Novel Metabolic Pathway for *N*-Methylpyrrolidone Degradation in *Alicyclophilus* sp. Strain BQ1

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ABSTRACT The molecular mechanisms underlying the biodegradation of *N*-methylpyrrolidone (NMP), a widely used industrial solvent that produces skin irritation in humans and is teratogenic in rats, are unknown. *Alicyclophilus* sp. strain BQ1 degrades NMP. By studying a transposon-tagged mutant unable to degrade NMP, we identified a six-gene cluster (*nmpABCDEF*) that is transcribed as a polycistronic mRNA and encodes enzymes involved in NMP biodegradation. *nmpA* and the transposon-affected gene *nmpB* encode an *N*-methylhydantoin amidohydrolase that transforms NMP to γ -*N*-methylaminobutyric acid; this is metabolized by an amino acid oxidase (NMPase), either by demethylation to produce γ -aminobutyric acid (GABA) or by deamination to produce succinate semialdehyde (SSA). If GABA is produced, the activity of a GABA aminotransferase (GABA-AT), not encoded in the *nmp* gene cluster, is needed to generate SSA. SSA is transformed by a succinate semialdehyde dehydrogenase (SSDH) (NMPase) to succinate, which enters the Krebs cycle. The abilities to consume NMP and to utilize it for growth were complemented in the transposon-tagged mutant by use of the *nmpABCD* genes. Similarly, *Escherichia coli* MG1655, which has two SSDHs but is unable to grow in NMP, acquired these abilities after functional complementation with these genes. In wild-type (wt) BQ1 cells growing in NMP, GABA was not detected, but SSA was present at double the amount found in cells growing in Luria-Bertani medium (LB), suggesting that GABA is not an intermediate in this pathway. Moreover, *E. coli* GABA-AT deletion mutants complemented with *nmpABCD* genes retained the ability to grow in NMP, supporting the possibility that γ -*N*-methylaminobutyric acid is deaminated to SSA instead of being demethylated to GABA.

IMPORTANCE *N*-Methylpyrrolidone is a cyclic amide reported to be biodegradable. However, the metabolic pathway and enzymatic activities for degrading NMP are unknown. By developing molecular biology techniques for *Alicyclophilus* sp. strain BQ1, an environmental bacterium able to grow in NMP, we identified a six-gene cluster encoding enzymatic activities involved in NMP degradation. These findings set the basis for the study of new enzymatic activities and for the development of biotechnological processes with potential applications in bioremediation.

KEYWORDS *Alicyclophilus*, biodegradation, *N*-methylpyrrolidone

Alicyclophilus belongs to the *Comamonadaceae* family of the betaproteobacteria. Members of this genus have the ability to grow in recalcitrant carbon sources, such as indole and acetone, under denitrifying conditions (1, 2). This genus has also been identified as member of microbial communities involved in the oxidation of arsenite

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[As(III)] to arsenate [As(IV)], coupled to chlorate reduction (3), and in triclosan degradation (4). *Alicyclophilus denitrificans* strains K601^T and BC have been characterized extensively. Strain BC is able to degrade benzene and toluene in the presence of chlorate as an electron acceptor but is not able to degrade cyclohexanol (5). In contrast, strain K601^T utilizes cyclohexanol as a sole carbon source but does not use chlorate as an electron acceptor (6). Additionally, the presence of many mono- and dioxygenase genes in the genomes of both strains has suggested that this genus has a broad ability for xenobiotic degradation (7). On the other hand, *Alicyclophilus* sp. strain BQ1 is able to attack ester bonds in the commercial polyurethane varnish Hydroform and to utilize *N*-methylpyrrolidone (NMP), present as an additive in the Hydroform formulation. Moreover, it can grow in a mineral medium containing NMP (MM-NMP) as the sole carbon source (8).

