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# *Proteus mirabilis* Amino Acid Deaminase: Cloning, Nucleotide Sequence, and Characterization of *aad*

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***Proteus*, *Providencia*, and *Morganella* species produce deaminases that generate α-keto acids from amino acids. The α-keto acid products are detected by the formation of colored iron complexes, raising the possibility that the enzyme functions to secure iron for these species, which do not produce traditional siderophores. A gene encoding an amino acid deaminase of uropathogenic *Proteus mirabilis* was identified by screening a genomic library hosted in *Escherichia coli* DH5α for amino acid deaminase activity. The deaminase gene, localized on a cosmid clone by subcloning and Tn*5*::*751* mutagenesis, was subjected to nucleotide sequencing. A single open reading frame, designated *aad* (amino acid deaminase), which appears to be both necessary and sufficient for deaminase activity, predicts a 473-amino-acid polypeptide (51,151 Da) encoded within an area mapped by transposon mutagenesis. The predicted amino acid sequence of Aad did not share significant amino acid sequence similarity with any other polypeptide in the PIR or SwissProt database. Amino acid deaminase activity in both *P. mirabilis* and *E. coli* transformed with *aad*-encoding plasmids was not affected by medium iron concentration or expression of genes in multicopy in *fur*, *cya*, or *crp E. coli* backgrounds. Enzyme expression was negatively affected by growth with glucose or glycerol as the sole carbon source but was not consistent with catabolite repression.**

Among the most common human infections are those of the urinary tract. After *Escherichia coli*, *Proteus mirabilis* is a frequent etiological agent particularly in catheterized patients or individuals with structural abnormalities of the urinary tract (46). *P. mirabilis* appears to localize in the kidney in higher numbers than other uropathogens (13, 43), and there it can cause serious complications including acute pyelonephritis, bladder and kidney stones, and bacteremia (36, 40). Testing of isogenic mutants in a CBA mouse model of ascending urinary tract infection has demonstrated that urease (18, 19), mannose-resistant/*Proteus*-like fimbriae (3–5), and *P. mirabilis* fimbriae (25) contribute significantly to the ability of this organism to establish infection. Other putative virulence determinants include hemolysin (28, 32, 33, 44), flagella associated with swarming motility (6, 31), a uroepithelial cell adhesin (47), ambient temperature fimbriae (24), and an immunoglobulin A-degrading protease (38, 39).

The role of iron in infections has been well established (2, 7, 8, 48), and most bacterial species produce phenolate- and/or hydroxamate-type siderophores to compete with the host for iron (7, 8, 30). Most genera of the family *Enterobacteriaceae* produce either enterobactin (a phenolate siderophore) or aerobactin (a mixed phenolate-hydroxamate siderophore) (7, 30). These traditional-type siderophores are biosynthesized by the action of a number of enzymes whose genes exist in an operon (30). Despite evidence that the urinary tract is an iron-limited niche (41) and that iron limitation reduces susceptibility of animals to development of *P. mirabilis* pyelonephritis (17), no traditional siderophores have been unequivocally demonstrated in *Proteus*, *Providencia*, or *Morganella* species (12, 30, 34).

Drechsel et al. (11) recently proposed that the *Proteus*, *Providencia*, and *Morganella* species use α-keto acids as siderophores. The α-keto acids are derived from the deamination of amino acids by the enzyme amino acid deaminase. Ironically, this reaction, which may contribute to virulence, has been used for years in the clinical laboratory as a biochemical marker for what was formerly designated the *Proteeae* tribe. It has been demonstrated that α-keto acids complex with iron and that these chelates could be transported, thus relieving iron restriction. The findings of Drechsel et al. (11) represent the first identification of a potential siderophore that is produced by a minor modification of a preexisting compound. *E. coli* does not produce an amino acid deaminase and cannot utilize α-keto acids as siderophores.

To better understand this novel putative siderophore system, we have cloned and sequenced the amino acid deaminase gene (*aad*) of *P. mirabilis* and have studied expression of the gene product.

## MATERIALS AND METHODS

**Bacterial strains.** *P. mirabilis* HI4320 was isolated from the urine of an elderly long-term-catheterized patient (29). Additional bacterial strains were isolated from patients with catheter-associated bacteriuria or acute pyelonephritis and have been described previously (27). *E. coli* HB101 and DH5α (1) were used as recipients of recombinant cosmids and plasmids. *E. coli* χ289 (*cya*$^+$ *crp*$^+$), χ6161 (*cya*), and χ6162 (*crp*) were the generous gift of Roy Curtiss III (Washington University). *E. coli* SM796 (*fur*$^+$) and SBC796 (*fur*) were kindly provided by Stephen Calderwood (Harvard University). Bacteria were grown in Luria broth and Luria agar (23). Agar concentration was raised to 2.0%, and 0.5% glycerol was added to prevent swarming of *P. mirabilis* on agar plates (6).

**Amino acid deaminase assay.** For qualitative determination of amino acid deaminase activity, bacteria were grown in modified phenylalanine broth (per liter, 3 g of yeast extract, 3 g of tryptone, 1 g of phenylalanine, 1 g of $Na_2HPO_4$, and 5 g of NaCl, pH 7.0). After 18 h of incubation, the addition of 20 μl of 10% ferric chloride (bioMérieux Vitek, Inc., Hazelwood, Mo.) to the bacterial suspension (200 μl) results in the formation of a green color if phenylalanine has been deaminated and a yellow color if no product is present.

