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Mutations Influencing the Assimilation of Nitrogen by *Yersinia pestis*[1]

R. R. BRUBAKER AND A. SULEN, JR.[2]

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823

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Cells of 20 isolates of *Yersinia* (*Pasteurella*) *pestis* exhibited an unusual nutritional requirement which could be fulfilled by glycine or L-threonine. Meiotrophic mutants which required neither of these amino acids (Gly/Thr$^+$) were isolated from cultures of all 20 strains at a frequency of 10^{-7}. Wild-type and Gly/Thr$^+$ cells of 14 strains failed to utilize L-amino acids or urea (0.01 M) as primary sources of nitrogen and grew slowly in the presence of low concentrations of NH_4^+ ($\leq$ 5 mM). Cells of six strains (termed N$^+$) utilized certain L-amino acids and urea (0.01 M) as primary sources of nitrogen and grew rapidly in the presence of $\leq$ 5 mM NH_4^+. N$^+$ but not N$^-$ organisms cultivated with NH_4^+ (0.01 M) as a primary source of nitrogen excreted a complete spectrum of naturally occurring amino acids; under this condition of growth the aspartase and particulate nicotinamide adenine dinucleotide phosphate transhydrogenase activities of N$^+$ and N$^-$ cells were repressed. N$^+$ meiotrophs arose at a frequency of 10^{-6} in cultures of all 14 N$^-$ isolates, and urease-positive meiotrophs could be selected at a frequency of 10^{-7} from N$^+$ but not N$^-$ cells of all 20 strains on a medium containing urea (0.01 M) as a primary source of nitrogen. These findings illustrate a reversible loss of genetic potential which has occurred during the evolution of *Y. pestis* as an obligate parasite and suggest that this organism is unable to efficiently remove dispensable deoxyribonucleic acid from its chromosome.

It is established that the primary fixation of NH_4^+ into α-amino groups occurs in most microorganisms via the action of glutamate dehydrogenase (EC 1.4.1.4) and that glutamate, the product of this reaction, can then undergo various transaminations which facilitate the biosynthesis of other amino acids (24, 32). Accordingly, glutamate dehydrogenase functions primarily as an anabolic enzyme, and its specific activity increases in *Escherichia coli* during growth in media lacking preformed α-amino groups. Aspartase (EC 4.3.1.1) can also catalyze the de novo synthesis of α-amino groups, but this enzyme is generally repressed during cultivation in minimal media indicating that it is primarily associated with the catabolism of aspartate (13, 34). Glutamate dehydrogenase can also function as a catabolic enzyme by catalyzing the oxidative deamination of glutamate, a reaction which serves as an important source of energy for a number of obligate and facultative intracellular bacterial parasites (2, 5, 19, 21, 22, 29, 31).

These organisms, which normally grow within highly enriched fluids of the mammalian host, often possess limited biosynthetic abilities and utilize preformed amino acids during their residence in vivo (25). A few facultative intracellular parasites, however, do exhibit considerable anabolic potential as judged by the ability to grow in vitro when supplied with NH_4^+ as the sole or primary source of nitrogen. Such species, which include *Yersinia* (*Pasteurella*) *pestis* and *Y. pseudotuberculosis*, synthesize α-amino groups via glutamate dehydrogenase during growth in minimal media. The same enzyme, however, evidently accounts for much of the rapid destruction of exogenous glutamate which occurs during cultivation in enriched media (5). Accordingly, the yersiniae might be expected to possess unique regulatory mechanisms which control the anabolic or catabolic potential of glutamate dehydrogenase by influencing its equilibrium.

As noted in this paper, biosynthesis of glutamate by *Y. pseudotuberculosis* was associated with repression of aspartase and particulate nicotinamide adenine dinucleotide phosphate (NADP) transhydrogenase activities. Similar changes occurred in the case of *Y. pestis* during cultivation in media containing NH_4Cl as a

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[2] Present address: Eli Lilly & Co., Indianapolis, Ind. 46227.

primary source of nitrogen. However, cells of most strains of the latter were unable to grow in the presence of low levels of NH_4^+ released by oxidation of amino acids or supplied directly by diffusion through agar. The primary purpose of this manuscript is to characterize this inability of *Y. pestis* to assimilate NH_4^+ and to describe an unusual nutritional requirement for glycine or L-threonine. A secondary objective is to define environmental conditions that are selective for three types of meiotrophic mutants which acquire the abilities to assimilate low concentrations of NH_4^+, synthesize glycine and threonine, and express the enzyme urease (EC 3.5.1.5); mutants of the latter type were previously isolated by Mollaret et al. (23) from lysates of cultures treated with bacteriophage.

