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Pathway and Enzyme Redundancy in Putrescine Catabolism in *Escherichia coli*

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Putrescine as the sole carbon source requires a novel catabolic pathway with glutamylated intermediates. Nitrogen limitation does not induce genes of this glutamylated putrescine (GP) pathway but instead induces genes for a putrescine catabolic pathway that starts with a transaminase-dependent deamination. We determined pathway utilization with putrescine as the sole nitrogen source by examining mutants with defects in both pathways. Blocks in both the GP and transaminase pathways were required to prevent growth with putrescine as the sole nitrogen source. Genetic and biochemical analyses showed redundant enzymes for γ-aminobutyraldehyde dehydrogenase (PatD/YdcW and PuuC), γ-aminobutyrate transaminase (GabT and PuuE), and succinic semialdehyde dehydrogenase (GabD and PuuC). PuuC is a nonspecific aldehyde dehydrogenase that oxidizes all the aldehydes in putrescine catabolism. A *puuP* mutant failed to use putrescine as the nitrogen source, which implies one major transporter for putrescine as the sole nitrogen source. Analysis of regulation of the GP pathway shows induction by putrescine and not by a product of putrescine catabolism and shows that putrescine accumulates in *puuA*, *puuB*, and *puuC* mutants but not in any other mutant. We conclude that two independent sets of enzymes can completely degrade putrescine to succinate and that their relative importance depends on the environment.

Polyamines are ubiquitous and important biological molecules. The major polyamine in *Escherichia coli* is the diamine putrescine (1,4-diaminobutane), although the triamine spermidine (aminopropyl-putrescine) and the diamine cadaverine (1,5-diaminopentane) are also present (6). Intracellular polyamine concentrations positively correlate with growth rate (2, 51). A variety of functions have been attributed to the polyamines. Polyamines affect ribosomal structure, protein and nucleic acid elongation rates, translational fidelity, chromosomal structure, and interactions between mRNA and ribosomes (8, 9, 15, 53). In *Escherichia coli*, polyamines, especially putrescine, affect the expression of several hundred genes, often affecting translation of specific genes (3, 52, 53). A polyamine-deficient mutant still grows aerobically, albeit about twice as slowly, but does not grow anaerobically or in 95% oxygen and is more sensitive to reactive oxygen species (5, 33). An appropriate level of polyamines would seem to be important for a variety of functions during growth. In addition, polyamine accumulation can be detrimental (1, 11, 28). Polyamines are also involved in several stress responses, and not surprisingly stress responses can affect intracellular polyamine concentrations (1, 34, 49, 50).

Putrescine can also be used a the sole nitrogen source. Growth with a nitrogen source other than ammonia limits growth and is considered nitrogen limited. Such growth induces at least 100 genes whose products assimilate ammonia and scavenge nitrogen-containing compounds (40, 54). This coordinated expression is called the nitrogen-regulated or Ntr response. Twenty Ntr proteins transport and degrade arginine (which can be degraded to putrescine), γ-aminobutyric acid (GABA) (a product of putrescine catabolism), and possibly putrescine (54).

Two pathways have been proposed to degrade putrescine via GABA to succinate (Fig. 1). Transaminase-dependent deamination initiates one pathway (48), while glutamylation of putrescine initiates a second pathway (23). The glutamylated putrescine (GP) pathway is essential for utilization of putrescine as the sole carbon source (22–26). Putrescine as the sole nitrogen source may involve the transaminase-dependent pathway, since nitrogen limitation appears to induce proposed enzymes of the transaminase-dependent pathway but not enzymes of the GP pathway (54). Furthermore, genetic analyses have not established which enzymes catalyze which reactions *in vivo*. We characterized utilization of putrescine as the sole nitrogen source by examining growth in mutants lacking genes of both pathways. We show that both pathways contribute to putrescine catabolism, redundant enzymes catalyze several reactions, and one enzyme, PuuC, catalyzes three separate reactions. We also characterize regulation of the GP pathway. A separate communication will describe the complex stress-induced regulation of the transaminase pathway. We conclude that two independent pathways can degrade putrescine, and their relative importance depends on growth conditions.

MATERIALS AND METHODS

Strains and plasmids. All strains used for growth rate determinations and enzyme assays are derivatives of *E. coli* K-12 strain W3110. Strains and plasmids used in this study are listed in Table 1. Deletions were constructed and verified as described previously (7). All deletions are in-frame deletions if the antibiotic resistance gene is removed. The extent of deletions in individual genes is shown in Table 2. The deletion of *ydcSTUV* left codons 1 to 5 of *ydcS* and codons 228 to 264 of *ydcV*. The deletion of *ydcSTUV-patD* left codons 1 to 5 of *ydcS* and 468 to 474 of *patD*. A derivative of PKD13 (7), pKD13cat, in which the chloramphenicol marker replaced the original kanamycin marker, was used to construct strains with chloramphenicol resistance. P1 phage transductions were done as described previously (32). The strains were tested for the appropriate antibiotic resistance, and the deletion or insertion was verified by PCR.

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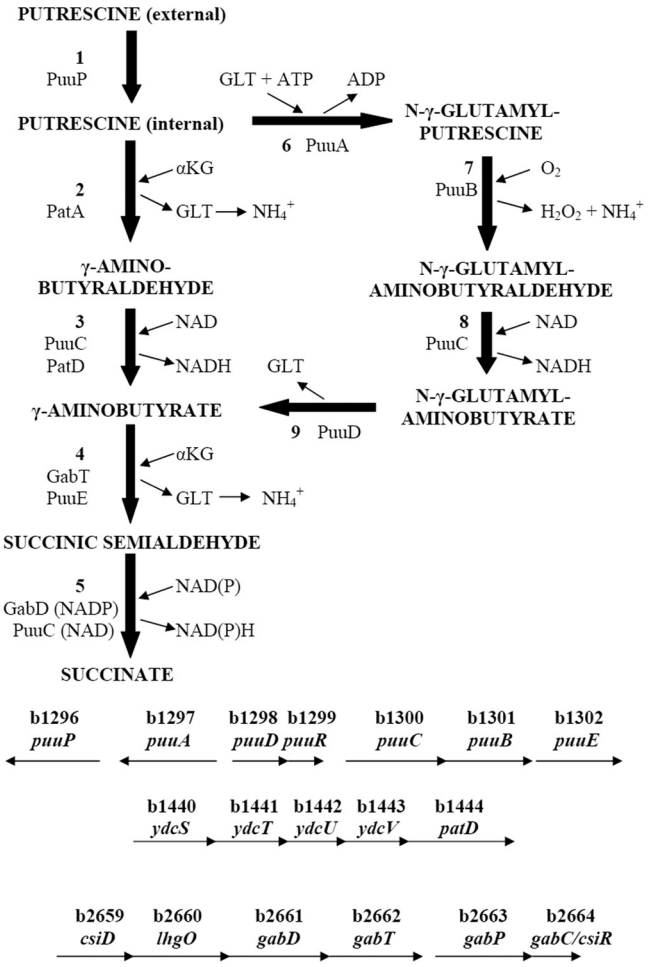