NMP is a five-membered cyclic amide that is industrially produced by a typical ester-to-amide conversion by reaction of γ -butyrolactone with methylamine. The exceptional chemical properties exhibited by NMP have rendered it useful in a variety of applications across diverse industries, such as electronics, petrochemical processing, agrochemicals, pharmaceuticals, industrial cleaners, paints and coatings, and others. In addition, NMP has a favorable toxicological and environmental profile in comparison to those of other solvents; therefore, its use as a substitute for chlorinated hydrocarbons has increased. In rats and humans, NMP can be absorbed by inhalation and by oral and dermal exposition and then is successively oxidized to several compounds (9), which are excreted in urine (10, 11). Although NMP shows low acute toxicity, it produces skin and mucosal irritation, and its metabolites show teratogenic effects in rats (12). For these reasons, NMP use has been restricted in the European Union and Japan, where the maximal concentrations allowed in formulations and products are 5% and 1 ppm, respectively (11, 13).

NMP is not transformed by chemical hydrolysis in the environment but is rapidly biodegraded under aerobic conditions. It also shows low NMP acute toxicity on aquatic and terrestrial species and therefore is not expected to present a significant risk to the environment (14). NMP biodegradation has been observed in static die-away and semicontinuous activated sludge systems, in which infrared (IR) spectroscopy analysis revealed a shift in the NMP pattern after biodegradation, suggesting that the C-N bond between the nitrogen atom and the carbonyl group is cleaved (15). Moreover, high levels of NMP degradation (>99.5%) were reported for members of the genera *Acinetobacter*, *Cupriavidus*, and *Pseudomonas* after 3 days of culture (16). Recently, *Paracoccus* sp. strain NMD-4 was reported to be able to utilize NMP as a carbon and nitrogen source; after 12 h and 24 h of cultivation in a mineral salts medium containing 500 mg liter⁻¹ (5 mM) NMP, the following two compounds were detected by high-pressure liquid chromatography-mass spectrometry (HPLC-MS): 1-methyl-2,5-pyrrolidinedione and succinic acid. Based on these results, it was proposed that NMP might be oxidized to 1-methyl-2,5-pyrrolidinedione, followed by a possible cleavage of the two C-N bonds to form succinaldehyde, which may be oxidized to succinate for entry into the Krebs cycle (17). Nonetheless, the enzymes performing the reactions responsible for these transformations remain unknown. In this work, by using transposon-based mutagenesis of *Alicyclophilus* sp. strain BQ1, we found a six-gene cluster, *nmpABCDEF*, encoding proteins whose activities could transform NMP to succinate for entry into the Krebs cycle. Moreover, after transfer of some of these genes to *Escherichia coli*, it acquired the novel ability to grow in NMP.

RESULTS AND DISCUSSION

***Alicyclophilus* sp. strain BQ1, but not *A. denitrificans* strains BC and K601^T or *E. coli*, is able to grow with NMP as the sole carbon source.** To determine whether other members of the *Alicyclophilus* genus showed the ability to degrade NMP, strains BC, K601^T, and BQ1, as well as *E. coli*, were cultivated in MM-NMP (25 mM) as the sole carbon source and in Luria-Bertani medium (LB) as a reference. We did not use simple carbon sources, such as glucose, acetate, citrate, malate, or glycerol, as references

FIG 1 Growth and NMP consumption of different strains in MM-NMP and LB. (A) Growth of *Alicyclophilus* sp. strain BQ1 (circles), *A. denitrificans* BC (squares), *A. denitrificans* K601^T (diamonds), and *E. coli* (triangles) in MM-NMP (25 mM) (closed symbols) and LB (open symbols). (B) Growth (open symbols) and NMP consumption (closed symbols) of *Alicyclophilus* sp. strain BQ1 (circles) and the NMP⁻A.IV.74 mutant clone (squares). Error bars indicate standard deviations for three independent experiments. (C) Growth of BQ1, BQ1 Nal^r, and NMP⁻A.IV.74 strains on MM-malate (10 mM) and MM-NMP (25 mM) solid media.

because BQ1 grows very poorly on them compared to its growth on NMP (data not shown). *A. denitrificans* strains BC and K601^T and *E. coli* were not able to grow with NMP (25 mM) as the sole carbon source, but *Alicyclophilus* sp. strain BQ1 grew in NMP to the same extent as that in LB. Although it showed a longer lag phase in MM-NMP (8 h) than in LB (4 h), it presented similar rates in the log phase in NMP (0.43 h⁻¹) and in LB (0.44 h⁻¹), and its maximal growth was slightly less in NMP (optical density at 600 nm [OD₆₆₀] = 2.84 ± 0.20) than in LB (OD₆₆₀ = 3.24 ± 0.14). These results indicate that for BQ1, NMP is as good a carbon and energy source as LB and that the enzymes involved in NMP degradation are present in *Alicyclophilus* sp. strain BQ1 but not in the other tested strains (Fig. 1A).

