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Role of Adenine Deaminase in Purine Salvage and Nitrogen Metabolism and Characterization of the *ade* Gene in *Bacillus subtilis*

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The isolation of mutants defective in adenine metabolism in *Bacillus subtilis* has provided a tool that has made it possible to investigate the role of adenine deaminase in adenine metabolism in growing cells. Adenine deaminase is the only enzyme that can deaminate adenine compounds in *B. subtilis*, a reaction which is important for adenine utilization as a purine and also as a nitrogen source. The uptake of adenine is strictly coupled to its further metabolism. Salvaging of adenine is inhibited by the stringent response to amino acid starvation, while the deamination of adenine is not. The level of adenine deaminase was reduced when exogenous guanosine served as the purine source and when glutamine served as the nitrogen source. The enzyme level was essentially the same whether ammonia or purines served as the nitrogen source. Reduced levels were seen on poor carbon sources. The *ade* gene was cloned, and the nucleotide sequence and mRNA analyses revealed a single-gene operon encoding a 65-kDa protein. By transductional crosses, we have located the *ade* gene to 130° on the chromosomal map.

When preformed purines are present in the growth medium of *Bacillus subtilis*, they are readily taken up by specific transport systems and used for nucleotide synthesis. Both adenine, guanine, and hypoxanthine and their nucleoside derivatives serve as sole purine sources, indicating efficient interconversion pathways. Under these conditions, de novo purine synthesis is shut down (37). Adenine is transported by a low-affinity and by a high-affinity transport system (K_m = 3 μM); the latter system is important when the concentration of adenine is low (4). A key reaction in adenine salvage is the phosphoribosylation of adenine to AMP (see Fig. 1), a reaction that is common to all organisms. However, the conversion of adenine to GMP involves several steps and also different pathways. In *Escherichia coli*, *Salmonella typhimurium* (34), and *Bacillus cereus* (17), adenine is converted to hypoxanthine, with the intermediate formation of adenosine and inosine catalyzed by purine nucleoside phosphorylase and adenosine deaminase. The resulting hypoxanthine then reacts with 5-phosphoribosyl-α-1-PP_i (PRPP) to form IMP, which is converted to GMP via xanthosine monophosphate. The direct deamination of adenine occurs only in bacteria and lower eukaryotes and has been reported for *B. subtilis* (15), *Azotobacter vinelandii* (19), *E. coli* (24), *Candida utilis* (19), *Schizosaccharomyces pombe* (40), *Saccharomyces cerevisiae* (14, 53), *Crithidia fasciculata*, and four *Leishmania* species (23). Adenine deamination is an essential step in the utilization of adenine as the total purine source in *S. pombe* (40) and *S. cerevisiae* (14). An alternative and possible route is the direct deamination of AMP catalyzed by AMP deaminase, with ATP as an allosteric activator and GTP as an inhibitor. This reaction has not yet been demonstrated in bacteria (30, 31). A completely different interconversion pathway includes the first steps of the histidine biosynthetic pathway and the final steps in the IMP biosynthetic pathway. Histidine is synthesized from ATP and PRPP, resulting in the formation of 5-aminoimidazole-4-carboxamide ribonucleotide as a by-product which is an intermediary compound in the biosynthesis of IMP (34, 37). The histidine interconversion pathway is blocked when cells are grown in the presence of histidine. The levels of the enzymes responsible for the conversion of adenine to hypoxanthine in the various organisms seem to be regulated differently. In *B. cereus*, adenosine deaminase synthesis is induced by adenine and adenosine, and the inosine formed induces inosine phosphorylase (33). The adenosine deaminase in *E. coli*, but not in *S. typhimurium*, is induced by adenine and hypoxanthine, while adenosine and inosine induce the synthesis of purine nucleoside phosphorylase in both *E. coli* and *S. typhimurium* (34). The level of adenine deaminase activity is uniquely regulated in *S. cerevisiae*. The enzyme level is reduced when cells are grown in media containing proline in place of ammonia as the sole nitrogen source and is not affected by adenine in the growth medium (14). In addition to serving as a nucleotide precursor, adenine can serve as the nitrogen and carbon source. Such catabolic reactions are important in many bacteria (52) and have been demonstrated for *B. subtilis*, which will utilize purine bases as nitrogen source (42). However, in *B. subtilis*, nothing is known about the reactions involved except for the deamination of adenine. In the present paper, we report on metabolic studies with wild-type cells and mutants defective in enzymes involved in adenine metabolism and on the genetic mapping and characterization of the *ade* gene encoding adenine deaminase (adenine aminohydrolase EC 3.5.4.2).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* and *B. subtilis* strains used are described in Table 1. Growth medium for *E. coli* was AB medium (10) supplemented with glucose (0.2%) and thiamine (1 mg/liter). If not otherwise stated, *B. subtilis* was grown in Spizizen salts-buffered glucose-citrate-glutamate medium as described before (16, 46). Both media were supplemented with amino acids (50 mg/liter) and purine bases (15 mg/liter) when required by auxotrophic mutants. When alternative nitrogen sources were included in the medium, ammonia and glutamate were omitted from the medium. Amino acids were added

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TABLE 1. Strains of bacteria used