FIG 1 Pathways and genes of putrescine catabolism. The transaminase pathway is reactions 2 to 5 (PatA, PatD, GabT, and GabD), and the glutamylated putrescine (GP) pathway is reactions 6 to 9, 4, and 5 (PuuA, PuuB, PuuC, PuuD, PuuE, and PuuC). Reactions 2, 4, and 7 generate ammonia for assimilation. The abbreviations are GLT for glutamate and α-KG for α-ketoglutarate. All the genes considered in this work are indicated in the bottom half, except for *patA* (b3073). The solid arrows indicate the genes and the direction of transcription, drawn to scale. There are documented promoters preceding *puuA*, *puuD*, *ydcS*, *csiD*, and *gabD*. Other promoters may exist. Evidence for a *puuAP* operon is presented in this work. Evidence for a nitrogen-regulated *gabDTPC* operon has been presented (46). Evidence for the larger *csiD-lhgO-gabDTPC* operon controlled by σ^S has been described (30). The *patA* gene (not shown) is monocistronic.

Plasmids from the ASKA collection were used for complementation studies. These plasmids contained *puuA*, *puuB*, *puuC*, *puuD*, *puuP*, *puuR*, *patA*, *patD*, and *gabD* under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. These plasmids code for chloramphenicol resistance. When the mutant was also chloramphenicol resistant, the *cat* gene from the ASKA plasmid was deleted by digesting with SspI and replacing it with the pUC18-derived ampicillin resistance marker *bla*. The AlwNI-BsrBI fragment from pUC18 was blunt-ended using T4 DNA polymerase before ligation into the ASKA plasmid. The ampicillin-resistant plasmid was constructed for plasmids containing *gabD*, *puuC*, *puuD*, and *puuR*. Detectable, and often abundant, protein was observed for PuuB, PuuC, PuuD, PuuE, PatD, and GabD but not PuuA, PuuC, PuuP, and PatA (http://ecoli.naist.jp/GB8). Despite the failure to observe PuuC and PuuP, each complemented the appropriate mutant. Plasmids containing *puuA* and *patA* did not complement the appropriate mutant, but there is no evidence that these plasmids code for a functional protein.

The plasmid pBLS17 was used to construct *lacZ* translational fusions (20). pBLS18, a derivative of pBLS17 with *lacZ* from pAH125 with the *trp* terminator deleted, was used to construct *lacZ* transcriptional fusions (20). To construct the *puuA-lacZ* and *puuD-lacZ* fusions, a PCR product containing 53 bases upstream from the start codon of *puuA* to 59 bases upstream from the start codon of *puuD* was inserted into plasmids pBLS17 and pBLS18. The *lacZ* fusions were integrated into the chromosomal lambda attachment site and checked for single insertion by PCR (12). pBLS17 and pBLS18 were also inserted into the chromosome as negative controls.

Media and growth conditions. W salts minimal medium contained 0.4% glucose as the carbon source and 0.2% of each nitrogen source (41). All cultures for growth studies and assays were grown at 30°C and aerated

TABLE 1 Strains and growth rates in glucose-putrescine medium[a]

Line no.	Strain	Description[b]	Generation time (min ± SEM)
1	W3110	*lacL8 lacI*^{q}	225 ± 7 (18)
2	CP2	Δ*patA*	220 ± 11 (6)
3	CP6	Δ*gabT*	221 ± 14 (4)
4	CP14	Δ*puuE*::*cat*	183 ± 10 (3)
5	CP36	Δ*gabT* Δ*puuE*::*cat*	305 ± 8 (3)
6	BLS71	Δ*patA* Δ(*ydcSTUV-patD*)::*kan*	240 ± 14 (9)
7	BLS73	Δ(*ydcSTUV-patD*)::*kan*	228 ± 7 (24)
8	BLS74	Δ*patA* Δ*patD*::*cat*	227 ± 8 (9)
9	BLS75	Δ*patD*::*cat*	241 ± 13 (10)
10	BLS77	Δ*puuR*::*cat*	NG, S
11	BLS79	Δ*puuP*::*cat*	NG
12	BLS80	Δ*puuA*::*cat*	NG
13	BLS81	Δ*puuA*	348 ± 9 (6)
14	BLS82	Δ*patA* Δ*patD*	210 ± 9 (9)
15	BLS83	Δ*patD*	241 ± 9 (12)
16	BLS84	Δ*puuR*	121 ± 6 (12)
17	BLS85	Δ*puuP*	NG
18	BLS88	Δ*patA* Δ*puuA*	NG
19	BLS89	Δ*puuC*::*cat*	305 ± 15 (8)
20	BLS90	Δ*puuC*	315 ± 12 (10)
21	BLS93	Δ*puuC* Δ*patD*	VSG
22	BLS96	Δ*puuB*::*cat*	320 ± 10 (6)
23	BLS97	Δ*puuB*	367 ± 18 (13)
24	BLS98	Δ*puuB patA*::*kan*	NG
25	BLS99	Δ*puuC* Δ(*ydcSTUV-patD*)::*kan*	VSG
26	BLS100	Δ*puuC* Δ*patD*::*cat*	VSG
27	BLS102	Δ*gabD*::*cat* Δ*patD*	235 ± 10 (3)
28	BLS103	Δ*puuC* Δ*patD* Δ*gabD*::*cat*	NG
29	BLS104	Δ*ydcSTUV*::*cat*	234 ± 7 (6)
30	BLS105	Δ*ydcSTUV*	225 ± 9 (6)
31	BLS106	Δ*puuC* Δ*gabD*::*cat*	443 ± 24 (3)
32	BLS108	Δ*csiD* Δ*puuA*	344 ± 16 (3)
33	BLS109	Δ*puuA* Δ*patD*	507 ± 33 (3)
34	BLS110	Δ*patA* Δ*puuC*	NG
35	PAJ2	Δ*puuD*	NG
36	SR4	Δ*gabD*::*cat*	184 ± 23 (4)

[a] NG, no growth; S, suppressors accumulate frequently on plates; VSG, very slow growth (10% increase per day) for several days, before a growth burst, which is assumed to be from accumulation of suppressors.