MATERIALS AND METHODS

Organisms. All strains of yersiniae were obtained through the courtesy of M. J. Surgalla or J. D. Marshall. The phenotypes of many of these isolates have been described (1); biotypes of *Y. pestis* were determined by the system of Devignat (8), and serotypes of *Y. pseudotuberculosis* were kindly identified by Marshall. Although cells of virulent *Y. pestis* were used in preliminary experiments, avirulent isolates were subsequently prepared by selection on magnesium oxalate-agar (16) or Congo red-agar (30) for investigation of physiological defects and meiotrophy.

Special media. The composition of nitrogen-deficient (ND) medium, a preparation modified from Higuchi and Carlin (15), Hills and Spurr (17), and Englesberg (10), is shown in Table 1. To prepare 1 liter of ND medium, 100 μmoles of the four amino acids were dissolved in 100 ml of 10-fold concentrated stock salt solution in a 2-liter Erlenmeyer flask. After addition of 870 ml of distilled water and 15 g of agar (Difco) in the case of solid medium, the flask was fitted with a cotton stopper and the contents were sterilized by autoclaving. After cooling, the *p*H was adjusted to neutrality with 5 N NaOH, and 10 ml of 100-fold concentrated stock solutions of sodium thiosulfate, sodium lactate, D-glucose, and $CaCl_2$ were added aseptically. Bromthymol blue or phenol red was occasionally autoclaved in the medium at a concentration of 0.001% to facilitate subsequent neutralization of *p*H. Calcium pantothenate (10 μM) often stimulated growth and was incorporated into solid media used for the primary isolation of meiotrophic mutants. ND medium alone supported little growth in the absence of a supplementary source of nitrogen.

TABLE 1. *Composition of nitrogen-deficient medium*[a]

Component	Millimoles per liter
Salt solution	
K_2HPO_4	25
Citric acid	10
$MgCl_2$	2.5
$FeCl_2$	0.1
$MnCl_2$	0.01
Amino acids	
L-Phenylalanine	0.1
L-Methionine	0.1
L-Isoleucine	0.1
L-Valine	0.1
Miscellaneous	
$Na_2S_2O_3$	2.5
Sodium lactate	10
D-Glucose	10
$CaCl_2$	2.5

[a] The *p*H was adjusted to neutrality with 5 N NaOH.

Cultivation. With the exception of assays for temperature-dependent determinants of virulence, the organisms were always incubated at 26 C. In order to define nutritional requirements, slopes of Blood Agar Base (Difco) were inoculated and, after incubation for 24 to 36 hr, the cells were removed and washed twice in sterile 0.033 M potassium phosphate buffer, *p*H 7.0 (phosphate buffer), and then used for inoculation of both liquid and solid media. In quantitative determinations, tubes (20 by 150 mm) containing 5 ml of plain or supplemental ND medium were slanted, and growth was recorded in terms of optical density at 620 nm with a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). In studies of growth in the presence of low levels of NH_4^+, the size of colonies on solid ND medium was determined with a no. 30.169 comparator (Edmund Scientific Co., Barrington, N.J.). Cells used as sources of enzymes were grown with aeration on a model R25 shaker (New Brunswick Scientific Co., New Brunswick, N.J.) in 100 ml of the enriched synthetic medium of Higuchi and Carlin (15) or in ND medium supplemented with glycine (0.25 mM) and NH_4Cl (0.01 M) per each 1-liter Erlenmeyer flask.

Enzyme determinations. Cells were harvested during the early stationary phase by centrifugation at 40,000 × *g* for 10 min, washed with phosphate buffer, and then suspended in 0.01 M tris(hydroxymethyl)aminomethane-chloride buffer, *p*H 7.8, containing 2-mercaptoethanol (0.01 M); dialyzed cell-free extracts were prepared by procedures described previously (6). The release of NH_3 from aspartate, as estimated by direct Nesslerization, was followed in order to determine aspartase (34). The method used to assay the particulate NAD(P) transhydrogenase activity has been described (6), and glutamate dehydrogenase was measured by the procedure of Vender, Jayaraman, and Rickenberg (34). Protein was estimated by the method of Lowry et al. (20).