Strain	Genotype	Origin or reference
B. subtilis		
ED1	*trpC2*	168 (C. Anagnostopoulus)
ED21	*ilvA1 sacA78*	QB16 (F. Kunst)
ED77	*trpC2 hisA1 thr5*	QB917 (13)
ED233	*trpC2 hisA1 purH1 guaA2*	Laboratory collection
ED250	*trpC2 hisA1 thr5 purA*	Laboratory collection
ED252	*trpC2 hisA1 ade-1 purH1*	ED233 TF[a] by ED250; Gua^+
ED265	*trpC2 ade-1*	ED252 TF[a] by ED1; Pur^+ His^+
ED279	*trpC2 ade-1 apt-7*	ED265; F-Adr[b]
ED287	*trpC2 hisA1 apt-7*	ED77 TF[a] by ED279; Thr^+ F-Adr[b]
ED292	*polC12 spcB3 strB3 pyrA26*	BD332 (27)
E. coli		
SØ446	*metB purE deoD apt*	20
MT102	F^- *araD139* Δ(*argF-leu*) *7697* Δ(*lac*)*X74 galU galK hsdR2* (r^-, m^+) *mcrB1 rpsL*	Laboratory collection

[a] TF, transformation of the first strain with DNA of the second strain.
[b] FAdr, resistant to 2-fluoroadenine (10 μM).

at a concentration of 0.2%, and purine compounds were added at a concentration of 0.05% (3.3 mM) when used as the nitrogen source. In some experiments, succinate-citrate or citrate served as the carbon source. L broth was used as complete medium. Bacteria were grown at 37°C with aeration by shaking, and growth was monitored by measuring the optical density at 436 nm (OD_{436}) in an Eppendorf photometer. An OD_{436} of 1 corresponds to 0.2 mg (dry weight)/ml. For plates, the media were solidified by adding 1.5% Difco agar. Auxotrophic mutants were scored on minimal plates with or without the required amino acid or nucleobase. The *strB* (45) and *spcB* (27) markers were scored on minimal agar plates containing 0.5% Casamino Acids and streptomycin sulfate (200 mg/liter) or spectinomycin dihydrochloride (100 mg/liter), respectively. Spontaneous *apt* mutants resistant to the toxic adenine analog 2-fluoroadenine were isolated on minimal plates (37) containing 10 μM 2-fluoroadenine (Drug Synthesis & Chemistry Branch, National Cancer Institute, NIH, Bethesda, Md.) and were assayed for adenine phosphoribosyltransferase (*apt*) activity (22).

Plasmids. The plasmids used in this work are described in the text and in Fig. 2. The cloning vectors used in *E. coli* were plasmid pUC19 (56) and pLNA2, which contains the *cat* gene from pC194 (29) ligated into pUN121 (35), which encodes a tetracycline resistance gene and the β-lactamase gene.

Enzyme assays. Exponentially growing cells at an OD_{436} of 1 were harvested by centrifugation at 6,000 × *g* for 5 min, washed with 0.9% NaCl, and frozen at −20°C. The cells were resuspended in 0.1 M Tris-HCl (pH 7.6) and disrupted by ultrasonic treatment for 1 min, and the extracts were cleared by centrifugation. Enzyme levels are given as nanomoles of product formed per minute at 37°C. Protein concentrations were determined by the method described by Lowry et al. (28). In some experiments, enzyme activities in whole cells were determined at an OD_{436} of 5. Adenine phosphoribosyltransferase activity was determined as described before (22). Adenine deaminase activity was assayed by determining the conversion of ^{14}C-labeled adenine to ^{14}C-labeled hypoxanthine, which were separated by ion-exchange chromatography. The assay mixture contained 0.1 M Tris-HCl (pH 7.6), 5 mM $MnCl_2$, 0.5 mM ^{14}C-labeled adenine (5 Ci/mol), and 20 to 60 μg of protein. The reaction was started by adding the cell extract. Samples (10 μl) were withdrawn at 4-min intervals, applied to polyethyleneimine-impregnated cellulose plates on plastic sheets, and chromatographed in H_3BO_3 (10 g/liter) and LiCl (1.5 g/liter) (pH 7.0). After autoradiography, the adenine and hypoxanthine spots were cut out and counted in a liquid scintillation counter.

Uptake, incorporation, conversion, and excretion of nucleobases. The rate of uptake was determined as described before (46), and the concentration of the base used was 1 μM. Conversion of adenine to hypoxanthine and incorporation into nucleotides and nucleic acids or the conversion of hypoxanthine into adenine were monitored for cells grown in the presence of 50 μM ^{14}C-labeled purine base (5 mCi/mol). Samples (20 μl) were removed and chromatographed as described for the adenine deaminase assay. The radioactivity incorporated into nucleotides and nucleic acids remained at the application point on the chromatogram, while adenine and hypoxanthine were moved from the application point and were separated.

Nucleotide pools. ATP, GTP, ppGpp, and pppGpp pools were extracted with formic acid and determined as described before (21, 47) for cells cultured in low-phosphate medium in the presence of [^{32}P]phosphate (10 Ci/mol).

DNA manipulation and genetic techniques. Chromosomal DNA was isolated from *B. subtilis* as described before (46). Small-scale plasmid preparations from *E. coli* were prepared as described by Birnboim and Doly (6), and large-scale preparations were prepared as described by Sambrook et al. (43). The procedures for transformation and transduction with AR9 phages were as described before (46). DNA was treated with restriction enzymes, T4 DNA ligase, and Klenow polymerase as recommended by the suppliers (Gibco BRL, Gaithersburg, Md., and Boehringer, Mannheim, Germany).

DNA sequencing. DNA sequence was obtained by the chain-termination reaction method with dideoxyribonucleotides as described by Sanger et al. (44). All sequence analyses were done on double-stranded plasmid DNA templates.