[b] Deletions are assumed to be polar for genes with an antibiotic resistance gene indicated. In-frame deletions are assumed to be nonpolar, and there is no associated antibiotic resistance gene.

TABLE 2 Extent of deletions in genes

Gene	Size (no. of residues)	Deleted residues
gabD	482	109–482
gabT	426	47–391
patD	474	2–467
patA	497	1–488
puuA	472	6–462
puuB	426	9–387
puuC	495	5–485
puuD	254	8–233
puuE	421	2–391
puuP	461	5–452
puuR	185	6–181

at 220 rpm. Growth was monitored with a Klett colorimeter, model 800-3, using a 42 filter. Luria-Bertani broth and agar plates were supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, or 20 μg/ml chloramphenicol where appropriate. The complementation analysis required determining the correct concentration of inducer for expression of the complementing gene from the appropriate ASKA plasmid. Initially, experiments were performed with 100 μM IPTG, and this worked well for the plasmids with *puuB*, *puuD*, *puuP*, *puuR*, *patD*, and *gabD*. If this failed, higher and lower concentrations were tested. The plasmid with *puuC* required a higher inducer concentration (200 μM) and did not generate detectable PuuC protein after induction and purification (http://ecoli.naist.jp/GB8). These results imply poor expression.

Enzyme assays. For all assays, 10-ml cultures were harvested in late exponential phase (~100 Klett units or A_{600} of ~0.6), pelleted at 4°C, washed twice with cold 150 mM NaCl, and frozen at −80°C. For β-galactosidase assays, thawed cell pellets were resuspended in 1 ml Z buffer (31) with 1 mM β-mercaptoethanol and sonicated on ice in three 5-s bursts. After centrifugation at 4°C, the supernatants were assayed as described previously (31). For dehydrogenase assays, thawed cells were resuspended in 0.1 M KPO_4 buffer (pH 7.5)-1 mM dithiothreitol and disrupted with three 5-s bursts of sonication. Succinic semialdehyde dehydrogenase activity was assayed in 0.1 M KPO_4 buffer (pH 7.8), 0.28 mM NADP or NAD, and 0.6 mM succinic semialdehyde at 37°C. The reaction was started with the addition of succinic semialdehyde, and the A_{340} was monitored. For the γ-aminobutyraldehyde dehydrogenase assay, crude extracts were ultracentrifuged for 90 min at 120,000 × *g* to remove NADH-oxidizing activity. This dehydrogenase was assayed in glycine buffer (pH 9.5), 0.28 mM NADP or NAD, and 0.5 mM γ-aminobutyraldehyde at 37°C. The reaction was started with the addition of γ-aminobutyraldehyde, and the A_{340} was monitored. The γ-aminobutyraldehyde substrate was freshly prepared from 4-amino-butyraldehyde diethylacetal (Sigma-Aldrich) by boiling for 10 min in 0.2 M HCl (47). All protein concentrations were determined using bovine serum albumin as a standard (29). Activities were expressed as nmol minute^{-1} mg protein^{-1}.

RESULTS

The genetics of putrescine catabolism to GABA. Our previous studies of GABA and arginine as the sole nitrogen sources were conducted for cells grown at 30°C (21, 45, 46). Therefore, we studied putrescine as the sole nitrogen source at this temperature. In contrast, analysis of putrescine as the sole carbon source was conducted at 20°C (22–26). These differences are significant and will be discussed later.

We first disrupted putrescine catabolic genes of the transaminase pathway, which are known to be nitrogen regulated (54). The transaminase pathway converts putrescine to γ-aminobutyrate by a transaminase-dependent deamination, catalyzed by PatA (also called YgjG), and an NAD-dependent aminobutyraldehyde dehydrogenase (ABDH), catalyzed by YdcW (also called Prr) (43, 44). Mutants with in-frame deletions of their genes have not been described. We will show that they are the major enzymes that catalyze their respective reactions. We redesignated YdcW as PatD (putrescine aminotransferase pathway, dehydrogenase). Colonies of CP2 (Δ*patA* strain) were smaller than those of W3110 (wild type) on agar plates containing either broth or glucose-putrescine minimal medium (not shown). However, CP2 in liquid minimal medium grew normally with putrescine, ammonia, or alanine as the sole nitrogen sources, slightly faster (≤20%) with ornithine or GABA, and slightly slower (≤20%) with agmatine (Fig. 2 and results not shown). A Δ*patD* strain grew normally with all nitrogen sources tested, including putrescine (Fig. 2 and results not shown). Two different Δ*patA* Δ*patD* double mutants also grew normally with putrescine as the nitrogen source (Fig. 2). The *patD* gene appears to be part of a larger *ydcSTUV-patD* operon. The first

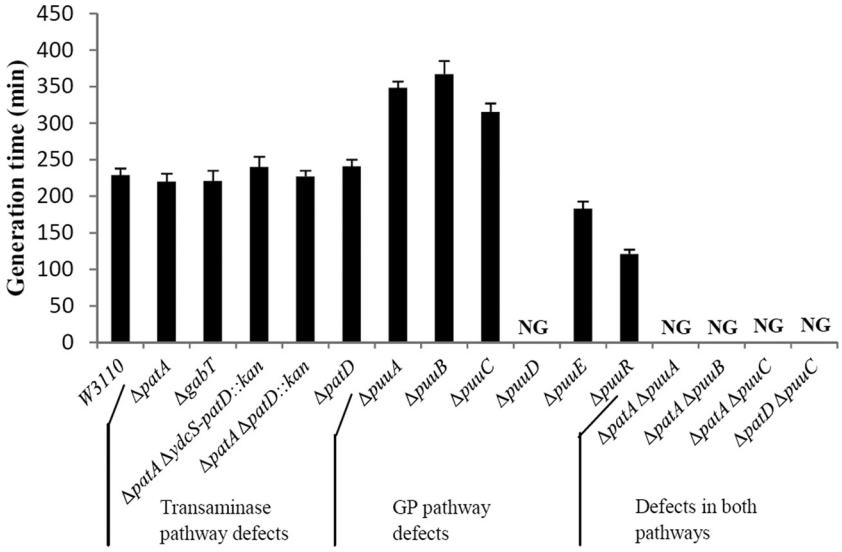

FIG 2 Growth with putrescine as the nitrogen source. The strains and doubling times are listed in Table 1. NG indicates no growth.