Excretion of amino acids. Cells grown in liquid ND medium supplemented with NH_4Cl (0.01 M) were collected by centrifugation, and the supernatant fluid was lyophilized. A 5% solution of the resulting powder was clarified by centrifugation and adjusted to *p*H 2 with H_2SO_4, and amino acids were removed

by exchange on a column containing Dowex 50 W, 200 to 400 mesh, in the H^+ form. After elution with 1 N NH_4OH, the amino acids were brought to dryness and then determined qualitatively by two-dimensional chromatography with phenol:water (4:1, w/v) and butanol:acetic acid:water (100:22:50, v/v).

Bacterial properties. Established determinants of virulence were estimated by methods described previously (1, 16, 30), and abilities to reduce nitrate and ferment sugars were assayed with Nitrate Broth (Difco) and Purple Broth (Difco) containing 1% carbohydrate, respectively. Motility was determined in tubes containing Heart Infusion Broth (Difco) plus 0.3% agar, and the production of murine toxin was estimated qualitatively by gel diffusion with monospecific antiserum. Ability to express urease was assayed with Christensen's urea agar (Difco).

Reagents. Inorganic compounds of highest available purity were purchased from the Fisher Scientific Co., Pittsburg, Pa. Pyridine nucleotides, glutathione, and sugar phosphates were obtained from the Sigma Chemical Co., St. Louis, Mo., and remaining organic compounds were products of Calbiochem, Los Angeles, Calif.

RESULTS

ND medium supplemented with NH_4Cl (0.01 M) as a primary source of nitrogen failed to promote significant growth of 20 typical strains of *Y. pestis*, whereas five strains of *Y. pseudotuberculosis* grew rapidly in this environment (Table 2). This finding was unexpected because ND medium contains all of the compounds which have been established as nutritional requirements of *Y. pestis* (10, 17), thus the possibility was considered that stasis was caused by an inability to perform de novo biosynthesis of α-amino groups. In order to test this hypothesis, NH_4^+ was replaced by an equimolar concentration of

TABLE 2. *Optical density of duplicate cultures of yersiniae after incubation for 3 days at 26 C in nitrogen-deficient medium supplemented with NH_4Cl (0.01 M) and glycine (0.01 M)*[a]

Strain	Phenotype or serotype[b]	Added source of nitrogen			
		None	NH_4Cl	Glycine	NH_4Cl plus glycine
Yersinia pestis					
Alexander	O	.000	.020	.000	.450
A1122	O	.000	.030	.000	.420
A12	O	.000	.020	.000	.410
A4X	O	.000	.020	.000	.450
D1	A	.000	.030	.150	.480
Dodson	O	.000	.040	.000	.510
G25	O	.000	.030	.000	.440
G32	O	.000	.030	.000	.400
G35	O	.000	.020	.000	.500
Java	O	.000	.020	.000	.450
KIM-9	M	.000	.050	.120	.450
KIM-10	M	.000	.030	.170	.500
Kuma	A	.000	.040	.170	.420
MP6	O	.000	.000	.000	.520
M23	O	.000	.010	.000	.480
Poona	O	.000	.000	.000	.490
Salazar	O	.000	.040	.120	.540
Shasta	O	.000	.040	.000	.390
Siam	A	.000	.020	.000	.490
TS	O	.000	.030	.000	.480
Yokohoma	A	.000	.040	.150	.540
Yersinia pseudotuberculosis					
PB1	I	.010	.420	.250	.640
EP2	II	.000	.390	.280	.590
MD31	III	.020	.450	.300	.550
Nielson	IV	.000	.420	.190	.640
24	V	.000	.440	.250	.590

[a] Cultures consisted of 5 ml of medium per slanted tube (20 by 150 mm) inoculated with 10^6 washed cells per ml.

[b] O = variety *orientalis*, M = variety *mediaevalis*, A = variety *antiqua*; numerals indicate serotype of *Yersinia pseudotuberculosis*.

α-amino groups in the form of L-glutamate, L-asparagine, L-aspartate, L-serine, or glycine. Only glycine was an effective source of nitrogen in that this amino acid permitted growth to occur in cultures of 6 of the 20 strains which were examined (Table 2). However, the addition of NH_4Cl (0.01 M) and glycine (0.01 M) to ND medium resulted in rapid growth of all 20 isolates (Table 2). This finding indicated that all strains of *Y. pestis* possessed a nutritional requirement for glycine but that this amino acid, unlike NH_4Cl, could only serve six strains as a primary source of nitrogen.

