, P+)</td>
<td>Hypoxanthine</td>
<td>1 hr 35 min</td>
</tr>
<tr>
<td>45 (VW+−, P−)</td>
<td>Guanine</td>
<td>1 hr 45 min</td>
</tr>
<tr>
<td>45 (VW−, P−)</td>
<td>None</td>
<td>&gt;24 hr</td>
</tr>
<tr>
<td>45 (VW−, P−)</td>
<td>Hypoxanthine</td>
<td>1 hr 20 min</td>
</tr>
<tr>
<td>45 (VW−, P−)</td>
<td>Guanine</td>
<td>1 hr 10 min</td>
</tr>
</tbody>
</table>

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and P− organisms produced labeled adenine residues of RNA during cultivation in the complex synthetic medium. Typical separations of mononucleotides and determinations of radioactivity in hydrolysates of ribonucleic acid (RNA) from P+ cells grown in minimal and complex synthetic media containing guanine-8-14C are shown in Fig. 3.

Table 6. Conversion of guanine-8-14C to adenine residues (isolated as adenine monophosphate) and guanine residues (isolated as guanosine monophosphate) of ribonucleic acid by growing cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>Specific activity of isolated mononucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIM-10 (VW+, P−)</td>
<td>Minimal synthetic</td>
<td>AMP 0.05 GMP 0.12</td>
</tr>
<tr>
<td>KIM-10 (VW−, P+)</td>
<td>Minimal synthetic</td>
<td>AMP 0.0 GMP 0.10</td>
</tr>
<tr>
<td>KIM-10 (VW+, P−)</td>
<td>Complex synthetic</td>
<td>AMP 0.04 GMP 0.11</td>
</tr>
<tr>
<td>KIM-10 (VW−, P+)</td>
<td>Complex synthetic</td>
<td>AMP 0.04 GMP 0.12</td>
</tr>
</tbody>
</table>

a Specific activity of exogenous guanine-8-14C was 0.13 μC/μm.

A search for single-step purine-auxotrophs capable of fulfilling their nutritional requirement with compounds other than hypoxanthine or inosine resulted in the isolation of strain 25 (VW+, P+). Cells of this mutant grew only in the presence of guanine or guanosine (Table 3) and lacked detectable guanosine monophosphate synthetase (EC 6.3.4.1) when grown under conditions which yielded a specific activity of 0.004 in extracts of strain KIM-10 (VW−, P+).

Mutants 46 (VW+, P+), 61 (VW+, P+), 45 (VW+, P+), and 25 (VW+, P+) were able to synthesize capsular antigen or fraction I and were pesticinogenic. Accordingly, they possessed all of the established determinants of virulence in P. pestis (6, 11) except purine independence. The intraperitoneal LD50 in mice of these isolates and their prototroph is listed in Table 7. It is evident that mutations preventing the de novo biosynthesis of IMP promoted only a slight decrease in virulence in mice (LD50 102 cells), whereas a metabolic block which prevented the conversion of IMP to GMP resulted in outright avirulence (LD50 > 107 cells). The intraperitoneal

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**Fig. 3.** Radioautographs of hydrolysates of ribonucleic acid isolated from cells of strain KIM-10 (VW−, P+) after growth with guanine-8-14C and paper electrophoresis; the time constant, counting range, and chart speed were 10 sec, 10^4 counts per min, and 1 cm per min, respectively. A, cells grown in complex synthetic medium; B, cells grown in minimal synthetic medium; C, location of mononucleotides as observed under ultraviolet light.
TABLE 7. Virulence of purine- auxotrophs of Pasteurella pestis obtained from strain KIM-10

<table>
<thead>
<tr>
<th>Strain</th>
<th>Metabolic block</th>
<th>Intrapertitoneal LD50 in white mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIM-10</td>
<td>(Prototroph) Early block in de novo synthesis</td>
<td>&lt;10 cells ~10^9 cells</td>
</tr>
<tr>
<td>46 (VW+, P+)</td>
<td>Formation of AICAR*</td>
<td>~10^9 cells</td>
</tr>
<tr>
<td>61 (VW+, P+)</td>
<td>Conversion of AICAR to inosine monophosphate</td>
<td>~10^9 cells</td>
</tr>
<tr>
<td>45 (VW+, P+)</td>
<td>Conversion of xanthine monophosphate to guanosine monophosphate</td>
<td>&gt;10^9 cells</td>
</tr>
</tbody>
</table>

a Aminimidazolecarboxamide ribonucleotide.

LD50 of strain 25 (VW+, P+) in guinea-pigs was >10^8 cells.

DISCUSSION

Mutational loss of AICAR formyltransferase in E. coli merely results in a general nutritional requirement for purine which can be satisfied by adenine, hypoxanthine, xanthine, or guanine. It is also necessary to prevent the deamination of adenine to hypoxanthine and the conversion of xanthine and guanine to IMP in this organism to produce a mutant which exhibits a monospecific requirement for hypoxanthine (25). Nevertheless, this phenotype was expressed by P. pestis after the loss of AICAR formyltransferase; mutants blocked prior to the formation of AICAR could utilize adenine, AICA, or hypoxanthine as a source of purine. Xanthine and guanine were never observed to support the growth of single-step mutants blocked in the de novo biosynthesis of IMP.