For quantitative determination of activity, cells were harvested by centrifugation (3,500 × *g*, 5 min, 4°C), washed with 20 mM sodium phosphate buffer (pH 7.0), resuspended in 1/10 of the original volume in 20 mM sodium phosphate buffer (pH 7.0), and ruptured in a French pressure cell at 20,000 lb/in$^2$. Cellular debris and membranes were removed by centrifugation (30,000 × *g*, 30 min, 4°C). A sample (200 μl) of the cytoplasmic extract was added to 2 ml of 0.1 M phenylalanine in 20 mM sodium phosphate buffer, pH 7.0. At 5-min intervals, 0.5

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ml of the reaction mixture was added to 0.5 ml of distilled $H_2O$ containing 50 μl of 10% $FeCl_3$. The $A_{614}$ was determined within 30 s, since the color formation was rapidly lost after 90 s. Amino acid deaminase activities were determined from the linear portion of the plot of time versus concentration of product; values are reported as micromoles of phenylpyruvic acid generated from phenylalanine per minute per milligram of protein. Commercially available phenylpyruvic acid (Sigma) served as a standard. The α-keto acid phenylpyruvic acid, complexed with iron, has a green color that has an absorbance peak at 614 nm. An $A_{614}$ of 1.0 was determined to be equal to 1.0 mM phenylpyruvic acid.

**Siderophore detection.** The qualitative production of siderophores was assayed by culturing bacteria on siderophore detection agar as described by Schwyn and Neilands (37). *E. coli* HB101 and *E. coli* SAB11 (*ent* mutant) were used as positive and negative controls, respectively. The production of siderophores is indicated by a yellow or orange color surrounding the colony; the lack of siderophore production results in no change in the green color of the agar.

**EDDA assay.** To assess the abilities of *P. mirabilis* and *E. coli* containing recombinant plasmids to chelate and internalize iron, the inhibition of bacterial growth was monitored on solid medium containing the synthetic iron chelator ethylenediamine-di-orthohydroxyphenyl acetic acid (EDDA). The procedure was modified from the method of Miles and Khimji (26) by inclusion of only one bacterial strain. Briefly, 9-cm-diameter petri plates were filled with 20 ml of Phe broth, solidified with 1.5% agar, containing EDDA (≥10 μg/ml) and about $10^4$ CFU/ml. Plates were observed after 20 h at 37°C for bacterial growth.

**Genomic library construction and screening of clones for amino acid deaminase activity.** A genomic library of *P. mirabilis* HI4320 chromosomal DNA was generated as previously described (20). Ampicillin-resistant cosmid clones were isolated and stored in 25% glycerol at −70°C in the wells of microtiter plates. For screening clones for amino acid deaminase activity, the stored library was thawed, inoculated into phenylalanine broth (200 μl in the wells of microtiter plates) containing ampicillin (100 μg/ml), and incubated at 37°C for 18 h. The addition of 5 μl of 10% ferric chloride to a well containing 200 μl of culture allowed the detection of amino acid deaminase-positive clones by the generation of a green color.

**Transposon mutagenesis.** Transposon mutagenesis was employed to map the amino acid deaminase activity on pSPH8, a *Sph*I-generated subclone. Plasmid pME9, which carries transposon Tn*5*::*751* and contains a temperature-sensitive replicon (41), was transferred by conjugation into *E. coli* DH5α transformed with deaminase-encoding clones. Transconjugants were plated onto medium containing chloramphenicol (marker for deaminase clone) and kanamycin (marker for Tn*5*::*751*) and incubated at 30°C. Plasmid DNA was isolated from pooled transconjugants by standard methods (1) and transformed into *E. coli* DH5α. Transformants were plated onto medium containing chloramphenicol (20 μg/ml) and kanamycin (50 μg/ml) and incubated at 43°C to select for the deaminase clone with Tn*5*::*751* insertions. Transformants were tested for amino acid deaminase activity. The insertion sites of Tn*5*::*751* were determined by restriction analysis with *Eco*RI, *Eco*RV, and *Bgl*II.

**Nucleotide sequencing.** Sequencing was performed by the dideoxy chain termination method with double-stranded DNA as the template (1). Reagents from the Prism Ready Reaction Dye Deoxy Termination Kit (Applied Biosystems) were used in conjunction with *Taq* polymerase (Boehringer Mannheim Corporation). Reactions were run on a model 373A DNA sequencer (Applied Biosystems).

**DNA and amino acid sequence analyses.** Genepro DNA sequence analysis software (Riverside Scientific Enterprises) was used for identification of open reading frames (ORFs) and restriction enzyme sites and for analysis of the deduced amino acid sequence. Putative promoters, control elements, and ribosomal binding sites were identified by visual inspection (45). The Genetics Computer Group sequence analysis software package, version 7.3.1 (University of Wisconsin), was used to screen the PIR and SwissProt databases for related polypeptides.

**PCR.** PCR was used to amplify a DNA fragment containing only the *aad* ORF. Oligonucleotides based on the acquired DNA sequence were synthesized by the phosphorimidite method on an Applied Biosystems automated DNA synthesizer (model 380B). Reactions were performed in an Ericomp thermocycler with Deep Vent DNA polymerase (New England Biolabs). Reactions were run for 30 cycles (94°C, 1 min; 45°C, 1 min; 72°C, 2 min). PCR products (5 μl) were analyzed on a 2.0% agarose gel.