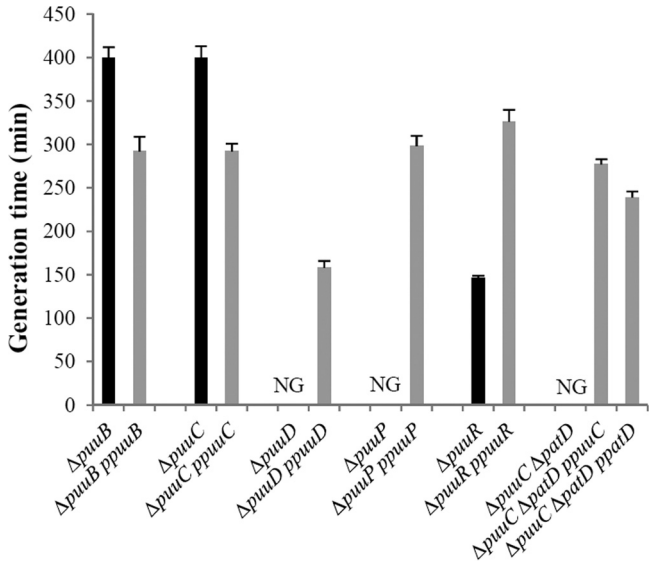

FIG 3 Complementation analysis of mutants with defects in putrescine catabolism. The strains are listed in Table 1. The designation of *ppuuB*, etc., indicates an ASKA plasmid with the indicated gene, e.g., *puuB*. NG indicates no growth. The black bar indicates a mutant, while the gray bar indicates a complemented strain.

four genes of the operon specify a proposed transporter for putrescine/spermidine. Growth with putrescine was not affected by deletion of these four genes in wild-type, Δ*patD*, or Δ*patA* Δ*patD* backgrounds (Fig. 2 and Table 1, lines 6, 7, 29 and 30). These results indicate that PatA and PatD are not required for putrescine catabolism.

The enzymes of the GP pathway catalyze ATP-dependent glutamylation of putrescine (PuuA), oxygen-dependent deamination (PuuB), dehydrogenase-dependent oxidation (PuuC), and deglutamylation of *N*-γ-glutamyl-γ-GABA (PuuD) (Fig. 1). Strains with in-frame deletions of *puuA*, *puuB*, or *puuC* had a doubling time 50% greater than the wild type with putrescine as the sole nitrogen source (Fig. 2). ASKA plasmids containing *puuB* and *puuC* restored wild-type growth to Δ*puuB* and Δ*puuC* strains, respectively (Fig. 3). (The ASKA plasmid containing *puuA* did not restore wild-type growth to a Δ*puuA* strain. This is a problem with the ASKA plasmid, which does not produce detectable PuuA after induction and purification [http://ecoli.naist.jp/GB8]. A different *puuA* plasmid has been shown to complement the defect in a Δ*puuA* strain [25]. We did not reconstruct the plasmid.) Unlike the *puuA*, *puuB*, and *puuC* mutants, a Δ*puuD* mutant did not grow with putrescine (Fig. 2). The ASKA plasmid containing *puuD* not only restored growth to a Δ*puuD* strain but also resulted in faster growth than a wild-type strain (Fig. 3). We conclude that the enzymes of the GP pathway (except for PuuD) are not required for putrescine catabolism as the nitrogen source, although their absence detectably impaired growth.

We then examined mutants with defects in both pathways. Strains with deletions of *patA* and any of the following—*puuA*, *puuB*, or *puuC*—did not grow with putrescine as the nitrogen source (Fig. 2). Putrescine supplementation in either glucose-ammonia (nitrogen-rich) or glucose-glutamine (nitrogen-limited) minimal medium did not affect growth of these double mutants, which suggests that putrescine toxicity cannot account for the mutant phenotype (results not shown). A Δ*puuA* Δ*patD* strain had a doubling time that was 50% greater than that of a Δ*puuA* strain (Table 1, lines 13 and 33). This suggests that PatD is not the only enzyme with ABDH activity in the transaminase pathway. A Δ*puuC* Δ*patD* strain, which lacks the proposed aldehyde dehydrogenases of both pathways, failed to grow (Fig. 2). This suggests that PuuC may be the second enzyme with ABDH activity. ASKA plasmids containing either *puuC* or *patD* restored a wild-type growth rate to the Δ*puuC* Δ*patD* mutant (Fig. 3). Defects in both pathways generally prevent putrescine utilization, which implies that either pathway is sufficient for putrescine utilization.

It is possible that other pathways may contribute to putrescine catabolism, although not sufficiently for putrescine utilization as the sole nitrogen source. We specifically tested whether CsiD (also called YgaT) contributes to putrescine catabolism. CsiD is one product of a complex *csiD-lhgO-gabDTPC* operon (30). The last four genes specify proteins involved in GABA metabolism (30, 46). Homology and structural analysis suggests that CsiD is a nonheme iron II-dependent oxygenase, possibly with α-ketoglutarate as one substrate (4). LhgO (previously called YgaF) is a flavin adenine dinucleotide-dependent L-2-hydroxyglutarate oxidase (18). Together, CsiD and LhgO could conceivably degrade putrescine to GABA by an oxygen-dependent pathway that is known to exist in some bacteria (27). We transferred a deletion of *csiD* into a Δ*puuA* mutant, which is detectably impaired in putrescine utilization. The deletion of *csiD* did not further impair the growth of a Δ*puuA* mutant (Table 1, lines 13 and 32), which suggests that CsiD does not contribute to putrescine catabolism.

There are several polyamine transport systems, including products of the *potFGHI* and *ydcSTUVW* operons, that are induced by nitrogen limitation and the Ntr response (14, 54). Deletions of these operons had no effect on growth with putrescine as the nitrogen source (results not shown). The *puuP* gene, which is adjacent to *puuA*, specifies a transport system that is not regulated by nitrogen limitation. The Δ*puuP* strains failed to utilize putrescine as a nitrogen source at 30°C (Fig. 3 and Table 1, lines 11 and 17). A *puuP* plasmid restored a wild-type growth rate to the mutant (Fig. 3). PuuP appears to be the major transporter for putrescine as the sole nitrogen source. PuuP has been previously shown to be required for putrescine utilization as a carbon source at 20°C (26).

In summary, three types of defects prevent utilization of putrescine as the sole nitrogen source: loss of PuuD, loss of PuuP, and defects in both the GP and transaminase pathways.