The possibility was considered that the presence of a metabolic block which prevented the de novo biosynthesis of IMP might indirectly promote stasis in the presence of exogenous adenine. For example, under certain conditions adenine is known to repress formyltetrahydrofolate synthetase (EC 6.3.4.3) in Streptococcus faecalis (1). Similarly, toxic concentrations of intermediates can accumulate in mutants lacking enzymes which catalyze the interconversion of purine nucleotides (13, 25). These or related phenomena might account for the observed inability of cells of strain 45 (VW+, P+) to utilize adenine as a source of purine. However, it seemed equally probable that loss of AICAR formyl-

transerase, which is necessary for both the de novo biosynthesis of IMP and the conversion of AMP to IMP via AICAR, might directly account for the nutritional response of strain 45 (VW+, P+). This possibility was tested by determining the rates of incorporation of adenine-2-14C and adenine-8-14C into guanine residues of RNA. Both carbon 8 and carbon 2 would be expected to remain in the purine moiety during deamination. However, carbon 2 but not carbon 8 would be transferred to imidazoleglycerol phosphate after metabolism via the histidine cycle (24). The fact that incorporation from adenine-2-14C was no more than 15% of that obtained with adenine-8-14C indicates that the histidine cycle represents the major route in P. pestis for the conversion of adenine to IMP. This interpretation was strengthened by the inability to detect adenine deaminase in cell-free extracts of the organisms.

This result does not, of course, prove that P. pestis lacks the genetic potential to synthesize adenine deaminase; further study of extracts of organisms grown and prepared under different conditions will be necessary to clarify this situation. The significant point is that these experiments, in contrast to those of Mayskiy (27), demonstrate that cells of P. pestis can convert exogenous adenine to guanine residues of RNA. A possible explanation for this discrepancy would be the presence of histidine in the medium used by Mayskiy. This amino acid is known to repress the initial enzyme of the histidine cycle (25) and might thus prevent the conversion of AMP to IMP via AICAR in P. pestis. However, preliminary attempts to demonstrate such an effect of histidine in strain KIM-10 (VW+, P+) have been unsuccessful (unpublished observations).

Of considerable interest was the finding that P+ mutants blocked in the de novo pathway of purine biosynthesis were unable to grow in the presence of xanthine or guanine under conditions which favored the phenotypic expression of the pigmentation reaction. Although this property is an important determinant of virulence in P. pestis, little is known about the mechanism of hemin absorption or its biological significance. Pesticin I (5) and certain artificial dyes (22, E. D. Beesly, personal communication) are also absorbed by P+ cells, and various modifications of the environment such as elevated temperature or use of complex media can inhibit the absorption of hemin (10, 22). Burrows and his colleagues have suggested that expression of the P+ phenotype in vivo is associated with the ability of P. pestis to derive iron for growth (11, 23). This
hypothesis is supported by the fact that the lethal-
ity of P− isolates which have retained the remain-
ing determinants of virulence is restored in mice
by concomitant injection with iron or hemin (23).
However, the injection of iron with cells which
are avirulent due to the loss of the pesticin I
determinant also results in the restoration of
virulence (6). Accordingly, the possibility exists
that some common mechanism which defends
the host against P− and nonpesticinogenic cells
is inactivated by iron and that this defense me-
chanism is in turn overcome by pesticinogenic cells
of the P+ phenotype.

A correlation between P+ and sensitivity to
pesticin I has been described (5). However, the
discovery that P+ but not P− pesticide-avirulent
blocked in the de novo biosynthesis of IMP fail
to grow on exogenous guanine is the first in-
dication that metabolic differences exist between
the two types of cell. Of additional interest is the
finding that the presumptive block between
guanine and AMP in P+ cells was only expressed
under conditions which favor the pigmenta-
tion reaction. The fact that strain 25 (VW+, P+)
blocked between XMP and GMP, was able to
grow in the presence of guanine indicated that
this purine was able to enter P+ cells. This sup-
position was verified by the results of isotope-
dilution experiments which showed that exoge-
nous guanine was efficiently incorporated into
guanine residues of RNA under all sets of ex-
perimental conditions. Obviously the block could
not occur between IMP and AMP; thus at-
tention has become focused on the activity of GMP
reductase, an enzyme which catalyzes the conver-
sion of GMP to IMP.

A deficiency of GMP reductase in P+ cells
grown under conditions which favor the pigmenta-
tion reaction could account for the results
described in Table 6. Alternatively, identical results
would be obtained if the GMP pool was de-
creased under these conditions, providing this
nucleotide had less affinity for GMP reductase
than for enzymes responsible for its conversion
to guanosine triphosphate. One mechanism that
might account for a decreased pool of GMP
would be the absorption of guanine to P+ cells
in a manner similar to that of hemin. Preliminary
attempts to distinguish between repression of
GMP reductase and decrease of the GMP pool
have been inconclusive. Although GMP reductase
was not detected in P+ cells, only slight activity
was observed in comparable preparations of P−
organisms. However, the P+ but not P− cells were
able to absorb guanine (R. R. Brubaker and M.

Further study of this problem is currently in
progress. The inability of Maysky (27) to detect
a metabolic block between guanine and AMP
could reflect use of P− strains or a medium which
would not support the pigmentation reaction.

It is evident from these studies that the viru-
ulence of pesticide-avirulent of P. pestis varies
considerably depending upon the location of the
metabolic block. Mutants which were unable to
synthesize IMP via the de novo pathway retained
a considerable degree of virulence in mice, whereas
strain 25 (VW+, P+), blocked between
IMP and GMP, was completely avirulent (Table
6). Attempts to isolate VW+, P+ adenine au-
xotrophs, blocked between IMP and AMP, have
so far been unsuccessful. Such mutants, how-
ever, might also be expected to be avirulent as
judged by the results reported for B. anthracis
(21). It is also of interest that guanine oxo-
trophs of the latter species, blocked between
IMP and GMP, were fully virulent. Presumably
this difference reflects a greater ability of B.
anthracis to obtain guanine in vivo.

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