Gly/Thr phenotypes. Further tests with over 30 potential growth factors showed that the nutritional requirement for glycine could be fulfilled by L-threonine but not by L-serine. During these determinations, which were performed on solid ND medium supplemented with NH_4Cl (0.01 M), colonies of apparent mutant cells became visible in the absence of added glycine or L-threonine after incubation for 4 to 6 days (Fig. 1A). After purification of these meiotrophs, termed Gly/Thr^+, a series of comparative determinations were performed in liquid media in order to further characterize the unusual nutritional requirement of the prototroph.

As shown in Fig. 2A, a concentration of at least 0.01 M NH_4^+ was necessary for optimal growth of a Gly/Thr^+ mutant, whereas five times this molarity of NH_4^+ did not influence the response of the prototroph. Growth of the latter was not affected by addition of L-methionine, L-phenylalanine, L-isoleucine, or L-valine at 10 times the concentrations (0.1 M) which were necessary to promote maximum growth of meiotrophic Gly/Thr^+ cells (Fig. 2B). However, concentrations of 0.25 mM glycine (Fig. 2C) or L-threonine (Fig. 2D) were sufficient to permit equivalent growth of prototrophic cells. The glycine requirement of all 20 strains of *Y. pestis* which were examined could be fulfilled by L-threonine, and all 20 strains yielded Gly/Thr^+ meiotrophic mutants at a frequency of 10^{-7}.

N phenotypes. As already noted, cells of 6 of the 20 isolates of *Y. pestis* grew in ND medium supplemented with glycine (0.01 M); equimolar concentrations of L-glutamate, L-glutamine, L-aspartate, or L-asparagine could also support the growth of cells of these six strains (termed

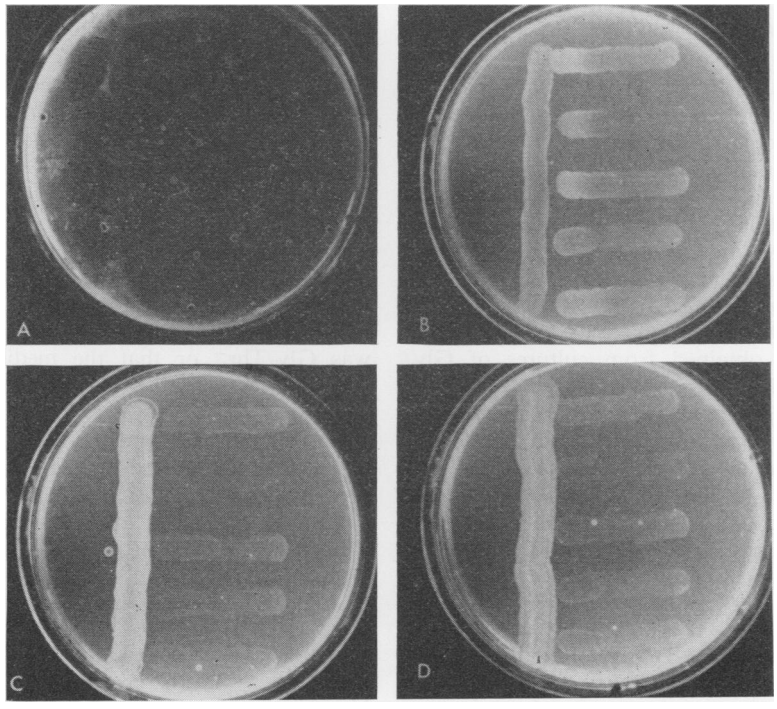

FIG. 1. *Expression of meiotrophic phenotypes of Yersinia pestis. (A) Colonies of Gly/Thr^+ cells of strain Yokohoma obtained in nitrogen-deficient medium supplemented with NH_4Cl (0.01 M) after heavy inoculation with wild-type cells and incubation for 7 days at 26 C. (B) Cross-feeding of five wild-type strains of Y. pestis (horizontal streaks) by a Gly/Thr^+, N^+ meiotroph of strain Kuma (vertical streak) after incubation on nitrogen-deficient medium supplemented with NH_4Cl (0.01 M) for 7 days at 26 C. (C) Repetition of the same experiment with Y. pseudotuberculosis strain PB1 (vertical streak) and (D) with Escherichia coli strain B (vertical streak).*