Northern (RNA) blot and primer extension analysis. Isolation of total RNA from *B. subtilis* and primer extension analysis with reverse transcriptase (SuperScript RNase H-Reverse Transcriptase, Gibco BRL) were performed exactly as described before (45). RNA (5 μl, corresponding to approximately 20 μg) for Northern blot analysis were mixed with 12.5 μl of formamide, 4 μl of formaldehyde (25%), 1 μl of ethidium bromide (1 mg/ml), and 2.5 μl of 10× morpholinepropanesulfonic acid (MOPS) buffer (0.2 M MOPS [pH 7.0], 0.05 M NaAc, 0.01 M EDTA). After being heated for 5 min at 65°C, the sample was chilled on ice and 5 μl of loading buffer (50% glycerol, 0.1 mg of bromophenol blue per ml) was added. The sample was loaded on a gel composed of 1% agarose in 1× MOPS buffer and 4.25% formaldehyde, and the gel was electrophoresed for 2.5 h at 100 V in 1× MOPS buffer. After separation, the RNA was transferred to a Qiabrane nitrocellulose membrane (QiaGen, Hilden, Germany) by the capillary technique. The RNA was then cross-linked to the membrane by being heated for 2 h at 80°C, and the membrane was prehybridized for 2 h at 65°C in phosphate-sodium dodecyl sulfate (SDS) buffer (0.5 M sodium phosphate [pH 7.2], 7% SDS). After prehybridization, the membrane was hybridized for 18 h at 65°C in phosphate-SDS buffer containing a ^{32}P-labeled DNA probe. The DNA probe was made radioactive by using the random-primed DNA Labeling kit (Boehringer) together with [α-^{32}P]dATP. After hybridization, the membrane was washed three times with 20 mM sodium phosphate (pH 7.2) and 1% SDS at 65°C. Finally, the membrane was sealed in a cellophane envelope, and the hybridization products were visualized by autoradiography.

Nucleotide sequence accession number. The sequence of the 2,120-bp DNA fragment has been submitted to the EMBL nucleotide sequence data bank and has been assigned the accession number X83795.

RESULTS

Mutants defective in adenine metabolism. During the construction of a *purH purA* mutant by congression, an *ade* mutant was fortuitously found among transformants in a genetic cross with DNA from ED250 (*purA*; requiring adenine) and ED233 (*purH guaA*; requiring guanine) as the recipient. Recombinants were selected as Gua^+ on minimal plates containing adenine and hypoxanthine as purine sources (Table 1). Of the 138 recombinants recovered, 1 recombinant, named ED252 (*purH ade*), could not grow on adenine as the sole purine source. Strain ED252, like the *purH ade*$^+$ recombinants, could grow on either hypoxanthine or guanine as the purine source. ED252 was defective in adenine deaminase, and the enzyme level was <0.1 nmol/min/mg (dry weight), compared with 7.5 nmol/min/mg (dry weight) in the *purH ade*$^+$ recombinants. The purine and histidine requirement of strain ED252 was cured by transformation, resulting in strain ED265 (*ade*) (Table 1).

To be able to study the role of adenine deaminase in adenine metabolism in more detail, we isolated an *apt* mutant defective in adenine phosphoribosyltransferase activity. In strain ED265

TABLE 2. Patterns of adenine metabolism in *ade* and *apt* mutant strains of *B. subtilis*[a]

Strain	Relevant genotype	Activity (nmol/min/mg [dry wt])[b]			Amt of adenine (nmol/mg [dry wt])[c]	
		Adenine PRTase[d]	Adenine deaminase[e]	Adenine uptake[e]	Excreted[f]	Incorporated[f]
ED77	Wild type	4.7	7.5	9	<2	148
ED265	*ade*	4.3	<0.2	8	<2	80
ED287	*apt*	<0.1	4.2	5	<2	124
ED279	*ade apt*	<0.1	<0.2	<0.1	15	<5

[a] Cells were grown in glucose minimal medium in the presence of histidine (50 mg/liter) as an amino acid supplement.
[b] Values are the averages from at least three experiments that differed by less than 15%.
[c] Values are the averages of three experiments that differed by less than 10%.
[d] Adenine phosphoribosyltransferase (PRTase) activity was determined in cell extracts.
[e] Determined in whole cells.
[f] Determined in cells growing from an OD_{436} of 0.05 to an OD_{436} of 1.

(*ade*), an *apt* mutation was introduced by selecting for resistance to 2-fluoroadenine as described in Materials and Methods, resulting in strain ED279 (*ade apt*). The *apt* mutation was introduced into strain ED77 by congression and scoring for resistance to 2-fluoroadenine (Table 1). One recombinant, ED287 (*apt*), was picked. Metabolic studies with the wild type and mutants defective in adenine metabolism (*ade*, *apt*, and *ade apt* double mutants) showed that either adenine deaminase or adenine phosphoribosyltransferase activity is required for adenine uptake and incorporation (Table 2), indicating a strong coupling between adenine uptake and adenine metabolism. In the *ade apt* double mutant (ED279), we could demonstrate excretion of ^{14}C-labeled adenine when cells were grown in the presence of ^{14}C-labeled hypoxanthine. On solid medium, the double mutant was able to cross-feed the adenine auxotrophic strain ED250 (*purA*). These experiments indirectly provide evidence for an otherwise substantial reutilization of endogenously formed adenine in wild-type cells, catalyzed either by adenine deaminase together with hypoxanthine phosphoribosyltransferase or by adenine phosphoribosyltransferase alone, and support evidence for the pathways of adenine metabolism shown in Fig. 1. To determine the role of adenine deaminase and adenine phosphoribosyltransferase in the salvaging of adenine, we determined the overall contribution from exogenously supplied adenine to purine nucleotide synthesis in the wild-type strain and in *ade* and *apt* mutant strains. To avoid interference from the histidine biosynthetic pathway, by which adenine can be converted to GMP through the formation of the intermediary purine precursor 5-aminoimidazole-4-carboxamide ribonucleotide, the pathway was blocked by including histidine in the growth medium. *B. subtilis* cells grown in adenine-supplemented medium (15 mg/liter) will synthesize all purine nucleotides from adenine (37). The amount of adenine incorporated in wild-type cells (ED77) (Table 2) corresponds to that observed when both adenine and guanine nucleotides are synthesized from the exogenously supplied adenine (37). The efficiency of adenine utilization in mutants blocked in adenine utilization compared with that for wild-type cells revealed somewhat reduced incorporation in the *apt* mutant (ED279), which can synthesize both AMP and GMP from adenine (Fig. 1). This indicates that conversion of adenine to hypoxanthine and the further metabolism of hypoxanthine to adenine and guanine nucleotides do not completely reduce the endogenous purine biosynthesis. Adenine utilization was significantly reduced in the *ade* mutant (ED265), in which adenine cannot be converted to guanine nucleotides. Very little incorporation of adenine occurred in the *ade apt* mutant ED279. This finding indicates that conversion of adenine to adenosine catalyzed by adenosine phosphorylase followed by phosphorylation of adenosine is not an alternative pathway under the conditions described. In *S. cerevisiae*, adenine can be converted to adenosine, which can accumulate in the cytoplasm (26). Such an accumulation could not be demonstrated for *B. subtilis* (data not given).