The NMP⁻A.IV.74 mutant, which is unable to grow in NMP, has a transposon-interrupted gene in a cluster not present in *A. denitrificans* BC and K601. The ability of *Alicyclophilus* sp. strain BQ1 to utilize NMP as a carbon source was demonstrated by the consumption of 40% of NMP at 12 h and 90% of NMP at 24 h in supernatants of cultures grown in MM-NMP (Fig. 1B). To identify the genes responsible for NMP degradation in *Alicyclophilus* sp. strain BQ1, a mini-Tn5::Km^r transposon (18)-tagged library of 7,132 mutants was constructed and screened for clones that had lost the ability to grow in NMP. We isolated a mutant clone (NMP⁻A.IV.74) that was unable to grow in NMP (Fig. 1B) but able to grow in MM-malate (10 mM), to the same extent as

FIG 2 Genetic organization of the *nmpABCDEF* gene cluster in *Alicyclophilus* sp. strain BQ1 and their homologous genes in other proteobacteria. (A) Arrows represent the locations and orientations of genes homologous to the *nmpABCDEF* gene cluster (colored). The genomic context is presented, with conserved (gray) and nonconserved (white) genes for different bacterial species included. Sizes and positions of genes are drawn to a scale relative to the original data reported for the genome of each organism. (B) Putative proteins encoded by the *nmpABCDEF* gene cluster in *Alicyclophilus* sp. strain BQ1, with locus tags, nucleotide positions of coding regions, and closest identity matches to different bacterial species.

that of the wild-type (wt) BQ1 strain, and a Nal^r mutant, used as the genetic background for constructing the transposon-tagged library, which discounted the possibility of an auxotrophic mutation (Fig. 1C). NMP levels in the MM-NMP medium inoculated with the NMP⁻A.IV.74 mutant did not change, indicating that the mutant did not consume NMP and that NMP was not abiotically degraded during the cultivation time (Fig. 1B). A unique transposon insertion in the NMP⁻A.IV.74 chromosome was demonstrated by Southern blotting (data not shown). The transposon insertion site was located in a 1,776-bp open reading frame (ORF) encoding the α subunit of *N*-methylhydantoin amidohydrolase (NMHase) (*nmpB*) in the *Alicyclophilus* sp. strain BQ1 genome (GenBank accession number [NZ_NKDB00000000.1](#)) were analyzed, and a six-ORF cluster (*nmpABCDEF*) (GenBank accession number [KY695150](#)) with the same transcriptional orientation and a GC content (61 to 66%) and preferential use of codons similar to those of the rest of the genome was identified. This cluster is absent from *A. denitrificans* BC and K601^T (7) but is present in *Alicyclophilus* sp. strain B1 (Fig. 2A). Sequence similarity search analysis revealed that the genes flanking the