**Southern and colony blot hybridization.** Southern blots of *P. mirabilis* chromosomal DNA and colony blots of various organisms were prepared by standard methods (1). Blots were hybridized with a 2.7-kb *Eco*RI-*Sph*I *aad*-encoding fragment that was labeled with the ECL random prime labeling kit (Amersham); blots were developed as instructed by the manufacturer.

**Nucleotide sequence accession number.** The nucleotide sequence reported here has been deposited at GenBank under the accession number U35383.

## RESULTS

**Evidence for lack of traditional siderophores in *P. mirabilis*.** To demonstrate that *P. mirabilis* does not produce the traditional enterobactin or aerobactin-type siderophores, 16 strains were plated onto siderophore detection agar (37). After 48 h of growth at 37°C, no indication of siderophore production, indicated by a yellow or orange color, was noted in comparison with *E. coli* HB101 and SAB11, as positive and negative controls, respectively.

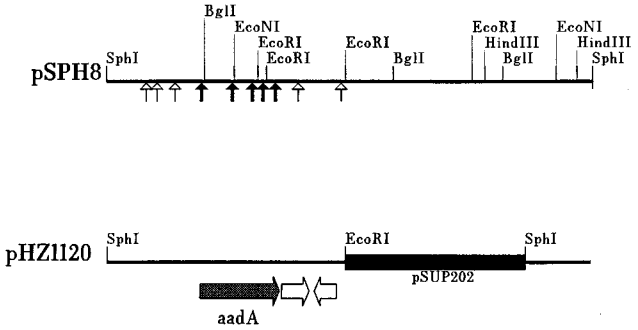


FIG. 1. Restriction maps of *aad*-containing plasmids. A restriction map of the 8.9-kb *Sph*I fragment of pSPH8, a 22-kb deaminase-positive subclone, is shown with the insertion points of 10 Tn*5*::*751* mutants. Filled arrows indicate the points of insertions that resulted in the loss of amino acid deaminase activity; open arrows indicate the points of insertions that remained deaminase positive. The map of pHZ1120 shows the locations of the *aad* ORF and two downstream ORFs.

**Isolation of the *P. mirabilis* amino acid deaminase gene *aad*.** *E. coli* does not produce an amino acid deaminase. This allowed for screening of a *P. mirabilis* genomic library hosted in *E. coli* DH5α for amino acid deaminase activity. One clone among approximately 1,500 tested repeatedly demonstrated qualitative amino acid deaminase activity. Cosmid p6G12 was isolated from this clone and transformed into *E. coli* DH5α and HB101. The resulting transformants were both positive for amino acid deaminase, verifying that p6G12 contained the gene sequences necessary for this activity.

**Mapping *aad*.** To localize the deaminase-encoding gene, subcloning of the amino acid deaminase activity was attempted by digestion of p6G12 with a variety of restriction enzymes (*Eco*RI, *Eco*RV, *Pst*I, *Pvu*I, *Sph*I, *Bgl* II, and *Sal* I-*Bam*HI) and subsequent ligation into the corresponding sites of pSUP202, a mobilizable cloning vector (41). The only positive subclones that were obtained resulted from incomplete digestion of p6G12 with *Sph*I and *Sal*I-*Bam*HI. The smallest partial *Sph*I-digested subclone (the 22-kb pSPH8) contained two *Sph*I fragments of 8.9 and 6.1 kb (the largest restriction fragment is shown in Fig. 1). Subclones generated with the other enzymes listed did not result in positive subclones. Partial *Eco*RI digestion and religation of pSPH8 resulted in a 7.2-kb amino acid deaminase-positive clone, pHZ1120, which carries a 4.2-kb insert (Fig. 1).

Transposon mutagenesis of pSPH8 was used to further localize the amino acid deaminase activity. The position of Tn*5*::*751* insertions and the resulting phenotypes (Fig. 1) demonstrated that sequences necessary for deaminase activity mapped to a 2.2-kb region, 1.2 kb downstream of the *Sph*I site.

**Nucleotide sequence of *aad*.** Restriction fragments of pSPH8 were cloned into pBlueScript for nucleotide sequencing; pHZ1120 was also used as template. The nucleotide sequence of the region identified as encoding amino acid deaminase by mapping with Tn*5*::*751* was determined for both strands (Fig. 2). An ORF of 1,419 nucleotides corresponded to the same location that was mapped with Tn*5*::*751* insertions and was designated *aad* (amino acid deaminase) (Fig. 1). Two other ORFs of 495 and 402 nucleotides were also identified downstream of *aad*.