To determine whether adenine deaminase is important for the utilization of adenine as a nitrogen source, we analyzed the growth of ED265 and ED1 on plates containing glucose as a carbon source and different nitrogen sources. The *ade* mutant (ED265) compared with wild-type cells (ED1) had lost the ability to grow on adenine as a nitrogen source, while growth on hypoxanthine or uric acid was not affected.

Some properties of adenine deaminase. Initially, we could determine adenine deaminase activity only for whole cells. Extraction of cells with a variety of buffers and additions resulted in the loss of enzyme activity. A systematic search for an ion requirement led to the finding that the enzyme specifically required Mn^{2+} for activity. No activity with Mg^{2+} (0.05 to 50 mM concentration) was seen. Determination of K_ms for adenine in whole cells and in cell extracts revealed the same value, namely, 0.05 mM. Preliminary analysis of adenine deaminase activity in cell extracts at concentrations of 0.05 and 0.5 mM adenine in the presence of compounds that might feedback inhibit or activate the enzyme revealed no significant effects on enzyme activity. The compounds tested were hypoxanthine, NH_4^+, IMP, AMP, ATP, and GTP at a concentration of 1 mM, P_i at a concentration of 10 mM, and glutamine at concentrations of 1 and 20 mM (data not shown).

Regulation of adenine utilization. The pattern of adenine metabolism in wild-type cells indicates some kind of control of adenine utilization. The transport of adenine was not affected by the addition of purine compounds to minimal growth medium (data not given). Little is known about the regulation of the deamination of adenine. In an earlier study, we measured the level of purine biosynthetic enzymes in strain ED77 (*trp his thr*) as affected by the addition of purines to cells grown in glucose minimal medium. A result of this was that the specific adenine-repressing effect on purine gene repression depends on the functioning of the *apt*-encoded enzyme and that maximal repression by adenine requires the functioning of both adenine phosphoribosyltransferase and adenine deaminase activity (47). In these experiments, we did not determine whether

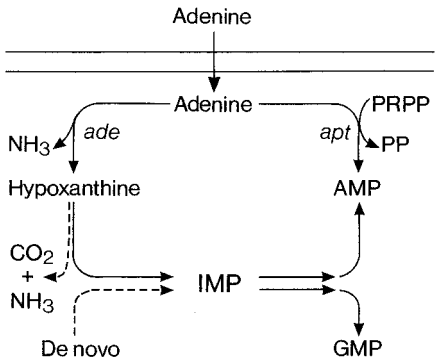

FIG. 1. Adenine salvage and degradation pathways in *B. subtilis*. Relevant enzymes are identified by their gene symbols (*ade*, adenine deaminase; *apt*, adenine phosphoribosyltransferase). The double line indicates the cytoplasmic membrane.

TABLE 3. Effects of purine bases and carbon and nitrogen sources on growth and on the level of adenine deaminase activity in *B. subtilis* ED77 and ED1[a]

Strain and carbon source	NH_4^+ or amino acid addition	Purine addition	Doubling time (min)	Amt of adenine deaminase (nmol/min/mg of protein)
ED77				
Glucose	NH_4^+ and glutamate	None	54	36
Glucose	NH_4^+ and glutamate	Hypoxanthine	48	23
Glucose	NH_4^+ and glutamate	Adenine	50	35
Glucose	NH_4^+ and glutamate	Guanosine	54	16
Glucose	NH_4^+ and glutamate	Adenine + guanosine	48	33
ED1				
Glucose	NH_4^+ and glutamate	None	42	25
Glucose	Glutamate	None	58	24
Glucose	Glutamine	None	40	17
Glucose	Casamino Acids	None	36	29
Glucose	None	Adenine	300	29
Glucose	None	Uric acid	128	22
Succinate	NH_4^+ and glutamate	None	90	13
Succinate	NH_4^+ and glutamate	Adenine	88	11
Succinate	NH_4^+ and glutamate	Uric acid	86	13
Citrate	Glutamate	None	180	20
Citrate	Glutamine	None	89	9
Citrate	Proline	None	98	13

[a] Cells were grown in minimal medium with the indicated additions. Purine was added at concentrations of 1 and 3.3 mM (the latter when serving as the sole nitrogen source). Adenine deaminase activity was determined for cell extracts. Values are the averages of three different experiments that differed by less than 15%.

the levels of adenine deaminase were altered when different purine compounds were added to the growth medium. The result of such an experiment (Table 3) now shows that addition of either guanosine or to a lesser extent, hypoxanthine to the growth medium resulted in decreased levels of adenine deaminase. Under these conditions, deamination of adenine is not required for GMP synthesis. The level of adenine phosphoribosyltransferase was not affected by the addition of purine compounds to minimal growth medium. In a few experiments, we determined adenine phosphoribosyltransferase activity; the level varied between 23 and 29 nmol/min/g of protein. However, the phosphoribosylation of adenine is controlled by feedback inhibition by AMP (5).