nmpABCDEF cluster in *Alicyclophilus* sp. strain BQ1 are contiguously located in *A. denitrificans* BC (ALIDE_RS12310 and ALIDE_RS12315) and K601^T (ALIDE2_RS13245 and ALIDE2_RS13250), indicating that the cluster in BQ1 is placed in a genome region that is conserved in this genus (Fig. 2A). The gene cluster *nmpABCDEF* is present in the betaproteobacteria *Thauera* sp. strain SWB20 and *Burkholderia contaminans* and in the alphaproteobacteria *Paracoccus pantotrophus* and *Paracoccus yeei* but absent in *Acidovorax* sp. strain JS42, another *Comamonadaceae* family member (not shown) (Fig. 2A). Interestingly, in *Alicyclophilus* sp. strain B1, the *nmpABCDEF* cluster is present (ALISP_RS13440 to ALISP_RS13415) and has a transposase-encoding gene (ALISP_RS13445) next to the *nmpA* gene, suggesting a horizontal transfer mechanism for the acquisition of this cluster in *Alicyclophilus* sp. strain B1. This transposase gene is not present in *Alicyclophilus* sp. strain BQ1, though. The species we identified as containing the *nmp* cluster have been studied for their potential in different bioremediation applications. Some *Thauera* species degrade aromatic compounds under aerobic and anaerobic conditions (19, 20) and oxidize organic acids and alcohols (21). *B. contaminans* is part of the *Burkholderia cepacia* complex (22), formed by 17 species that are genetically different but phenotypically similar, some of them with abilities to degrade aromatic compounds and chloride solvents present in herbicides and pesticides (23, 24). In addition, some *Paracoccus* sp. strains can degrade organic compounds, such as acetone (25), methylamine, and *N,N*-dimethylformamide (26). With the exception of *Paracoccus* sp. strain NMD-4, which has been reported to degrade NMP, there is no information on the ability of the above-mentioned bacteria to grow with NMP as the sole carbon source (17). Unfortunately, the genome of *Paracoccus* sp. strain NMD-4 has not yet been sequenced.

The sequence analysis of the *nmpABCDEF* cluster identified five structural genes (*nmpA*, *-B*, *-C*, *-D*, and *-F*) and a possible regulator gene (*nmpE*) (Fig. 2A). *nmpA* and *nmpB* (CE154_RS17095, CE154_RS17100) encode two polypeptides, of 73.5 kDa and 63.5 kDa, with high identities to the β and α subunits, respectively, of the NMHases from *Thauera* sp. strain SWB20 and *B. contaminans* (Fig. 2B). NMHases, initially studied in *Pseudomonas putida* 77, catalyze the ATP-dependent hydrolysis of the amide bond (N-3–C-4) in *N*-methylhydantoin (NMH), yielding *N*-carbamoylsarcosine, and are also able to hydrolyze hydantoin, L-5-methylhydantoin, glutarimide, and succinimide (27), but the hydrolysis of NMP has not been tested. In an ATP-dependent hydantoin amidohydrolase from *Pseudomonas* sp. strain NS671 (28), formed by the subunits HyuA (76 kDa) and HyuB (65 kDa) (29), the ATPase motif was identified in the HyuA subunit (30). In the *Alicyclophilus* sp. strain BQ1 NMHase, an ATPase motif was detected in the β subunit, encoded by *nmpA*, suggesting that BQ1 NMHase activity might be ATP dependent.

nmpC (CE154_RS17105) encodes a 50-kDa protein with several conserved domains of amino acid oxidases (AAO) from *Thauera* sp. strain SWB20 and *B. contaminans* (Fig. 2B). These proteins show a characteristic Rossmann-type fold that covalently binds flavin adenine dinucleotide (FAD) by two motifs, the dinucleotide-binding and adenine-binding motifs (31). Both motifs were identified in ORFC, the first with the characteristic sequence GGGFVG between residues Gly39 and Gly44 and the second comprising the Thr215, Val214, and Ala213 residues and the hydrophobic residue Ile38 (data not shown).

nmpD encodes a 12-kDa protein (ORFD) (CE154_RS17110) present in all the analyzed clusters (Fig. 2B). A conserved domain of unknown function (DUF861) was identified in the C-terminal region. This conserved domain is present in proteins belonging to the functionally diverse RmLC-like cupin superfamily. Members of this superfamily have very low sequence identity but exhibit a conserved six-stranded β -barrel fold (32). This superfamily comprises 20 families, including nonenzymatic proteins, such as plant seed storage proteins, transcription regulators, and stress-related proteins, and enzymatic proteins, such as isomerases/epimerases, decarboxylases, and dioxygenases (33). The remarkable functional diversity of this protein superfamily does not allow us to suggest a function for NMPD or a putative role for this protein in NMP metabolism.

nmpE (CE154_RS17115) encodes a 58-kDa protein exhibiting identity to a PucR transcriptional regulator (Fig. 2B). The amino-terminal region shows a conserved domain present in PucR regulatory proteins, whereas in the carboxy-terminal region, a helix-turn-helix DNA binding domain present in LysR-type transcriptional regulators was observed. In *Bacillus subtilis* under nitrogen-limiting conditions, PucR controls the expression of the *puc* and *ureABC* operons, which encode proteins involved in purine catabolism and in the conversion of urea to ammonia and CO₂ by urease, respectively (34). In both pathways, the PucR regulator recognizes nitrogenous compounds as effector molecules. Based on the chemical nature of NMP, it seems possible that the putative PucR-type transcriptional regulator encoded by *nmpE* may recognize NMP or a close derivative as the effector molecule to mediate *nmpABCDEF* gene cluster expression.