The redundancy and specificity of enzymes that degrade putrescine to GABA. Loss of PuuA, PuuB, or PuuC, in a strain without PatA, prevents putrescine utilization. Since these four enzymes catalyze different reactions, the phenotypes imply that each is a nonredundant enzyme. We confirmed this conclusion for PatA. Cell extracts of W3110 (wild type) and CP2 (Δ*patA* mutant) grown in glucose-putrescine minimal medium had 2.36 ± 0.07 and 0.09 ± 0.02 units of putrescine transaminase activity, respectively, which implies that PatA is essentially the only putrescine transaminase (39).

The ABDH reaction of the transaminase pathway oxidizes γ-aminobutyraldehyde to GABA. Purified PatD (formerly YdcW) has NAD-specific dehydrogenase activity (44). Genetic results in the preceding section suggested that PuuC might have ABDH activity. Assays of NAD-dependent ABDH activity from cell extracts confirmed this possibility (Fig. 4). A Δ*patD puuC*$^+$ strain pos-

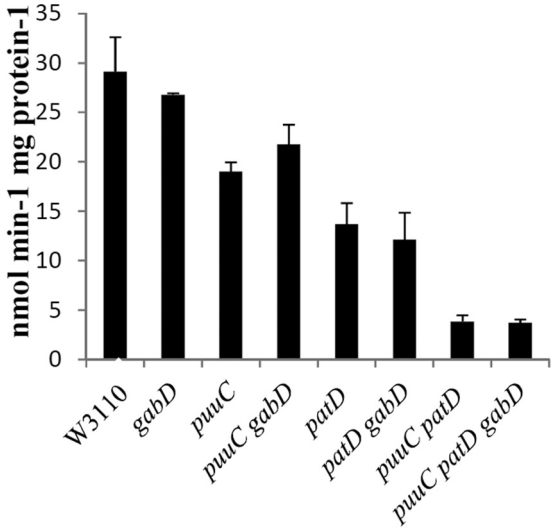

FIG 4 The activity of γ-aminobutyraldehyde dehydrogenase (ABDH). Cells were grown in glucose-aspartate-putrescine minimal medium, which is inducing but eliminates differences in growth rates.

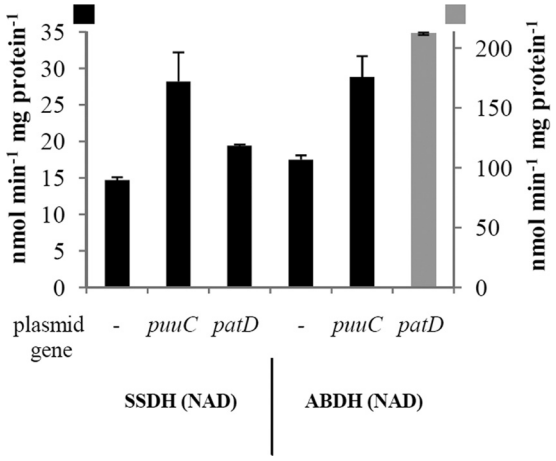

FIG 5 The activity of succinic semialdehyde dehydrogenase (SSDH) and γ-aminobutyraldehyde dehydrogenase (ABDH). Strain BLS93 (Δ*puuC* Δ*patD*) was grown with ASKA plasmids containing the indicated gene. Cells were grown in LB medium. The black-bar values use the scale on the left, while the gray-bar value uses the scale on the right.

sessed about 45% of wild-type activity, a *patD* $^{+}$ Δ*puuC* strain had about 70% activity, and a Δ*patD* Δ*puuC* strain had about 15% activity. These results suggest that PatD and PuuC account for 55% and 30%, respectively, of ABDH activity, and that another enzyme or enzymes have 15% residual activity. A plasmid with either *patD* or *puuC* increased ABDH activity from a Δ*patD* Δ*puuC* strain 2 or 14 times over the background level, respectively (Fig. 5). The *puuC* gene appears to be poorly expressed from the ASKA plasmid (see Materials and Methods, "Strains and plasmids"). Nonetheless, biochemical and genetic results indicate that PuuC and PatD have ABDH activity *in vivo*. GabD, the remaining aldehyde dehydrogenase in the two putrescine catabolic pathways, does not contribute to ABDH, since a Δ*patD* Δ*puuC* Δ*gabD* strain still possessed residual activity (Fig. 4), and cells with a plasmid with *gabD* which had 50 times more NADP succinic semialdehyde dehydrogenase (SSDH) activity did not have increased ABDH activity (not shown).

The redundancy and specificity of enzymes degrade GABA. The transaminase and GP pathways convert putrescine to GABA, which is then metabolized to succinate. GabT converts GABA to succinic semialdehyde, and the extent of homology between GabT and PuuE suggests that PuuE probably catalyzes the same reaction. We examined growth of mutants lacking these enzymes. CP6 (Δ*gabT*) (Table 1, line 3) and CP14 (Δ*puuE*) (Table 1, line 4) grew normally with putrescine as the sole nitrogen source, whereas CP36 (Δ*gabT* Δ*puuE*) (Table 1, line 5) grew 30% slower than the wild type. To determine if CP36 can catabolize the GABA generated from putrescine, we measured growth yields with limiting nitrogen. With 1 mM NH_4Cl as the only nitrogen source, the wild-type strain and the three mutants grew to a density of 81 Klett units. With 1 mM putrescine (which has two nitrogens), W3110, CP14, CP6, and CP36 grew to 151 ± 3, 144 ± 2, 176 ± 1, and 78 ± 2 Klett units, respectively. (Each value is the average of three determinations.) We conclude that CP36 (Δ*gabT* Δ*puuE*), which grew to half the level of the other strains, can remove only one nitrogen from putrescine and therefore cannot degrade GABA. Extracts of W3110 (wild type), CP14 (Δ*puuE* *gabT*$^{+}$), CP6 (Δ*gabT puuE*$^{+}$), and CP36 (Δ*puuE* Δ*gabT*) had 430 ± 22, 214 ± 11, 111 ± 8, and −1.0 ± 2.6 units of GABA transaminase activity, respectively. We conclude that (i) PuuE and GabT are the only transaminases that degrade GABA to succinic semialdehyde *in vivo* and (ii) loss of both enzymes allows utilization of one, but not two, nitrogens of putrescine.