To see whether the metabolism of adenine was coupled to protein synthesis, ED21 cells (*ilv*) were starved for isoleucine, which results in the formation of uncharged isoleucine-tRNA, which unleashes the stringent response characterized by ppGpp and pppGpp accumulation and a severe reduction in protein synthesis. Under these conditions, we observed an increase in the ATP pool, and adenine incorporation was arrested while adenine deamination was unaffected (Table 4). This means that purine salvage is inhibited while the transport of adenine and use of adenine as the nitrogen source continues. The kinetic properties of adenine deaminase described above may indicate that the rate of deamination of exogenously supplemented adenine is mainly dependent on the available concentration of adenine and not on a low-molecular activator or inhibitor.

To determine whether the nitrogen or the carbon source had any effect on the level of adenine deaminase, we used strain ED1, which is auxotrophic only for tryptophan. Cells were grown in liquid medium and on a variety of combinations of carbon and nitrogen sources, including ammonia, amino acids, and purine compounds. In glucose medium, a twofold variation in the level of adenine deaminase was found; the lowest level was seen when glutamine was the nitrogen source. Reduced levels were found when succinate and citrate served as the carbon source (Table 3). Purine compounds could not serve as the sole nitrogen source when succinate and citrate served as the carbon source. No relation between enzyme activity and the growth rate was observed. In a few experiments, we determined adenine phosphoribosyltransferase activities. The levels varied between 12 and 14 nmol/min/mg of protein. To see whether another adenine deaminase exists under some growth conditions, we grew strain ED265 (*ade*) in the media described in Table 3. Growth occurred in all media except for the medium in which adenine was the only nitrogen source, but we could not detect the adenine deaminase activity. This suggests that there is only one enzyme in *B. subtilis.*

Cloning of the *ade* gene. The *ade* gene was cloned by functional complementation in *E. coli*. The *E. coli* purine auxotrophic strain SØ446 (*purE deoD apt*) is unable to metabolize adenine and, therefore, it cannot use adenine as the purine source and thus requires hypoxanthine for growth. If *B. subtilis* adenine deaminase could be expressed in *E. coli*, adenine would serve as the purine source after conversion to hypoxanthine. A genomic library of *Eco*RI restriction fragments from *B. subtilis* ED1 cloned into pLNA2 and propagated in *E. coli* (MT102) was used. *E. coli* (SØ446) was transformed with plasmids of this library, and transformants were selected on L broth tetracycline (5 mg/liter). Among 3,000 tested colonies, 1 was able to utilize adenine as the purine source. The genotype of the transformant was verified, and it contained a plasmid (pHH1010) with a 2.8-kb *Eco*RI fragment in pLNA2. The ability to complement SØ446 on adenine was preserved (Fig. 2). The adenine deaminase activity determined for strain SØ446/pHH1010 was 180 nmol/min/mg of protein, compared with <0.2 for SØ446. Plasmid pHH1010 was transformed into *B. subtilis* ED252 (*purH ade*), which cannot grow on adenine as the sole purine source, selecting for recombinants that would grown on plates containing adenine as the sole purine source. Transformants which could grow on adenine as the sole purine source appeared on the plate and were picked up, and a few were analyzed. They were *purH* and had wild-type adenine deaminase levels. Because plasmid pLNA2 cannot replicate in *B. subtilis*, transformants could arise only by recombination with the chromosome. Transformation with cloned DNA fragments in pLNA2 in both *E. coli* and *B. subtilis* made it possible to identify the region of the cloned fragment that contained the *ade* gene (Fig. 2). The difference in complementation furthermore helped in the localization of the gene on the cloned

TABLE 4. Effects of amino acid starvation on nucleotide pools and adenine metabolism in *B. subtilis*[a]

Presence of isoleucine	Amt (nmol/mg [dry wt])				Metabolism (nmol/min/mg [dry wt])	
	pppGpp	ppGpp	ATP	GTP	Adenine deamination	Adenine incorporation
Yes	0.1	0.1	5.2	2.3	7.3	2.5
No	0.6	0.4	17.2	1.2	6.6	0.2

[a] Strain ED 21(*ile*) was grown in the presence of isoleucine and was shifted to isoleucine-free medium. Nucleotide pool sizes and adenine metabolism were determined for whole cells before and after 20 min of starvation for isoleucine. Values are the averages of two experiments that differed by less than 15%.

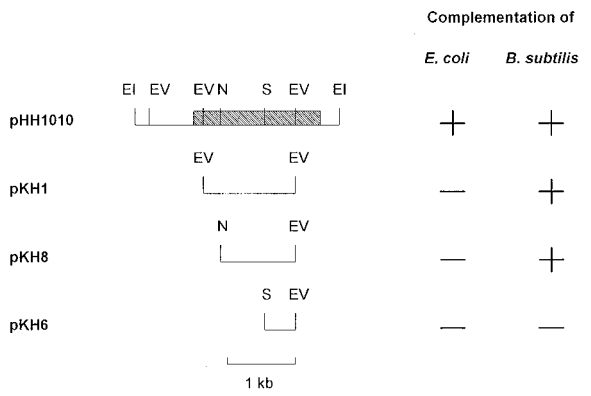

FIG. 2. Complementation of *B. subtilis* ED252 (*purH ade*) and *E. coli* SØ446 (*purE apt deoD*) mutant strains with cloned chromosomal *B. subtilis* DNA fragments. The location of the *ade* gene is indicated by a hatched box. Restriction enzymes: EI, *Eco*RI; EV, *Eco*RV; N, *Nru*I; and S, *Sma*I. Plasmid pHH1010 contained a 2.8-kb *Eco*RI fragment cloned in pLNA2. pKH1 contained a 1.3-kb *Eco*RV fragment from pHH1010 in pUC18. In pKH8, a 0.25-kb *Hin*cII-*Nru*I fragment is deleted from pKH1; in pKH6, a 0.9-kb fragment is deleted from pKH1.

fragment, because complementation in *B. subtilis* required only that the fragment could complement the *ade* mutation.