The *nmpF* gene (CE154_RS17120) encodes a 51-kDa protein whose sequence exhibits high identity to succinate semialdehyde dehydrogenase (SSDH) from *Thauera* sp. and *B. contaminans* (Fig. 2B). This enzyme catalyzes the irreversible oxidation of succinate semialdehyde (SSA) to succinate and CO₂.

The *nmpABCDEF* cluster is transcribed as a polycistronic mRNA. Short intergenic distances and the same transcription direction as that of the BQ1 *nmpABCDEF* gene cluster were found in homologous gene clusters from other proteobacteria, suggesting that these genes may be organized as an operon. However, by *in silico* analysis of the *nmpABCDEF* cluster, four putative σ^{70} promoters were identified: three in the upstream regions of *nmpA* (P_A), *nmpC* (P_C), and *nmpD* (P_D) and one in the *nmpE* coding region (P_E). Rho-independent terminators were also identified downstream of *nmpB*, *nmpC*, and *nmpE* (Fig. 3A). According to these predictions, the following four putative transcriptional units could be expressed: two dicistronic transcripts, *nmpA-nmpB* and *nmpD-nmpE*, and two monocistronic transcripts, *nmpC* and *nmpF*. In order to define whether these genes are expressed as a single transcriptional unit or as multiple transcripts, as the bioinformatic analysis suggested, RNA extracted from bacteria grown in MM-NMP (25 mM) was used to synthesize cDNA for reverse transcription-PCR (RT-PCR) analysis with primers spanning different positions along the gene cluster. Control PCRs using primers for the DNA gyrase subunit A gene, a housekeeping gene, demonstrated the complete elimination of DNA in DNase-treated RNA, as well as the proper synthesis of cDNA (Fig. 3B). Intragenic RT-PCRs using this cDNA as the template produced amplicons for all the genes of the cluster (Fig. 3C), proving that the entire *nmpABCDEF* cluster is transcribed under NMP growth conditions. Intergenic amplicons of the expected sizes were obtained by using genomic DNA (gDNA) as the template, guaranteeing that the primers were useful for analysis of the transcriptional units (Fig. 3D). With cDNA as the template, all the pairs of intergenic primers, which linked one transcript to the contiguous one, produced amplicons which matched the sizes of those obtained with gDNA as the template, demonstrating that the genes are expressed as one polycistronic mRNA and that P_A controls the expression of the genes as an operon (Fig. 3E).

***nmpB* encodes a protein involved in the utilization of NMP as a carbon source.** To demonstrate that the interruption of *nmpB* by Tn5 in the mutant strain NMP⁻A.IV.74 was responsible for its inability to degrade NMP, the mobilizable plasmid pBBR1MCS-5, bearing the wild-type (wt) *nmpA* and *nmpB* genes and the putative regulatory region P_A (pBBR1:: P_A *nmpAB*), was transferred to the NMP⁻A.IV.74 mutant in order to reconstitute this ability. Since the mini-Tn5::Km^r cassette has transcription termination signals of phage T4D gene 32 and a translation stop box containing stop codons in the three reading frames flanking the antibiotic resistance gene, expression of genes downstream of *nmpB* could be affected. Another construct, containing the *nmpA*, *nmpB*, *nmpC*, and *nmpD* genes and the putative regulatory region P_A (pBBR1:: P_A *nmpABCD*), was also generated and transferred to the NMP⁻A.IV.74 mutant. When mutants transfected with these constructs were challenged to grow on MM-NMP agar plates, only the clone complemented with the *nmpABCD* genes recovered the ability to grow (Fig. 4A). In order to measure NMP consumption, and since the complemented mutants exhib-