The *aad* ORF predicts a 473-amino-acid polypeptide of

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AAT ACT TAT TTC GAC TCT TTT GTT CCA CTT TAT TAT CAA TAG GTT TTT TTT ATA AGT AAC      60

CAT AAT GTT TCT AAT GGT ATT ATG TCT TAT TAG TAT TAT TAA CAA ATT ATT ATA TTC ATT     120

                  -35                                 -10
AAC ATT ATA ATA AAA CAT GAT ATT ATA AAG CCT CTA AAA AAT AAT CTA ATA ACA ATA TAA     180
                  SD
ATA GTA AGA CTA TTA CTG AGG TAA AAG ATG AAC ATT TCA AGG AGA AAG CTA CTT TTA GGT     240
                                M  N  I  S  R  R  K  L  L  L  G
GTT GGT GCT GCG GGC GTT TTA GCA GGT GGT GCG GCT TTA GTT CCA ATG GTT CGC CGT GAC     300
V  G  A  A  G  V  L  A  G  G  A  A  L  V  P  M  V  R  R  D
GGC AAA TTT GTG GAA GCT AAA TCA AGA GCA TCA TTT GTT GAA GGT ACG CAA GGG GCT CTT     360
G  K  F  V  E  A  K  S  R  A  S  F  V  E  G  T  Q  G  A  L
CCT AAA GAA GCA GAT GTA GTG ATT ATT GGT GCC GGT ATT CAA GGG ATC ATG ACC GCT ATT     420
P  K  E  A  D  V  V  I  I  G  A  G  I  Q  G  I  M  T  A  I
AAC CTT GCT GAA CGT GGT ATG AGT GTC ACT ATC TTA GAA AAG GGT CAG ATT GCC GGT GAG     480
N  L  A  E  R  G  M  S  V  T  I  L  E  K  G  Q  I  A  G  E
CAA TCA GGC CGT GCA TAC AGC CAA ATT ATT AGT TAC CAA ACA TCG CCA GAA ATC TTC CCA     540
Q  S  G  R  A  Y  S  Q  I  I  S  Y  Q  T  S  P  E  I  F  P
TTA CAC CAT TAT GGG AAA ATA TTA TGG CGT GGC ATG AAT GAG AAA ATT GGT GCG GAT ACC     600
L  H  H  Y  G  K  I  L  W  R  G  M  N  E  K  I  G  A  D  T
AGT TAT CGT ACT CAA GGT CGT GTA GAA GCG CTG GCA GAT GAA AAA GCA TTA GAT AAA GCT     660
S  Y  R  T  Q  G  R  V  E  A  L  A  D  E  K  A  L  D  K  A
CAA GCG TGG ATC AAA ACA GCT AAA GAA GCG GCA GGT TTT GAT ACA CCA TTA AAT ACT CGC     720
Q  A  W  I  K  T  A  K  E  A  A  G  F  D  T  P  L  N  T  R
ATC ATT AAA GGT GAA GAG CTA TCA AAT CGC TTA GTC GGT GCT CAA ACG CCA TGG ACT GTT     780
I  I  K  G  E  E  L  S  N  R  L  V  G  A  Q  T  P  W  T  V
GCT GCA TTT GAA GAA GAT TCA GGC TCT GTT GAT CCT GAA ACA GGC ACA CCT GCA CTC GCT     840
A  A  F  E  E  D  S  G  S  V  D  P  E  T  G  T  P  A  L  A
CGT TAT GCC AAA CAA ATC GGT GTG AAA ATT TAT ACC AAC TGT GCA GTA AGA GGT ATT GAA     900
R  Y  A  K  Q  I  G  V  K  I  Y  T  N  C  A  V  R  G  I  E
ACT GCG GGT GGT AAA ATC TCT GAT GTG GTG AGT GAG AAA GGG GCG ATT AAA ACG TCT CAA     960
T  A  G  G  K  I  S  D  V  V  S  E  K  G  A  I  K  T  S  Q
GTT GTA CTC GCT GGG GGT ATC TGG TCG CGT TTA TTT ATG GGC AAT ATG GGT ATT GAT ATC    1020
V  V  L  A  G  G  I  W  S  R  L  F  M  G  N  M  G  I  D  I
CCA ACG CTC AAT GTA TAT CTA TCA CAA CAA CGT GTC TCA GGG GTT CCT GGT GCA CCA CGT    1080
P  T  L  N  V  Y  L  S  Q  Q  R  V  S  G  V  P  G  A  P  R
GGT AAT GTG CAT TTA CCT AAT GGT ATT CAT TTC CGC GAA CAA GCG GAT GGT ACT TAT GCC    1140
G  N  V  H  L  P  N  G  I  H  F  R  E  Q  A  D  G  T  Y  A
GTT GCA CCA CGT ATC TTT ACG AGT TCA ATA GTC AAA GAT AGC TTC CTG CTA GGG CCT AAA    1200
V  A  P  R  I  F  T  S  S  I  V  K  D  S  F  L  L  G  P  K
TTT ATG CAC TTA TTA GGT GGC GGA GAG TTA CCG TTG GAA TTC TCT ATT GGT GAA GAT CTA    1260
F  M  H  L  L  G  G  G  E  L  P  L  E  F  S  I  G  E  D  L
TTT AAT TCA TTT AAA ATG CCG ACC TCT TGG AAT TTA GAT GAA AAA ACA CCA TTC GAA CAA    1320
F  N  S  F  K  M  P  T  S  W  N  L  D  E  K  T  P  F  E  Q
TTC CGA GTT GCC ACG GCA ACA CAA AAT ACG CAA CAC TTA GAT GCT GTT TTC CAA AGA ATG    1380
F  R  V  A  T  A  T  Q  N  T  Q  H  L  D  A  V  F  Q  R  M
AAA ACA GAA TTC CCA GTA TTT GAA AAA TCA GAA GTT GTT GAA CGT TGG GGT GCC GTT GTG    1440
K  T  E  F  P  V  F  E  K  S  E  V  V  E  R  W  G  A  V  V
AGT CCA ACA TTT GAT GAA TTA CCT ATC ATT TCT GAG GTC AAA GAA TAC CCA GGC TTA GTG    1500
S  P  T  F  D  E  L  P  I  I  S  E  V  K  E  Y  P  G  L  V
ATT AAC ACG GCA ACA GTG TGG GGT ATG ACA GAA GGC CCG GCA GCG GGT GAA GTG ACC GCT    1560
I  N  T  A  T  V  W  G  M  T  E  G  P  A  A  G  E  V  T  A
GAT ATT GTC ATG GGC AAG AAA CCT GTT ATT GAT CCA ACG CCG TTT AGT TTG GAT CGT TTT    1620
D  I  V  M  G  K  K  P  V  I  D  P  T  P  F  S  L  D  R  F
AAG AAG TAA ACC CTT GTT TTA CTG TAA TTA AAC ACA TTA AGG AGT TTT CAT GCG CTA TAA    1680
K  K
AAG TCT ACT TGT TGC TTT ACC TTT CGT GTT CGG TGT AGC AAG TGC AAA TGC AGC GGA GAA    1740