Nucleotide and derived amino acid sequences and homology. The deletion analysis shown in Fig. 2 revealed that part of the *ade* gene most likely was contained in an 840-bp *Sma*I-*Nru*I fragment. The nucleotide sequences of this fragment and of the flanking regions were determined as described in Materials and Methods. The sequenced DNA fragment (Fig. 3) contained 2,120 bp and revealed an open reading frame which we assigned to the *ade* gene on the basis of the complementation analysis described above (Fig. 2). Four potential translational initiation codons were found in the N-terminal part of the *ade* reading frame (Fig. 3). The TTG codon at nucleotide 335 is the most likely start codon because of the presence of a putative ribosome binding site [(317)AAAAAGGAGGGG(329)] 7 nucleotides upstream (25). This open reading frame encodes a putative polypeptide with a calculated molecular mass of 63 kDa. Located immediately downstream of the translational stop signal is a candidate for a factor-independent transcription terminator sequence capable of forming a secondary stem and loop structure with a calculated free energy of −28.8 kcal (ca. −120 kJ)/mol (51). Interestingly, the dyad symmetric se-

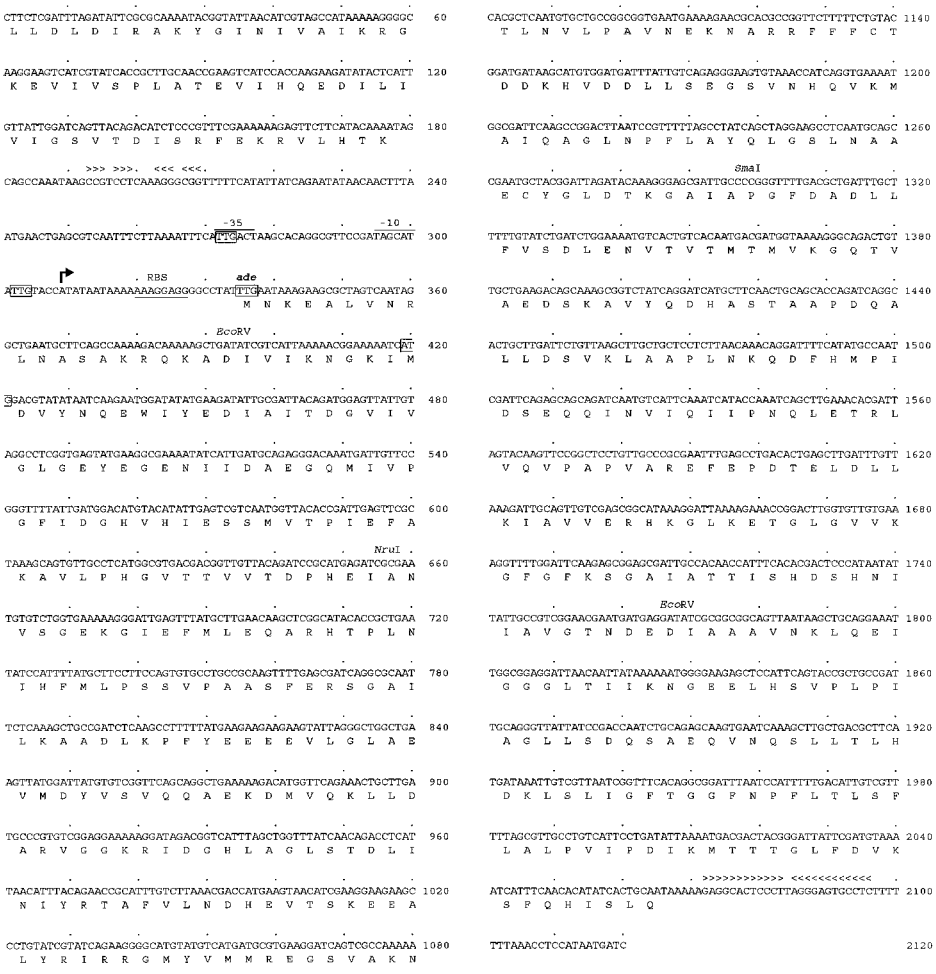

FIG. 3. Nucleotide and deduced amino acid sequences of the *B. subtilis ade* gene and an upstream open reading frame. Lines above the nucleotide sequence show the −35 and −10 regions of the *ade* gene promoter. Underlined sequence indicates a putative ribosome-binding site (RBS). The arrow at position 309 shows the start point of the *ade* transcript. Boxed nucleotides at positions 272 to 274, 302 to 304, 335 to 337, and 419 to 421 indicate putative translational start codons. The most likely is positions 335 to 337. The positions of inverted repeat sequences downstream and upstream of the *ade* gene are indicated (>>><<<). Only relevant restriction sites are shown.