FIG 3 Cotranscription analysis of the *nmpABCDEF* gene cluster. (A) Positions of putative promoters (bent arrows), Rho-independent terminators (hairpin loops), and intragenic and intergenic regions to be amplified by specific primers between the *nmpA-nmpB*, *nmpB-nmpC*, *nmpC-nmpD*, *nmpD-nmpE*, *nmpE-nmpF*, and *nmpF-nmpG* genes. (B to E) Total RNA isolated from *Alicyclophilus* sp. strain BQ1 cells growing in MM-NMP was reverse transcribed and the cDNA synthesized used as the template for PCRs. (B) Positive and negative controls amplified using the primer set *gyrA-gyrAR*, targeting the housekeeping *gyrA* gene; the templates were genomic DNA (gDNA) from *Alicyclophilus* sp. strain BQ1, RNA treated (DNase +) or not (DNase -) with RNase-free DNase, and synthesized cDNA. (C) *nmpABCDEF* intragenic RT-PCR amplifications using the primer sets HydAF-HydAR (lane A), NMHydF-NMHydR (lane B), AaOXiF-AaOXiR (lane C), CupF-CupR (lane D), PucF-PucR (lane E), and SSDHF-SSDHR (lane F). (D and E) Intergenic PCR (D) and RT-PCR (E) amplifications using gDNA and cDNA, respectively, as the templates, with the primers sets HydAF-*nmpABR* (lanes A-B), *nmpBCF*/*nmpBCR* (lanes B-C), AaOXiF/*nmpCDR* (lanes C-D), CupF/PucR (lanes D-E), AaOXiF/*nmpDER* (lanes E-F), and *nmpEFF*/*nmpEFR* (lanes E-F). Negative-control (C-) reaction mixtures contained no DNA template. M, DNA marker (λ DNA EcoRI + HindIII ladder [Thermo Scientific]).

ited very little growth in liquid MM-NMP, we considered that the addition of a small supplementary carbon source would be helpful to support the complemented mutant's initial growth to subsequently express the proteins needed to consume NMP. Malate was tested as a carbon source in liquid cultures, but cell growth was very poor. Therefore, we looked for a condition under which *Alicyclophilus* sp. strain BQ1 would be able to grow to a measurable amount but not to the maximum growth observed in complete LB or MM-NMP (25 mM) (Fig. 1). We tested different LB dilutions (80%, 60%, 40%, 30%, and 20%) (data not shown) and determined that LB diluted to 30% sustained bacterial growth to a maximum OD_{660} of 1.5, almost half of the growth observed in 100% LB and in MM-NMP (25 mM). Based on this, the growth of the wt *Alicyclophilus* sp. strain BQ1 and the NMP consumption measured by gas chromatography-MS (GC-MS) were followed in MM-NMP with 30% LB (MM-NMP/LB30) and in LB30 medium alone. During the first 10 h of incubation, wt *Alicyclophilus* sp. strain BQ1 growth levels were identical in LB30 and MM-NMP/LB30, reaching a stationary phase with a maximal OD_{660} of 1.5. After that time, a second exponential growth phase was developed only in MM-NMP/LB30, reaching a maximum OD_{660} of 3.2 at 16 h of incubation (Fig. 4B). Concomitant with the second growth phase, a rapid decrease in NMP levels occurred from 12 h of incubation, to an undetectable level after 20 h. In contrast, when the NMP⁻A.IV.74 mutant was cultivated in MM-NMP/LB30, it showed only the first exponential phase, reaching a maximal OD_{660} of 1.5, with no changes in NMP concentration in the medium, showing the inability of NMP⁻A.IV.74 to metabolize NMP and to use it as a carbon source. These results indicate that the carbon source present in LB30 sustained growth only up to the first exponential phase and that the second exponen-