TGT TGT GCA TCA TAA ATT AGA CGG TAT GCC TAT TTC AGA AGC AGT TGA AAT TAA TGC AGG    1800
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FIG. 2. Nucleotide sequence of *aad* from *P. mirabilis* HI4320. The amino acid translation is represented by a single-letter code under the first nucleotide of each codon. Putative promoter (−35 and −10) and Shine-Dalgarno (SD) sequences are overlined. The 1,419-bp *aad* ORF predicts a 473-amino-acid polypeptide of 51,151 Da. Underlined sequences indicate locations of forward and reverse (complement of nucleotides shown) primers.

51,151 Da with a net charge of −1. Putative promoter sequences, identified as (−35) A*TGAT*A and (−10) A*ATA AT (mismatch from consensus is followed by an asterisk [45]), are separated by 17 nucleotides. A putative ribosomal binding site of AGG was identified 6 bp upstream of the start codon (Fig. 2). The G+C content of the *aad* ORF was 43.5%, slightly higher than the overall G+C content of *P. mirabilis* genomic DNA (39%) (14).

The hydropathy plot of Aad predicts a soluble protein with a hydrophobic region at the N terminus. The characteristics of this sequence, however, do not conform to the specifications of the traditional leader peptide (45) (Fig. 3).

No significant amino acid sequence similarity (≥20%) was found between the predicted polypeptide predicted by *aad* and any polypeptide in the PIR and SwissProt databases when

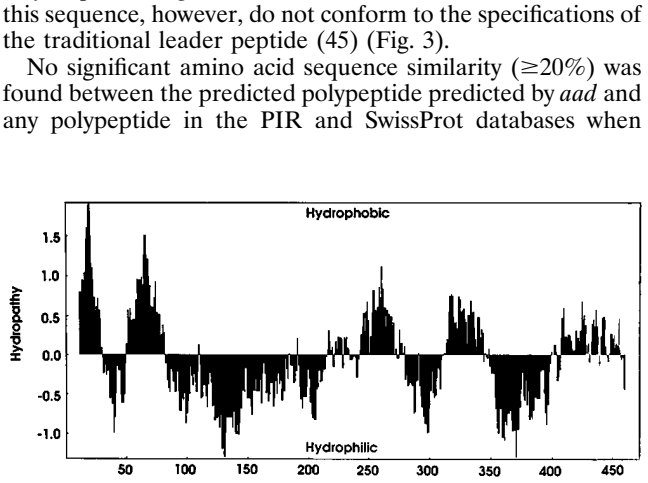


FIG. 3. Hydropathy plot of Aad. Hydropathy was calculated for Aad by the algorithm of Kyte and Doolittle (21) with a window of 20 amino acid residues. The N-terminal hydrophobic region does not possess the characteristics of a cleavable leader peptide.

searched with the Genetics Computer Group sequence analysis package, version 7.3.1 (University of Wisconsin). The polypeptide predicted by ORF134, downstream of *aad*, shares 61.7% identity with a hypothetical 18.9-kDa protein in the mob (mobilization) 5′ region of the *E. coli* chromosome (accession no. P32125).

**Deaminase activity is encoded by a single gene.** With pHZ1120 as template, PCR was used to amplify a DNA fragment containing only the *aad* ORF. The upstream primer (TTTCTAATGGTATTATGTCTTATT, nucleotides 67 to 92) allowed for 140 bp upstream of the putative start codon. The downstream primer (CGAACACGAAAGGTAAAGC, complementary to nucleotides 1693 to 1712) allowed for 70 bp downstream of the putative stop codon (Fig. 2). To confirm that the ORF designated *aad* was sufficient for amino acid deaminase activity, the 1,640-bp fragment generated by PCR was cloned into the *Srf* I site of pCR-SCRIPT KS(+). This clone, pAAD31, which contains the 1,419-bp ORF with 140 bp upstream and 70 bp downstream, was positive for amino acid deaminase activity, demonstrating that this one gene is both necessary and sufficient for enzyme activity.