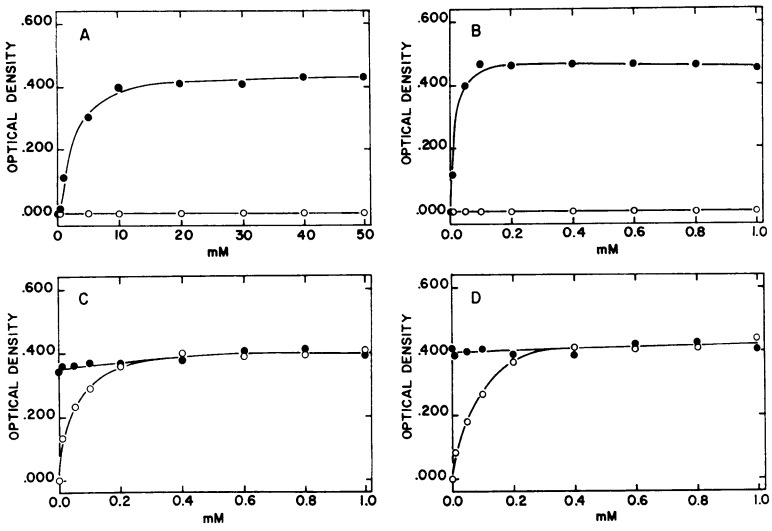

FIG. 2. *Response of Gly/Thr⁻ (○) and Gly/Thr⁺ (●) cells of Yersinia pestis strain A4X in nitrogen-deficient medium supplemented with (A) NH_4Cl, (B) an equimolar mixture of L-methionine, L-phenylalanine, L-isoleucine, and L-valine (values refer to concentrations of individual amino acid), (C) glycine, and (D) L-threonine. Cultures consisted of duplicate slanted tubes (20 by 150 mm) containing 5 ml of medium inoculated with 10^6 washed cells per ml; the cultures were incubated for 3 days at 26 C.*

N^+) in the presence of sufficient glycine or L-threonine (0.25 M) to fulfill the normal requirement for these amino acids. Identical results were obtained with Gly/Thr^+ meiotrophs obtained from these six N^+ strains in the absence of added glycine or L-threonine. Furthermore, Gly/Thr^+ mutants of the N^+ but not N^- phenotype were capable of cross-feeding wild-type cells on solid ND medium supplemented with NH_4Cl (0.01 M) as a primary source of nitrogen (Fig. 1B); this phenomenon did not occur in the case of *Y. pseudotuberculosis* (Fig. 1C) or *E. coli* (Fig. 1D). An examination of the supernatant fluids obtained from cultures of Gly/Thr^+, N^+ cells of strains Kuma and Yokohoma disclosed the presence of a complete spectrum of the naturally occurring amino acids. Similar preparations obtained from Gly/Thr^+, N^+ cells of strains A1122 and TS contained only trace amounts of glutamic acid, ornithine, and arginine in addition to the four amino acids which were components of the medium.

Meiotrophic N^+ mutants could be selected at a frequency of 10^{-6} from all 14 N^- strains listed in Table 2 on solid ND medium supplemented with glycine (0.01 M); colonies of N^+ cells became visible after incubation for 4 to 6 days. These mutants, like the six naturally occurring N^+ isolates, could also utilize L-glutamate, L-aspartate, or L-asparagine as primary sources of nitrogen provided that they were Gly/Thr^+ or that the medium contained glycine or L-threonine (0.25 M). Further study of an N^- strain and an isogenic N^+ meiotroph disclosed that cells of the former grew slowly when supplied with small concentrations of NH_4^+ ($\leq$ 5 mM) by diffusion through agar. In contrast, cells of the mutant formed large colonies when plated on ND medium containing equivalent concentrations of NH_4^+ (Table 3).