FIG 4 Growth, NMP consumption, and complementation analysis of the NMP⁻A.IV.74 mutant. (A) Growth on solid MM-malate (10 mM) and MM-NMP (25 mM) media of wt *Alicyclophilus* sp. strain BQ1, the NMP⁻A.IV.74 mutant transformed with the empty vector pBBR1 (A.IV.74), the NMP⁻A.IV.74 mutant transformed with pBBR1::P_AnmpAB (A.IV.74 nmpAB), and the NMP⁻A.IV.74 mutant transformed with pBBR1::P_AnmpABCD (A.IV.74 nmpABCD). (B) Growth (solid lines) of wt *Alicyclophilus* sp. strain BQ1 (circles) and NMP⁻A.IV.74 (squares) in LB30 (gray symbols) and MM-NMP/LB30 (white symbols) and NMP consumption (dashed lines, black symbols) of the same clones growing in MM-NMP/LB30. (C) Growth in MM-NMP/LB30 (solid lines, white symbols) of the NMP⁻A.IV.74 mutant transformed with the empty pBBR1MCS-5 vector (circles), pBBR1::P_AnmpAB (squares), or pBBR1::P_AnmpABCD (diamonds) and NMP consumption (dashed lines, black symbols) of the same clones growing in MM-NMP/LB30. Error bars indicate standard deviations for three independent experiments.

tial phase, observed for the wt, was sustained by the utilization of NMP as a carbon source (Fig. 4B). The diauxic behavior observed for wt *Alicyclophilus* sp. strain BQ1 growing in MM-NMP/LB30 suggests that a catabolite repression regulatory mechanism might control the expression of the *nmp* genes, as observed in several culture systems where two carbon sources were included (35). Having characterized MM-NMP/LB30 as a useful experimental system for analyzing the ability of *Alicyclophilus* sp. strain BQ1 to consume NMP, we cultivated the NMP⁻A.IV.74 mutant complemented with either the pBBR1::P_AnmpAB or pBBR1::P_AnmpABCD construct in this medium. The mutant transformed with the empty vector only exhibited the first exponential phase and did not consume NMP (Fig. 4C). When the mutant was complemented with pBBR1::P_AnmpAB, it showed only the first exponential phase, reaching an OD₆₆₀ of 1.5, but in contrast to what was expected, complete NMP consumption at 16 h was observed (Fig. 4C). The explanation for the consumption of NMP by the pBBR1::P_AnmpAB-complemented mutant is discussed in the following section. On the other hand, the mutant complemented with pBBR1::P_AnmpABCD exhibited diauxic growth and complete NMP con-

FIG 5 NMP degradative pathway by enzymes encoded in the *nmpABCDEF* gene cluster of *Alicyclophilus* sp. strain BQ1. Enzymatic activities proposed for the proteins encoded by the *nmpA-nmpB*, *nmpC*, and *nmpF* genes are indicated; the *nmpD* and *nmpE* genes encode a hypothetical protein and a putative PucR family transcriptional regulator, respectively. Based on protein sequence comparison, a demethylation reaction for the AAO encoded by the *nmpC* gene that transforms γ -N-methylaminobutyric acid into GABA and a putative GABA-AT activity required for GABA transformation to SSA are shown (dashed arrows). However, experimental data support the possibility that a deamination reaction directly transforming γ -N-methylaminobutyric acid into SSA is the most probable activity for NMPC (solid arrows).

sumption similar to those of wt *Alicyclophilus* sp. strain BQ1 (Fig. 4C), indicating that the *nmpABCD* genes are needed to fully restore the wt phenotype of the NMP⁻A.IV.74 mutant, both for growth and for NMP degradation. Since RT-PCR analysis showed that the *nmp* gene cluster is transcribed as a polycistronic mRNA, complementation with *nmpABCD*, the first four genes of the operon, represents a puzzling result. Two mechanisms might explain it. One is that not only P_A controls the expression of the genes in the cluster but also other promoters (P_D , P_F , or others not identified by the bioinformatic analyses performed) may regulate the expression of the genes downstream of *nmpD*; the second possibility is that other proteins encoded by homologous genes in the BQ1 genome may substitute for the activities of the *nmpE*- and *nmpF*-encoded proteins. Genes homologous to *nmpE* were not found in the *Alicyclophilus* sp. strain BQ1 genome, but 3 genes encoding SSDHs were found (CE154_RS11195, CE154_RS11480, and CE154_RS16960). Further experiments are needed to define the mechanism behind this result.