Strains without succinic semialdehyde dehydrogenase (SSDH) activity might be impaired in utilization of one nitrogen from putrescine, or they might grow slower if succinic semialdehyde is toxic. GabD is known to have NADP-dependent SSDH activity. A Δ*gabD* mutant and a Δ*gabD* Δ*patD* mutant grew as well as the wild type with putrescine as the nitrogen source (Table 1, line 27, and not shown). The doubling time of a Δ*puuC* Δ*gabD* mutant (Table 1, line 31) was 100% greater than that of the wild type, while the doubling time of a Δ*puuC* mutant (Table 1, line 20) was only 40% greater. These results are consistent with the possibility that PuuC has SSDH activity *in vivo*. Therefore, we examined SSDH activity in crude extracts from various mutants. For cells grown in glucose-putrescine minimal medium, GabD accounted for 85% of NADP-dependent SSDH activity (Fig. 6B). Loss of both PuuC and PatD did not diminish NADP-dependent activity (Fig. 7). (Cells for this assay had to be grown in glucose-ammonia-putrescine medium, since the double mutant does not grow in glucose-putrescine medium.) Unexpectedly, PuuC accounted for 75% of NAD-dependent SSDH activity (Fig. 6A). Confirmation of these results was sought from cells containing plasmids with *puuC*, *patD*, or *gabD*. For NADP-dependent SSDH, a Δ*puuC* Δ*gabD* mutant with an empty ASKA plasmid had 11 U of activity, while the mutant with an ASKA-*gabD* plasmid had 655 U of activity. Plasmids with *patD* or *puuC* had no effect on NADP-dependent SSDH (not shown). In other words, only GabD has NADP-dependent activity. For NAD-dependent SSDH, cells with *puuC* or *patD* on a plasmid had 100% and 30% more activity than cells with an empty plasmid (Fig. 5). Genetic and biochemical evidence indicates that GabD and PuuC have NADP- and NAD-dependent SSDH activity, respectively.

Polarity in GP pathway genes. In the course of mutant construction, we isolated mutants with both insertions, which are

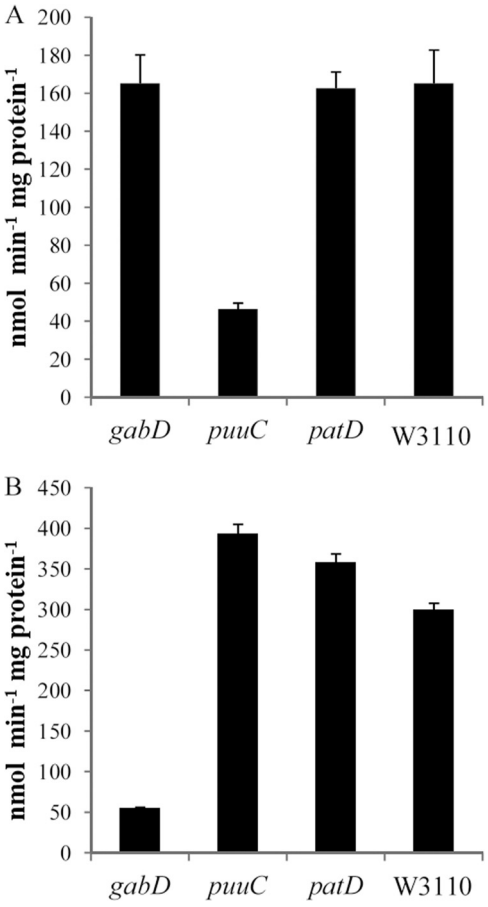

FIG 6 Succinic semialdehyde dehydrogenase activity in various mutants. Cells were grown in glucose-putrescine minimal medium. (A) NAD-dependent activity; (B) NADP-dependent activity.

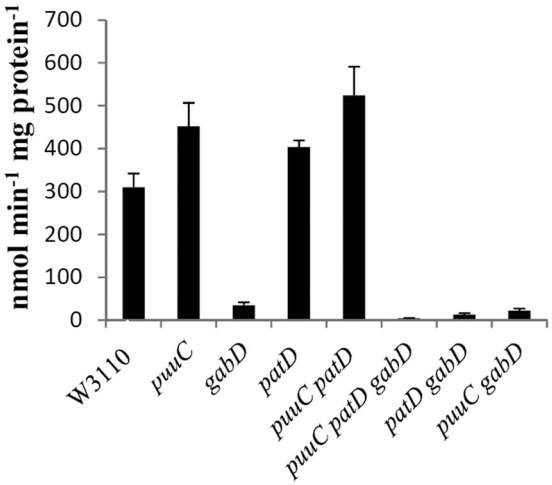

FIG 7 NADP-dependent succinic semialdehyde dehydrogenase activity. Cells were grown in glucose-aspartate-putrescine minimal medium, which allows growth of mutants that cannot grow in glucose-putrescine medium and eliminates growth differences but still allows induction.

presumably polar, and in-frame deletions. There were two examples of differences in growth for the two types of mutants. First, an in-frame deletion of *puuA* only modestly impaired growth, whereas an insertion in *puuA* eliminated growth (Table 1, lines 12 and 13). The insertion presumably prevents *puuP* expression, which is required for putrescine catabolism. This suggests the existence of a *puuAP* operon. Second, an in-frame deletion of *puuR* stimulated growth (Table 1, line 16), whereas an insertion in *puuR* eliminated growth (Table 1, line 10). Loss of PuuD, perhaps by destabilization of *puuD* mRNA, might explain this phenotype. Another possibility is high expression of the *puuAP* operon coupled with loss of PuuC, PuuB, and PuuE, resulting in either toxic accumulation of γ-glutamyl-γ-putrescine or depletion of substrates (glutamate and ATP). Strains with insertions in *puuB* and *puuC* (Table 1, lines 22 and 19, respectively) grew as well as strains with in-frame deletions of these genes (Table 1, lines 24 and 20, respectively). This is expected, since both the insertions and deletions affect only the GP pathway and not the transaminase pathway. Because of the complex phenotype of the strain with the insertion in *puuR*, it is difficult to interpret much about polarity in the *puuD-puuR-puuC-puuB-puuE* region.