**Prevalence and copy number of *aad*.** Southern blot analysis of *P. mirabilis* chromosomal DNA of six strains (two strains from patients with acute pyelonephritis [CFT295 and CFT37] and four strains from patients with catheter-associated bacteriuria [MA2489, HI4320, DR535, and HU2450]) demonstrated that *aad* was present in all strains tested and is conserved on a 2.7-kb *Sph*I-*Eco*RI restriction fragment (data not shown). The *aad* probe did not react with chromosomal DNA isolated from other bacterial species including urinary tract isolates of *E. coli* (five strains), *Providencia stuartii* (three strains), *Providencia rettgeri* (two strains tested), and *Morganella morganii* (five strains).

To determine the copy number of *aad*, Southern blots of *P. mirabilis* HI4320 chromosomal DNA, digested separately with several restriction enzymes, were prepared and hybridized with the *aad* probe. The pattern of the hybridizing bands corresponded to the known map of the cloned *aad*, with no additional reacting bands, suggesting that only one copy of *aad* is present on the *P. mirabilis* chromosome (data not shown).

**Expression of native and recombinant amino acid deaminases.** To investigate possible regulatory mechanisms, activity of the amino acid deaminase from cellular extracts and the amount of phenylalanine deaminated in the culture medium were quantitatively determined. During growth in phenylalanine broth without glucose, native deaminase activity in cytoplasmic extracts of *P. mirabilis* HI4320 rose fivefold during logarithmic phase growth ($A_{600}$ of 0.1 to 0.5) and remained at that level throughout stationary phase (Fig. 4). The appearance and subsequent increase of phenylpyruvic acid in the culture supernatant lagged slightly behind the appearance of the deaminase activity in the cytoplasmic extracts.

Deaminase activity was repressed by addition of glucose to the culture medium. Culture in medium containing 25 mM glucose resulted in a reduction of deaminase activity after the cells had reached stationary phase with a corresponding reduction in phenylpyruvic acid in the culture supernatant. The reduction of phenylpyruvic acid in the culture supernatant corresponded to a reduction of deaminase activity in cellular extracts (Fig. 4). The concentration of phenylpyruvic acid present in the culture supernatants of both *P. mirabilis* and *E. coli* HB101(pHZ1120) was inversely proportional to the amount of glucose in the medium (Fig. 5). Culture supernatant of *E. coli* HB101(pSUP202), the vector control, contained no phenylpyruvic acid. At 25 mM glucose, the amount of deaminated phenylalanine was 61-fold lower for *P. mirabilis* and

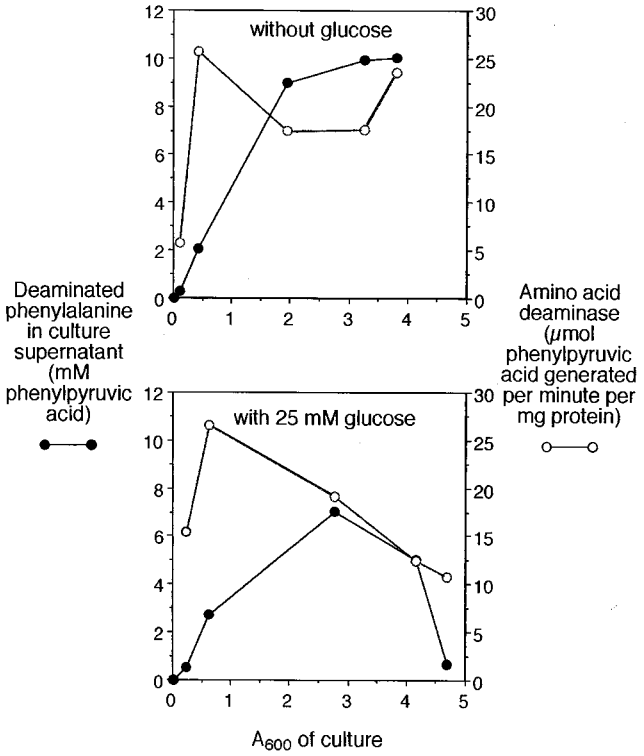


FIG. 4. Growth phase versus cytoplasmic amino acid deaminase activity and concentration of deaminated phenylalanine in the culture supernatant. *P. mirabilis* HI4320 was grown in phenylalanine broth without and with 25 mM glucose. Samples were removed at timed intervals. $A_{600}$ of the culture, the amount of phenylpyruvic acid in the supernatant, and the enzymatic activity of cytoplasmic extracts were determined.

13-fold lower for *E. coli* HB101(pHZ1120) compared with the respective strains cultured in the absence of glucose. The higher values observed for *E. coli* HB101(pHZ1120) cultured without glucose may be the result of higher copy number in the recombinant strain.

Deaminase activity was not affected by medium iron concentration. The amount of deaminase activity from cytoplasmic extracts of *P. mirabilis* HI4320, grown in a high iron concentration (Luria broth) or low iron concentration [Luria broth with 50 μg of ethylenediamine di(*o*-hydroxyphenylacetic acid) per ml], was not significantly different at 11.5 ± 1.7 versus 10.6 ± 0.85 μmol of phenylpyruvic acid generated per min per mg of protein, respectively.