Ure phenotypes. N^+ meiotrophs could also be selected on ND medium supplemented with 0.01 M L-glutamate, L-glutamine, L-aspartate, or L-asparagine provided that the prototroph was Gly/Thr^+ or that the medium contained glycine or L-threonine (0.25 M). Urea could also serve as a primary source of nitrogen for Gly/Thr^+, N^+ cells due, presumably, to spontaneous hydrolysis with release of NH_3 (Fig. 3B). However, when a population of Gly/Thr^+, N^+ organisms was plated on ND medium supplemented with urea (0.01 M) as a primary source of nitrogen, small colonies appeared after 3 or 4 days of incubation which were surrounded by large halos of growth (Fig. 3A); cells obtained from the central colony within these areas possessed the ability to express the enzyme urease. Urease-positive meiotrophs (Ure^+) could be obtained in this manner at a frequency of 10^{-7} from all of the strains of *Y. pestis* listed in Table 2.

Mutation to the Gly/Thr^+, N^+, or Ure^+ phenotype was not associated with gain or loss

of other properties associated with the expression of virulence or shared by *Y. pseudotuberculosis* Determinants which remained unchanged were abilities to synthesize virulence antigens, pesticin I, capsular antigen, glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and murine toxin and to ferment rhamnose, melibiose, and glycerol; no alteration in ability to absorb Congo red was noted and the meiotrophs remained nonmotile. Gly/Thr$^+$ and Ure$^+$ mutants were obtained from a *pur*$^-$*pyr*$^-$*his*$^-$auxotroph of the normally N$^+$ strain KIM-10, and the three nutritional requirements were retained throughout the process of selection.

Cells of wild-type *Y. pestis* and *Y. pseudotuberculosis*, after cultivation in liquid ND medium supplemented with glycine (0.25 mM) and NH_4Cl (0.01 M), lacked detectable aspartase and NADP transhydrogenase activities. Both of these enzymes were observed in cells cultivated in the enriched medium of Higuchi and Carlin (15); such organisms also contained an elevated level of glutamate dehydrogenase (Table 4). Repression of aspartase and transhydrogenase activities during growth in the absence of preformed α-amino groups was also

TABLE 3. *Size of colonies of N$^+$ and N$^-$ cells of Yersinia pestis strain A4X on solid nitrogen-deficient medium supplemented with glycine (0.25 mM) and NH_4Cl*[a]

Added NH_4Cl (mM)	Colony size (mm)[b]	
	N$^+$	N$^-$
None	1.1 (± 0.1)	0.2 (± 0.05)
0.1	1.3 (± 0.2)	0.5 (± 0.2)
0.5	1.9 (± 0.3)	1.0 (± 0.2)
1.0	2.2 (± 0.6)	1.5 (± 0.1)
5.0	2.0 (± 0.2)	1.6 (± 0.2)
10.0	2.0 (± 0.3)	2.0 (± 0.3)

[a] Plates were incubated for 6 days at 26 C.
[b] Values are averaged from 10 isolated colonies.

TABLE 4. *Specific activities of NAD(P) transhydrogenase system, glutamate dehydrogenase, and aspartase in yersiniae cultivated in nitrogen-deficient medium and the enriched medium of Higuchi and Carlin*[a]

Activity	Nitrogen-deficient medium	Higuchi and Carlin medium
Yersinia pseudotuberculosis strain PB1		
NAD(P) transhydrogenase	<.005	.155
Glutamate dehydrogenase	.030	.150
Aspartase	<.005	.355
Yersinia pestis strain A4X		
NAD(P) transhydrogenase	<.005	.170
Glutamate dehydrogenase	.045	.135
Aspartase	<.005	.245

[a] Results are expressed in terms of micromoles of product formed per minute per milligram of protein.