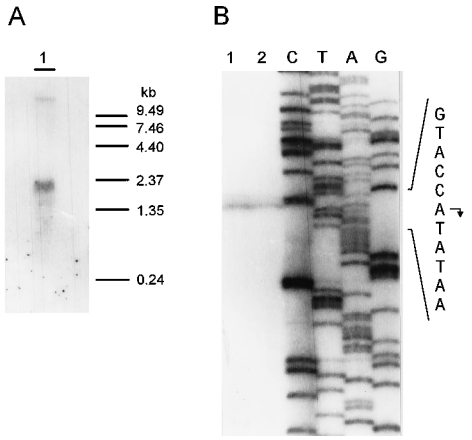

FIG. 4. Northern blot and primer extension analyses of the *ade* mRNA in strain ED1. (A) Northern blot analysis of total RNA. A 1.3-kb, ^{32}P-labeled *Eco*RV fragment containing most of the *ade* gene (Fig. 3) was used as a probe. The RNA size marker was the 0.24- to 9.5-kb RNA ladder from Gibco BRL (Gaithersburg). (B) Primer extension analysis of total RNA. The primer used corresponds to nucleotides 486 to 501 (Fig. 3). The sequence ladder (C, T, A, G) was obtained with the primer that was used for cDNA synthesis and has been converted to its complementary sequence for ease of comparison with Fig. 3. The position corresponding to the start of transcription is marked by an arrow. In lanes 1 and 2, 4 and 2 μl, respectively, of extension product were loaded on the gel.

quence is followed on both strands by a stretch of U residues and should therefore be able to terminate transcription coming from both directions. By the BLAST algorithm (1), homology to sequences deposited in the nucleotide database (GenBank release 86.0) was analyzed for the deduced amino acid sequence of the *ade* gene. The reading frame was found to be identical to reading frame ORF5 in the *B. subtilis* genomic region containing the *kinC* gene recently sequenced by Y. Kobayashi, Tokyo University of Agriculture and Technology, Tokyo, Japan (accession number D37799). Furthermore, the *ade* reading frame was 34% identical to an unidentified open reading frame from *E. coli*, designated o588, which encodes 588 amino acids (9). Two reading frames of 310 and 197 amino acids derived from nucleotide sequences from *Methanobacterium thermoautotrophicum* and *Methanobacterium thermoformicicum*, respectively, were 36% identical to the C-terminal part of the *ade* reading frame. The *Methanobacterium* sequences contain what is believed to be a mobile DNA element, which is called FR-I. The FR-I element in both sequences maps immediately downstream of the putative *ade* gene (36).

Determination of the *ade* transcription start site and the length of the *ade* mRNA. Total RNA from strain ED1 was subjected to Northern blot analysis, and the results are shown in Fig. 4A. A 1,373-bp *Eco*RV fragment (Fig. 3) containing most of the *ade* gene was used as a probe. The probe was found to hybridize to an mRNA molecule approximately 1.8 kb in length, indicating that we have cloned the whole *ade* operon and that it is monocistronic. The transcription start site was mapped by primer extension analysis. ED1 RNA was incubated with reverse transcriptase plus a single-stranded, ^{32}P-labeled DNA primer complementary to the sequence from nucleotides 486 to 501 (Fig. 3). The result shown in Fig. 4B indicates that the A residue at position 309 defines the first nucleotide in the *ade* mRNA molecule and confirms that the most likely start codon (335) is the start codon of the *ade* operon.

Chromosomal location of the *ade* gene. Preliminary phage AR9 transduction crosses with ED252 as recipient and AR9 lysates propagated on strains of the Dedonder mapping kit (13) located the *ade* gene close to the *pyr* operon (41). With ED252 (*ade purH*) as recipient and ED292 (*pyrA spcB strB*) as donor, *ade*$^+$ recombinants were scored on minimal plates with adenine as the sole purine source. Among 225 recombinants, 95% cotransduction with the *strB* marker was obtained. The distribution of the different recombinant classes indicates the following gene order: *strB-ade-pyrA-spcB*. This gene order was verified by several other crosses (data not given).

DISCUSSION

Two enzymes are involved in the reutilization of exogenously supplied or internally formed adenine in *B. subtilis*, namely, adenine phosphoribosyltransferase and adenine deaminase (Fig. 1). While the phosphoribosylation of adenine to AMP is regulated in response to the cellular demand by feedback mechanisms (5), there is no indication of regulation of the synthesis of the enzyme. Our findings suggest that the deamination of adenine seems to be regulated mainly by the available concentration of adenine and to a lesser extent by the level of adenine deaminase. The adenine taken up is either phosphoribosylated to AMP or deaminated to hypoxanthine which is converted into GMP. The activity of adenine deaminase is sufficient for making adenine just as good a purine source as hypoxanthine (37). The enzyme level is reduced twofold by the addition of guanosine to the growth medium, and under these conditions no conversion of adenine to guanine nucleotides occurs (Table 3). Synthesis of adenosine from adenine is of only minor if any importance for *B. subtilis*. Adenine can be formed from adenosine catalyzed by adenosine phosphorylase (37, 46). When protein synthesis is inhibited by the stringent response (Table 4), adenine metabolism is also affected, and the adenine taken up is predominantly deaminated and excreted as hypoxanthine. A strain unable to use adenine and adenosine as the purine source was isolated by mutagenesis and characterized by others (15). The authors suggested that *B. subtilis* has an enzyme that deaminates adenine, adenosine, and AMP. This adenosine and AMP deaminase activity can now be explained by the combined action of AMP nucleotidase, adenosine phosphorylase, and adenine deaminase activities (37, 46). The *ade* mutation reported here is the result of a single mutation that was mapped by transductional crosses. The mutation arose in a *purA* strain (ED250) most likely because it would be an advantage for an adenine-requiring mutant to avoid unnecessary deamination of adenine by becoming *ade*. Little is known about the endogenous formation of adenine in *B. subtilis*, for example, whether there is an enzyme that degrades AMP to adenine and ribose-5-phosphate, as in *E. coli*, or whether AMP is cleaved by nucleotidases (34). Important in the present context is that adenine is formed endogenously (Table 2) and that the deamination step does not appear to be regulated and is essential for the conversion of adenine compounds to GMP.