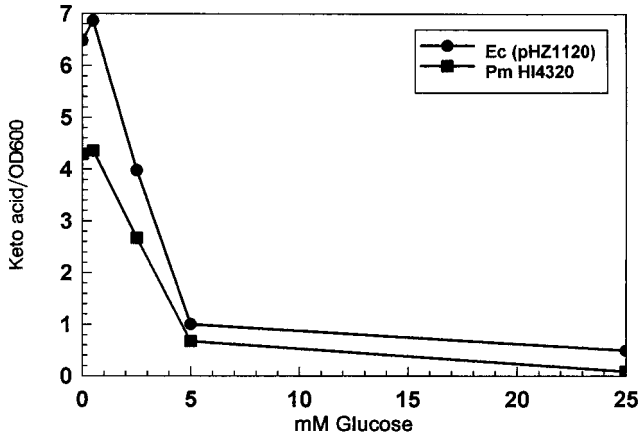


FIG. 5. Effect of culture medium glucose concentration on production of deaminated phenylalanine by *P. mirabilis* and *E. coli* HB101(pHZ1120). Concentration of deaminated phenylalanine was determined in the culture supernatant of cells grown with aeration at 37°C for 18 h in phenylalanine broth at various glucose concentrations. Ec, *E. coli*; Pm, *P. mirabilis*.

The activity of the amino acid deaminase expressed in *E. coli* containing multicopy deaminase-encoding plasmids was also not significantly affected by mutations in *fur* (iron regulation), *cya* (adenylate cyclase), or *crp* (catabolite repression). By comparing deaminase activity of French press lysates of *fur*$^+$ *E. coli* SM(pHZ120) and *fur E. coli* SBC(pHZ120), no apparent Fur-dependent regulation was noted. In a single experiment with duplicate samples, the results (in micromoles per minute per milligram of protein) were as follows: Luria broth, 12 versus 10.6; in Luria broth containing 200 μg of EDDA per ml, 7.8 versus 12.0; and in Luria broth plus 100 nM Fe, 9.4 versus 8.4. Likewise, the repression of deaminase activity by 25 mM glucose, observed in parent strain *E. coli* χ289(pAAD31) (10-fold repression by glucose) was not reversed either in the *cya* mutant *E. coli* χ6161(pAAD31) (6-fold repression) or in the *crp* mutant *E. coli* χ6162(pAAD31) (23-fold repression). These latter preliminary measurements suggested that the mechanism of glucose-induced repression of deaminase activity does not involve catabolite repression.

**Attempted construction of a deaminase-deficient mutant.** To assess the physiological consequences of the inactivation of amino acid deaminase, construction of a deaminase-negative strain of *P. mirabilis* H14320 was attempted by insertion of a *Hin*cII Kan$^r$ cassette into the T4 polymerase-treated *Eco*NI site within the *aad* ORF of pAAD31, cloning of the resulting 3.2-kb *Pvu*II fragment containing the interrupted gene into the *Eco*RV site of pSUP202, and electroporation of the construct (designated pARN3) into *P. mirabilis* HI4320. Kan$^r$ Amp$^r$ colonies were passaged repeatedly on nonselective medium, and then 7,500 colonies were screened. No Kan$^r$ Amp$^s$ colonies (i.e., clones in which allelic exchange had occurred and the plasmid had been cured) were isolated. Since these procedures have been used routinely to construct isogenic mutants in *mrp* (5), *pmf* (25), and *ure* (19) gene clusters, we concluded that mutation of the deaminase gene may represent a lethal event.

**Deaminase activity in an enterobactin-negative *E. coli* background.** To determine whether α-keto acids generated by amino acid deaminase were able to chelate iron and provide the iron to the cell in usable form, a siderophore-negative strain, *E. coli* SAB11, was used. Growth of this strain can be inhibited completely by EDDA-induced iron starvation at EDDA concentrations of ≥10 μg/ml. If amino acid deaminase products function as siderophores and can be transported into the bacterium, we postulated that *E. coli* SAB11 containing *aad* clones would be able to overcome the EDDA inhibition. To test this hypothesis, suspensions ($10^4$ CFU) of deaminase-positive *E. coli* SAB11(pHZ1120) were inoculated onto Phe broth agar containing EDDA at concentrations of 0, 10, 25, and 50 μg/ml. After 20 h of incubation, however, no bacterial growth was detected at EDDA concentrations of 10 μg/ml and higher, indicating that either the α-keto acids could not chelate the iron bound by EDDA or the bacterium could not transport the ferric-keto acid complexes into the cell or both.

**Utilization of ferric–α-keto acid complexes.** To determine whether the *aad* clone encodes a transport system, ferric–α-keto acids, including Fe-α-ketoisovaleric acid and Fe-α-ketoisocaproic acid, were applied as spots to the surfaces of EDDA plates containing *E. coli* SAB11(pHZ1120) ($10^4$/ml). The final concentration of ferric complexes was 0.2 mM. The

ratio of Fe to ligand was 1:30. No growth was observed on the plates, indicating a failure to transport ferric–α-keto acid compounds into the *E. coli* strain. Thus, the ability of amino acid deaminase to reverse EDDA-induced iron restriction could not be properly assessed because of the inability of the *E. coli* strain to transport ferric-keto acid complexes.