Regulation of GP pathway genes: PuuR and putrescine. We studied expression from the regions preceding *puuA*, *puuC*, and *puuD* with single-copy *lacZ* transcriptional fusions. (We also constructed *puuA-lacZ* and *puuD-lacZ* translational fusions. No differences were observed between the two types of fusions under a variety of conditions, and results are shown only for the transcriptional fusions.) We examined expression from the *puuD-lacZ* fusion in glucose-ammonia, glucose-ammonia-putrescine, and glucose-putrescine media and observed 1,400, 6,600, and 19,000 units of LacZ, respectively (open boxes, Fig. 8). Virtually identical results were observed for the *puuA-lacZ* fusions (not shown). The difference in expression from *puuD-lacZ* strains in glucose-ammonia-putrescine (nitrogen excess) and glucose-putrescine (nitrogen-limiting medium), 6,600 versus 19,000 units, might be due to control by nitrogen limitation. However, there was no difference in LacZ from cells grown in glucose-ammonia (nitrogen excess) versus glucose-glutamine (nitrogen limiting) (not shown), which argues against this possibility. These results suggest induction by putrescine.

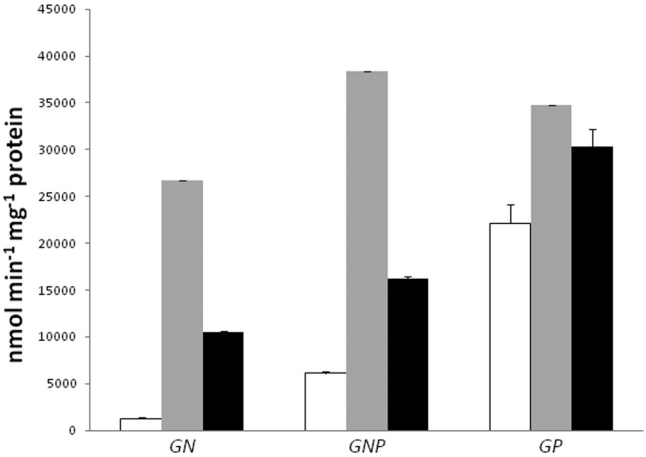

FIG 8 β-Galactosidase activity from a single-copy *puuD-lacZ* transcription fusion. Cells were grown in the indicated minimal medium: GN, glucose-ammonia; GNP, glucose-ammonia-putescine; and GP, glucose-putrescine. Activities in the wild type (blank), *puuR* (gray), and *puuR* strains with complementing plasmid (black) are shown.

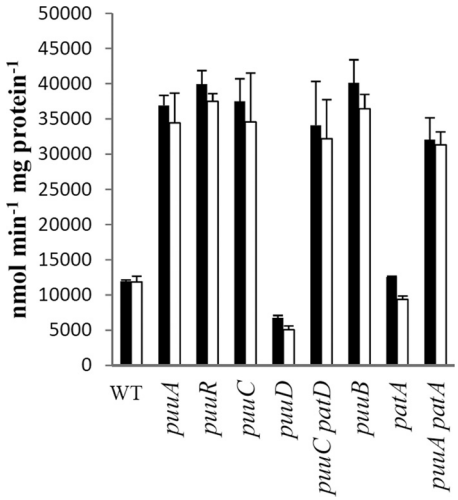

FIG 9 β-Galactosidase activity from strains with *puuD-lacZ* (black) and *puuA-lacZ* (white) fusions. Cells were grown in glucose-ammonia-putrescine minimal medium, which shows the largest differential between the wild-type and *puuR* backgrounds (see Fig. 8). This medium also eliminates the enormous growth rate differences of the various mutants.

The *puuC-lacZ* fusion contains 365 bases of the C terminus of *puuR*, the entire 274-base intergenic region, and 32 bases of *puuC*. The fusion gave 250, 310, and 480 units of LacZ in glucose-ammonia, glucose-ammonia-putrescine, and glucose-putrescine media, respectively. (A promoterless *lacZ* inserted into the chromosome generated 8 units of activity. All SEMs were ≤15% of the means.) These results are consistent with the possibility of a weak constitutive promoter but do not indicate the presence of a putrescine-inducible promoter. This region was not further analyzed.

The *puuR* gene codes for a potential 185-residue DNA-binding protein. It is a member of the XRE (xenobiotic response element) family which includes cro and cI of phage λ. The doubling time of a Δ*puuR* strain was half that of a wild-type strain in glucose-putrescine medium (Table 1, line 16). An ASKA-*puuR* plasmid restored a wild-type growth rate (Fig. 3). Loss of PuuR elevated expression from the *puuD* promoter 22-fold in glucose-ammonia medium, 6-fold in glucose-ammonia-putrescine medium, and 1.6-fold in glucose-putrescine medium (Fig. 8). The ASKA-*puuR* plasmid restored lower expression in glucose-ammonia and glucose-ammonia-putrescine media (black boxes, Fig. 8). Expression was higher in a *puuR* mutant than for wild-type cells grown in the most derepressing medium (glucose-putrescine medium) (Fig. 8). Results with the *puuA* fusion were virtually identical (not shown). These results suggest that PuuR is a repressor for both the *puuA* and *puuD* promoters and that PuuR mediates control by putrescine.

To test whether a derivative of putrescine is the inducer, we examined *puuA* and *puuD* expression in mutants with different blocks in putrescine catabolism. We grew cells in glucose-ammonia-putrescine medium, which allows growth of putrescine-nonutilizing mutants. In a Δ*puuA* Δ*patA* background, which does not degrade putrescine, expression of both *puuA* and *puuD* was as high as that in a Δ*puuR* strain (Fig. 9). These results are sufficient to suggest that putrescine, and not a derivative, is the inducer.

This conclusion allows an indirect test for putrescine accumulation in various mutants. Derepressed expression was observed for *puuA*, *puuB*, and *puuC* mutants but not for *patA* and *puuD* mutants (Fig. 9). The derepression implies putrescine accumulation in the former mutants. For these experiments, cells had been grown in glucose-aspartate-putrescine medium, which has the inducer but supports the same growth rate for wild-type and mutant strains. For cells grown in glucose-ammonia minimal medium, a Δ*puuA* Δ*patA* strain derepressed *puuA* expression about 2-fold but was only about 12% of the level in a wild-type strain grown in glucose-putrescine medium (data not shown). This implies that loss of the catabolic pathways only modestly increases intracellular putrescine in medium without exogenous putrescine.

DISCUSSION

Pathways of putrescine catabolism. The reactions of PatA, PatD, GabT, and GabD degrade putrescine to succinate (Fig. 1). Mutants lacking any of these enzymes grew normally with putrescine as the nitrogen source. Common regulatory features of these enzymes (discussed below) suggest that these four enzymes constitute a pathway, which we have called the transaminase pathway. The reactions catalyzed by PuuA, PuuB, PuuC, PuuD, and PuuE also completely degrade putrescine to succinate. PuuC catalyzes the third and sixth reactions of this pathway (reactions 8 and 5, Fig. 1). The latter reaction has not been previously described.