B. subtilis can use a number of compounds as the sole nitrogen source and is capable of growing on uric acid as the sole source of nitrogen (42). We have confirmed this and found that adenine, hypoxanthine, and uric acid supported the growth when added as the sole nitrogen source on agar plates. Because of the ability of *B. subtilis* to utilize purines as nitrogen sources, the effect of different nitrogen sources on adenine deaminase activity was examined. Compared with cells grown on minimal medium, a reduced doubling time was seen when purines served as the sole nitrogen source. The synthesis of enzymes required for the utilization of nitrogen-containing compounds such as histidine, proline, asparagine, and urea is induced by

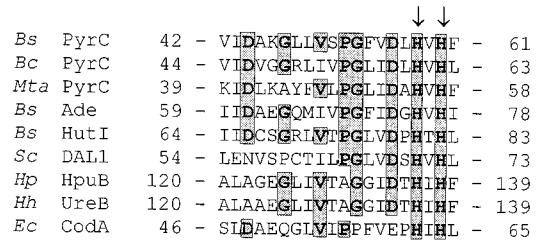

FIG. 5. Comparison of an N-terminal amino acid sequence of the *B. subtilis* adenine deaminase protein with similar sequences found in eight other proteins. Numbers indicate amino acid positions. Boldface letters indicate amino acids that are identical in at least six of the sequences. Histidine residues involved in metal ion binding in the dihydroorotase protein are indicated by arrows. Bs, *B. subtilis*; Bc, *Bacillus caldolyticus*; Mta, *M. thermoautotrophicum*; Sc, *S. cerevisiae*; Hp, *Helicobacter pylori*; Hh, *Helicobacter heilmannii*; and Ec, *E. coli*. The *B. subtilis* sequence was taken from Fig. 3. The *B. subtilis*, *B. caldolyticus*, and *M. thermoautotrophicum* PyrC sequences were from references 41, 18, and 38, respectively. The *B. subtilis Hut*I sequence was from GenBank (accession no. D31856). The *S. cerevisiae* DAL1 sequence was from reference 8. The *H. pylori* HpuB sequence was from reference 11. The *H. heilmannii* UreB (48) and *E. coli* CodA (12) sequences were obtained from GenBank.

their substrates or related compounds and is also affected by other nitrogen compounds in the growth medium (2, 3). This does not appear to be the case for adenine deaminase, which is only slightly affected by the nitrogen compounds present in the growth medium (Table 3); only a twofold reduction is seen when glutamine serves as the nitrogen source. Expression of the *hut* and the *put* operons is affected by the carbon source and is severely repressed when cells are grown in glucose medium (55). To determine whether catabolite repression or the carbon source had any effect, we grew the cells on different combinations of nitrogen and carbon sources. To our surprise, we found lower levels of adenine deaminase under most of these conditions (Table 3), which probably does not reflect a specific regulation of the expression of the *ade* gene but rather is an effect of the macromolecular composition in cells at low growth rates. Preliminary observations by us indicate that degradation of purines is induced when cells are grown on poor nitrogen sources. We have now initiated studies to see whether *B. subtilis*, like *Aspergillus nidulans*, possesses inducible pathways when purines serve as the nitrogen source (50).

The *ade* gene was cloned by complementation in *E. coli* and in *B. subtilis* (Fig. 2) and was identified as a single-gene operon (Fig. 3 and 4). In *E. coli*, there is no adenine deaminase activity and cells defective in adenine phosphoribosyltransferase activity and purine nucleoside phosphorylase cannot utilize adenine as a purine source. Mutants that express adenine deaminase activity have been isolated in *E. coli* (24). One likely candidate for an *ade* gene in *E. coli* is the open reading frame o588 (9), which shows homology to the *B. subtilis ade* sequence. Whether the *E. coli* enzyme is synthesized under some growth conditions remains to be established. The reading frame which showed similarity to the *B. subtilis ade* gene product found in two *Methanobacterium* species suggests that these organisms possess adenine deaminase activity. However, enzyme determinations revealed no adenine deaminase activity but adenosine and AMP deaminase activity (54).

A 20-amino-acid sequence in the N-terminal part of the *ade* reading frame (Fig. 5) showed 30 to 60% identity to sequences of reading frames known to encode the following enzymes: dihydroorotase from bacteria and archaea, urease from various bacterial species, cytosine deaminase from *E. coli*, allantoinase from *S. cerevisiae*, and imidazolone-5-propionate hydrolase from *B. subtilis*. The homologous sequence in these proteins is also located in the N-terminal part. Common for all enzymes is that they catalyze the hydrolysis of the carbon-nitrogen bond of either amide bonds (dihydroorotase, allantoinase, imidazolone-5-propionate hydrolase, and urease) or amidine bonds (adenine and cytosine deaminase). Adenine deaminase, dihydroorotase, urease, and cytosine deaminase have been found to require divalent metal ions, namely, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Fe^{2+}, respectively, for activity (7, 32, 39). The alignment of the homologous regions is shown in Fig. 5. Two histidine residues (marked by arrows) are believed to be involved in the binding of metal ions in the dihydroorotase enzyme (7). Therefore, we speculate that the 59- to 79-amino-acid region may be important for binding of metal ions and perhaps for the catalytic activity of the adenine deaminase enzyme.

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