**Cross-feeding of *P. mirabilis* by deaminase clones.** To determine whether increased availability of deaminase would allow *P. mirabilis* to overcome EDDA-induced iron restriction, different dilutions of *P. mirabilis* HI4320 ($10^4$, $10^5$, and $10^6$ CFU) were seeded onto EDDA (200 μg/ml)-containing plates. A suspension ($10^6$ to $10^7$/10 μl) of *E. coli* DH5α(pHZ1120) was inoculated as a spot onto the surface of the plates. Cross-feeding by the deaminase-producing recombinant clone did not allow growth of the *P. mirabilis* strain, suggesting that increasing the concentration of secreted amino acid deaminase was still not sufficient to overcome the iron limitation.

## DISCUSSION

The single *aad* gene from uropathogenic *P. mirabilis*, the product of which is involved in the deamination of amino acids, appears to be both necessary and sufficient to carry out this reaction. Deamination of phenylalanine distinguishes *Proteus*, *Providencia*, and *Morganella* species from all other genera of the *Enterobacteriaceae*, and it is postulated that the action of the deaminase may play a role in the acquisition of iron for the bacterium. Although there appears to be only one copy of this gene in the chromosome, it has not been determined whether *aad* represents the only gene which encodes an amino acid deaminase.

Drechsel et al. (11) proposed that the α-keto acids generated by the action of amino acid deaminase(s) represent a novel type of siderophore. *Proteus*, *Providencia*, and *Morganella* species do not produce the typical phenolate- or hydroxamate-type siderophores that are common among other genera of the *Enterobacteriaceae*. The production of enzymes required for the biosynthesis and transport of siderophores is regulated by the iron status of the cell. The Fur protein serves as the detector of iron availability and the repressor of siderophore genes (9, 10, 15, 16). When the environment becomes iron limited as in the host, bacteria respond by increasing the production of their siderophore(s). Along these lines, if the only role of the α-keto acids generated by the action of amino acid deaminase(s) is the role of siderophore, one would assume that the expression of *aad* would be regulated by iron availability. This does not appear to be the case. The nucleotide sequence upstream of *aad* does not contain the consensus Fur binding site (iron box), which is found upstream from iron-regulated genes (9, 10, 15, 16). In addition, the activity of the amino acid deaminase was not affected by iron restriction in either *P. mirabilis* or *E. coli* carrying *aad* on a plasmid. Amino acid deaminase activity expressed in *E. coli* from an *aad*-containing plasmid in low or high iron was also similar in *fur*$^+$ and *fur* backgrounds. The lack of iron regulation of *aad* does not necessarily rule out the role of α-keto acids as siderophores but rather suggests that these compounds may have additional functions in cellular metabolism.

The introduction of a preferred carbon source (e.g., glucose or glycerol) into the culture medium resulted in a reduction of deaminated phenylalanine in the supernatant and a reduction of amino acid deaminase activity in the cytoplasmic extracts. The effect on deaminase activity by glucose does not appear to be due to catabolite repression, however, since there was no difference between plasmid-encoded deaminase activities when genes were expressed in *E. coli cya* or *crp* mutants and when they were expressed in the corresponding wild-type host, grown in the presence or absence of glucose. Catabolite repression consensus sequences (22) were also not present upstream of *aad*. The finding that the deaminated phenylalanine disappears from the culture medium when the production and/or activity of the enzyme is decreased suggests that the product may be converted into another product or is unstable.

The deaminase-negative phenotype of *E. coli* allowed us to isolate an amino acid deaminase-positive clone from a genomic library of *P. mirabilis* HI4320 DNA. Traditional molecular cloning techniques and transposon mutagenesis mapped the amino acid deaminase activity to a specific region of the original cosmid. The nucleotide sequence was determined for this region, and an ORF of 1,419 nucleotides was identified that corresponded to the region mapped by transposon mutagenesis and was designated *aad* (amino acid deaminase). This gene predicts a polypeptide of 473 amino acids with a molecular size of 51,151 Da. The predicted polypeptide shared no significant amino acid sequence similarity with any polypeptide in the SwissProt or GenBank database. The lack of similarity between Aad and any other polypeptide is not surprising since *aad* represents the first amino acid deaminase gene for which the nucleotide sequence has been determined. It was surprising, however, that an *aad* gene probe did not hybridize with genomic DNA from *Providencia* or *Morganella* species. This observation suggests that these distinct genera produce a deaminase activity that is encoded by gene sequences that may not be closely related to the *aad* gene of *P. mirabilis*. Alternatively, the deaminase may be conserved at the level of amino acid sequence but not nucleotide sequence.

The role of Aad in either iron acquisition or basic cellular metabolism has not been elucidated. The generation of isogenic mutants of *aad* is paramount to addressing these concerns. Unfortunately, we have been unsuccessful in the construction of such mutants by standard mutagenesis methods that we have employed successfully for other putative virulence genes (*pmf* and *mrp* fimbrial genes and urease genes). This suggests but does not prove that inactivation of *aad* is lethal for *P. mirabilis*. If Aad is essential for cellular metabolism, it may prove difficult to construct a knockout mutation and definitively demonstrate its role in infection.

Additional attempts to demonstrate that deaminase products could overcome artifically induced iron restriction were made with a siderophore-negative *E. coli* strain transformed with the deaminase clone. The inability to overcome growth restriction may have been due in this case to the inability of *E. coli* to transport ferric-keto acid complexes. Alternatively, the affinity of α-keto acids for iron may be too low to overcome EDDA chelation under any circumstances and thus may require the use of lower-affinity iron chelators.

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