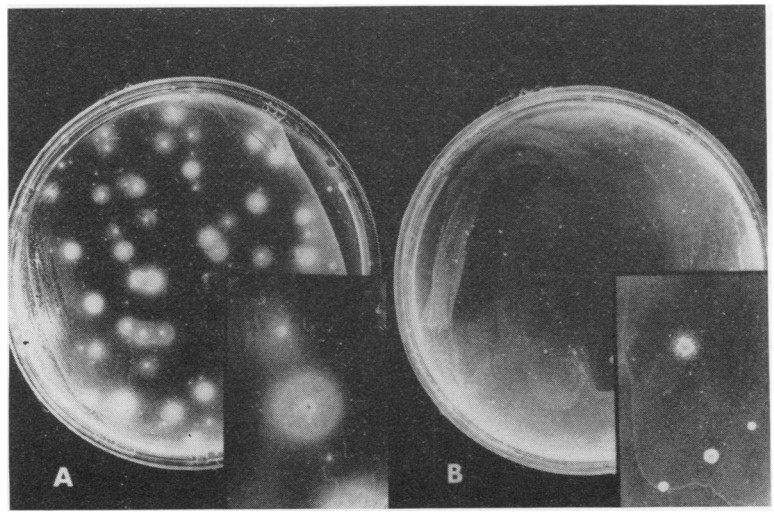

FIG. 3. *Expression of meiotrophic phenotypes of Yersinia pestis strain MP6 on nitrogen-deficient medium supplemented with urea (0.01 M) after incubation for 6 days at 26 C. (A) Colonies of urease-positive cells surrounded by halos of growth selected from a Gly/Thr$^+$, N$^+$ prototroph and (B) colonies of N$^+$ cells selected from a Gly/Thr$^+$, N$^-$ prototroph.*

noted in the case of Gly/Thr$^+$, N$^+$, and Ure$^+$ meiotrophs (*not illustrated*).

DISCUSSION

Although a particulate NADP transhydrogenase discovered in *Y. pestis* and *Y. pseudotuberculosis* (6) has not yet been characterized in detail, preliminary evidence indicated that this activity is similar to the energy-independent transhydrogenase system described in *E. coli* (3). Presumably this reversible system fulfills an important role in catalyzing the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) generated during the oxidative deamination of glutamate. The sum of the reactions catalyzed by glutamate dehydrogenase and the transhydrogenase system is given by the following equation:

$$\text{L-glutamate} + \text{NAD} + \text{H}_2\text{O} \rightarrow \alpha\text{-ketoglutarate} + \text{NADH} + \text{NH}_3 \quad (1)$$

where NADPH serves as a substrate for oxidative phosphorylation. Again, the sum of the reactions catalyzed by aspartase, aspartate aminotransferase (EC 2.6.1.1), fumarase (EC 4.2.1.2), and malate dehydrogenase (EC 1.1.1.37) is given by equation 1 illustrating a second mechanism for the catabolism of glutamate first suggested by Vender, Jayaraman, and Rickenberg (34).

A different situation would exist if transhydrogenase and aspartase activity was present under conditions where glutamate dehydrogenase was utilized for the de novo biosynthesis of α-amino groups. With this change of equilibrium, the sum of the reactions catalyzed by glutamate dehydrogenase and aspartate aminotransferase would be:

$$\text{oxalacetate} + \text{NADPH} + \text{NH}_3 \rightarrow \text{aspartate} + \text{NADP} + \text{H}_2\text{O} \quad (2)$$

and that catalyzed by the combined action of aspartase, fumarase, and malate dehydrogenase would be:

$$\text{aspartate} + \text{NAD} + \text{H}_2\text{O} \rightarrow \text{oxalacetate} + \text{NADH} + \text{NH}_3 \quad (3)$$

thus the sum of these expressions:

$$\text{NADPH} + \text{NAD} \rightarrow \text{NADP} + \text{NADH} \quad (4)$$

would be identical to the reaction reversibly promoted by the transhydrogenase system. Accordingly, the presence of aspartase and transhydrogenase activities in cells cultivated with NH_4^+ as a primary source of nitrogen would prevent net production of both α-amino groups and reduced pyridine nucleotides.

In view of this relationship, it was not surprising to find that aspartase and the transhydrogenase system were repressed in yersiniae cultivated with NH_4^+ as a primary source of nitrogen. However, despite the existence of this regulatory mechanism, cells of *Y. pestis* failed to grow in the absence of preformed α-amino groups suggesting that the minimal medium lacked essential growth factors or that the organisms were unable to aminate α-ketoglutarate. The existence of both of these potential restrictions was discovered during subsequent studies.

A favorable effect of glycine on the cultivation of *Y. pestis* was first noted by Rao (28). This observation was not verified by Hills and Spurr (17) or Englesberg (10) who reported that only L-isoleucine, L-valine, L-phenylalanine, L-methionine, and L-cysteine were stimulatory or essential for rapid growth at room temperature. Englesberg (10) also showed that L-cysteine could be replaced by $S_2O_3^{2-}$. More recently, following the general availability of highly purified amino acids, formulas for a number of useful synthetic media have appeared in the literature; those that lack added glycine generally contain L-threonine (4, 35) or vice versa (7, 18), and the significance of this interchangeability has remained unnoticed.