Loss of *puuA*, *puuB*, and *puuC* impaired but did not eliminate growth with putrescine as the nitrogen source (Fig. 2). In contrast, loss of *puuD* prevented growth in glucose-putrescine medium and a complementing plasmid stimulated growth that was faster than wild-type growth (Fig. 2 and 3). The transaminase pathway is intact in all of these mutants, which suggests that an incomplete GP pathway is creating a problem in the *puuD* mutant. It seems plausible that *N*-γ-glutamyl-γ-GABA accumulates. This can be a problem if *N*-γ-glutamyl-γ-GABA is toxic. However, this possibility would suggest impaired growth in glucose-ammonia-putrescine medium, which does not occur. An alternate possibility is that the glutamate in *N*-γ-glutamyl-γ-GABA is nonproductively sequestered; that is, it is not being recycled. This would create an effective glutamate starvation. This possibility predicts normal growth if glutamate or a precursor for glutamate (in this case ammonia) is provided and this prediction is met.

Specificity and redundancy of the aldehyde dehydrogenases in putrescine catabolism. PuuC oxidizes γ-aminobutyraldehyde, *N*-glutamyl-γ-aminobutyraldehyde, and succinic semialdehyde (Fig. 1, reactions 3, 5, and 8). This broad specificity is consistent with two characterizations of PuuC which were published before its role in putrescine catabolism was known. First, an *E. coli* aldehyde dehydrogenase that oxidizes acetaldehyde was sequenced (13), and current genome resources show that it is PuuC. It has 40% amino acid identity and 60% similarity to nonspecific mammalian aldehyde dehydrogenases (13). Second, a more recent study examined the substrate specificity of purified AldH (an alternate name for PuuC) and showed that it oxidizes a variety of aldehydes, including butyraldehyde (16). The pyridine nucleotide specificity of PuuC has been proposed to be flexible. GenBank and other annotations indicate that PuuC prefers NADP over NAD, which was suggested by the first characterization of PuuC (13). However, the ratio of NADP to NAD activity is not constant with cells from different media (13) or with different substrates (16). Our results show

that overexpression of *puuC* increased NAD-dependent SSDH activity but not NADP-dependent SSDH activity.

Our results identified the major dehydrogenases in the putrescine catabolism. Other dehydrogenases have been proposed to catalyze reactions in putrescine catabolism, and they may be important in different growth conditions. Sad (also called YneI) oxidizes succinic semialdehyde, and it has been reported that a *sad*/*yneI* mutant has a growth defect with putrescine as the carbon source (10, 22). We observed no steady-state growth defect for a Δ*yneI* strain with putrescine as the sole nitrogen source (unpublished observation).

An enzyme in addition to PatD and PuuC appears to oxidize γ-aminobutyraldehyde, since a double mutant still grew, albeit extremely slowly (Table 1), and this mutant still had 15% residual activity (Fig. 4). We did not specifically identify an enzyme with this residual activity, but several published results suggest that AdhE has ABDH activity. First, *E. coli* cannot utilize putrescine as a carbon source at ≥30°C. A mutant that could utilize putrescine as a carbon source at 37°C has elevated ABDH activity. This ABDH was purified and shown to have 95-kDa subunits, which excludes PatD (50.7-kDa subunits) and PuuC (53.2-kDa subunits) (38). The unusual size is sufficient to identify this ABDH as AdhE. Its subunits are 70% larger than those of the second largest aldehyde dehydrogenase, which is AldB (56.3-kDa subunits), and AdhE is the only soluble dehydrogenase in *E. coli*, with subunits of 95 kDa ± 10%. Second, purified AdhE has high activity with butyraldehyde, which suggests that activity with γ-aminobutyraldehyde is likely (42). Third, putrescine induces AdhE (52).

Regulation and relative contributions of the putrescine catabolic pathways. RNA polymerase complexed with σ^{54} controls expression of the operons of transaminase pathway genes, *gabDTPC*, *patA* (*ygjG*), and *ydcSTUV-patD* (40, 54). RNA polymerase complexed with σ^S also controls these operons. The σ^S-dependent control for the *csiD-lhgO-gabDTPC* operon has been described (30), and we will describe the complex σ^S-dependent control of *patA* and *ydcSTUV-patD* in a separate communication. Gene profiling suggests that high temperature increases their expression (17). It appears that these four operons constitute a stress-inducible pathway of putrescine catabolism.

Several factors have been reported to increase expression of GP pathway genes: putrescine, low temperature, and the transition from anaerobic to aerobic growth; other factors lower expression: succinate, high temperature, and low aeration (17, 22, 25, 26, 36). ArcA and PuuR have been implicated in GP pathway control (22, 25, 26, 36) (this paper). ArcA mediates repression during anaerobic growth (36) and probably with low aeration. In addition to these control mechanisms, a metal-catalyzed oxidation initiates posttranslational degradation of PuuA (25). PuuA inactivation is consistent with the importance of polyamines for growth with 95% oxygen (5). These mechanisms do not obviously account for the effects of temperature (described next) and succinate.

Gene profiling shows that expression of GP pathway genes is elevated at lower temperatures, and expression of transaminase pathway genes is repressed (17). This temperature effect can explain some unexpected aspects of putrescine catabolism. First, the GP pathway degrades putrescine as the sole carbon source at 20°C but not at 37°C (22–26). Our wild-type strain cannot use putrescine as the sole carbon source at 30°C. Diminished expression of the GP pathway may explain the failure to utilize putrescine at higher temperatures. Second, mutants that utilize putrescine as a carbon source at 37°C have been isolated, and they have elevated levels of both transaminase pathway enzymes (37). Several rationales can be suggested to account for this temperature effect. First, spermidine, which is derived from putrescine, is toxic at low temperatures (1, 11, 28). Putrescine catabolism could minimize the toxicity. Second, the growth rate of mutants suggests that the GP pathway is quantitatively more important than the transaminase pathway. This would generate more aldehydes, which could be a problem at higher temperatures, as has been proposed for another aldehyde-generating pathway (19, 35). Third, putrescine and other polyamines may be more important at higher temperatures.

We have shown that two pathways catabolize putrescine as the sole nitrogen source. The nonoverlapping regulatory factors and differential effect of temperature suggest that the relative importance of each pathway will depend on the environment. The GP pathway is essential for putrescine as the sole carbon source at low temperatures. It is not essential for putrescine catabolism as the sole nitrogen source, although it appears to be more quantitatively significant than the transaminase pathway.

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