Although this nutritional requirement is only casually related to questions concerning the assimilation of NH_4^+, its discovery is not trivial because glycine and threonine are synthesized by independent pathways (32), and it is difficult to envision a common metabolic block. One clue to this problem is the finding that radioactive serine was not detected in hydrolysates of *Y. pestis* cultivated in the presence of ^{14}C-glycine (9) suggesting that the cells lack serine hydroxylmethyltransferase (EC 2.1.2.1). A deficiency of this enzyme would also account for the observation that L-serine failed to fulfill the nutritional requirement for glycine as is the case in most glycine-auxotrophs of enteric bacteria (32). Preliminary studies indicate that serine hydroxylmethyltransferase is indeed absent in *Y. pestis* and that exogenous L-threonine may be converted to glycine by the mechanism described in *E. coli* by Van Lenten and Simmonds (33).

Even when the nutritional requirement for glycine or L-threonine had been satisfied, N$^-$ cells of *Y. pestis* grew slowly when supplied with low concentrations of NH_4^+ and failed to divide when single amino acids or urea were provided as primary sources of nitrogen. *Y. pestis* possesses transaminase systems which should conserve preformed α-amino groups assuming that a sufficient concentration of exogenous amino

acid had been able to enter the cell. This situation does not always occur as illustrated by the existence of a permeability barrier which prevents L-glutamate from serving as a sole source of carbon for the growth of *E. coli* (14). Cells of *Y. pestis* also contain an active L-amino acid oxidase system (26) which should generate low levels of NH_3 from exogenous amino acids; spontaneous hydrolysis of urea would cause the same result. Neither of these reactions evidently occurred at rates that were adequate to permit reductive examination of α-ketoglutarate. It is evident that stasis was not caused by a deficiency of NADPH or α-ketoglutarate because the sole addition of high concentrations of NH_4^+ was sufficient to promote growth. Furthermore, in the absence of NH_4^+, cells of an N^- strain excreted a significant level of α-ketoglutarate (K. Higuchi, Bacteriol. Proc., p. 58, 1958) indicating that production of this organic acid does not limit the subsequent synthesis of α-amino groups. The most likely interpretation of these findings is that N^- cells lack the ability to concentrate NH_4^+ and must be supplied with high levels of this cation when growth is dependent upon the biosynthesis of amino acids. It should be noted in this context that NH_4^+ exists in only trace amounts in the normal mammalian host which is rich in preformed amino acids, thus an inability of N^- cells to concentrate NH_4^+ would not limit growth in vivo.

Of significance was the discovery that meiotrophic Gly/Thr$^+$, N^+, and Ure$^+$ mutants could be selected from wild-type strains of *Y. pestis*. These mutants, as well as distinct meiotrophs described by Englesberg (10–12), have acquired single characteristics which are normally expressed by wild-type *Y. pseudotuberculosis*; the latter species is generally known to exhibit a close taxonomic relationship to *Y. pestis*. Accordingly, usage of the term meiotroph in this context implies that a defective gene in *Y. pestis* has undergone repair, either by true reversion or by an intracistronic suppressor mutation, to become an analogue of that normally present in *Y. pseudotuberculosis*. This assumption may be correct in the case of mutations which permit expression of enzymes such as urease or those associated with fermentation of carbohydrates. However, mutation to the N^+ phenotype results in ability to excrete large concentrations of α-amino acids, and this property is not shared by *Y. pseudotuberculosis*. Accordingly, meiotrophy in this context reflects a complex physiological change which could even be caused by an intercistronic suppressor mutation.

Cells of *Y. pseudotuberculosis* are generally transmitted in nature via the oral route, thus, the abilities to ferment uncommon sugars, synthesize amino acids, degrade urea, and concentrate NH_4^+ could favor long-term survival in soil and water. In contrast, these abilities are not essential to cells of *Y. pestis* which maintain an intimate contact with their mammalian host or arthropod vector (27). Nevertheless, meiotrophic mutants of *Y. pestis* which have acquired these properties can easily be obtained, indicating that the original enzymatic losses were not functions of large deletions and might therefore result from frameshift mutations or base transitions or transversions. At present, the possibility of a single nucleotide addition or deletion appears to be most likely because attempts to demonstrate cross-reacting proteins or to induce meiotrophy with alkylating agents have been unsuccessful (*unpublished observations*). In either event, it appears that the wild-type cell faithfully replicates a number of defective genes and thus lacks an efficient mechanism for the removal of extraneous deoxyribonucleic acid from its chromosome.

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