The *nmpABCDEF* gene cluster encodes enzymes that can transform NMP to succinate. The *P. putida* 77 cytosolic NMHase, induced when creatinine (CRT) or NMH is used as a nitrogen source, catalyzes the hydrolysis of the amide group of NMH to *N*-carbamoylsarcosine. Similarly, the *Alicyclophilus* sp. strain BQ1 NMHase, encoded by the *nmpA* and *nmpB* genes, is responsible for the first step in the NMP biodegradative pathway, hydrolyzing the unique amide group present in the NMP molecule to produce γ -N-methylaminobutyric acid (Fig. 5). The consumption of NMP by the mutant complemented with pBBR1:: P_A *nmpAB*, with no increase in growth, may be explained by considering that the enzymatic activity of *N*-methylhydantoin amidohydrolase was reconstituted in the pBBR1:: P_A *nmpAB*-complemented mutant, and hence NMP could be

hydrolyzed to γ -*N*-methylaminobutyric acid. Since the enzyme encoded by *nmpC* that transforms γ -*N*-methylaminobutyric acid into the following compound of the pathway should be absent in the pBBR1::P_A*nmpAB*-complemented mutant, it could not be channeled to the degradation pathway, and NMP could not be used as a carbon source. This explains why no growth was observed (Fig. 4A) and provides evidence for the proposed function of the enzyme encoded by the *nmpA* and *nmpB* genes. The second step in the proposed NMP degradation pathway involves the activity of an AAO, encoded by *nmpC*. This enzyme may catalyze either the oxidative demethylation of γ -*N*-methylaminobutyric acid to produce γ -aminobutyric acid (GABA) or a deamination reaction to directly produce SSA. γ -*N*-Methylaminobutyric acid is structurally similar to sarcosine, which in *P. putida* 77 is oxidatively demethylated by sarcosine dehydrogenase, an amino acid oxidase (36, 37). Also, in the nicotine catabolism of *Arthrobacter nicotinovorans* pAO1, the demethylation of γ -*N*-methylaminobutyric acid to produce GABA is performed by a FAD-dependent γ -*N*-methylaminobutyric acid oxidase (MABO) (38). If the demethylation reaction is performed and GABA is produced, it has to be transformed to SSA by a GABA aminotransferase (GABA-AT) activity, as it is metabolized in the GABA shunt in bacteria, plants, and mammals (39). A GABA-AT-encoding gene is absent from the *nmpABCDEF* gene cluster; nevertheless, genes encoding putative class III aminotransferase activities were identified in the *Alicyclophilus* sp. strain BQ1 genome (CE154_RS09420, CE154_RS13250, and CE154_RS21195). On the other hand, if AAO (NMPC) deaminates γ -*N*-methylaminobutyric acid, GABA-AT is not needed in the pathway to metabolize NMP (Fig. 5). In support of the deamination reaction, a FAD-dependent amino oxidase (AO) able to deaminate γ -*N*-methylaminobutyric acid to form SSA and methylamine has been reported for *A. nicotinovorans* pAO1 (40). Finally, SSDH (NMPF) converts SSA to succinate, which is channeled into the Krebs cycle (Fig. 5). Succinate has been detected after NMP degradation by *Paracoccus* sp. strain NMD-4 (17), supporting this reaction of our proposed NMP degradation pathway in *Alicyclophilus* sp. strain BQ1.

After functional complementation with *nmpABCD* genes, *E. coli* is able to utilize NMP as a carbon source. If the AAO performs the oxidative demethylation of γ -*N*-methylaminobutyric acid to produce GABA, the pathway couples two enzymatic activities of the CRT degradation pathway, NMHase and AAO, with activities from the GABA shunt, GABA-AT and SSDH. These activities participate in amino acid metabolism, acting in the arginine-proline and alanine-aspartate-glutamate pathways, respectively. An analysis of the metabolic maps available in the Kyoto Encyclopedia of Genes and Genomes (KEGG) showed that in *E. coli* MG1655, GABA-AT (*gabT* and *puuE*) and SSDH (*gabD* and *ynel*) are present, but the enzymatic activities involved in creatinine degradation are not, which explains the inability of *E. coli* K-12 MG1655 to utilize NMP as a sole carbon source (Fig. 1). Therefore, to provide evidence that the coupling of these enzymatic activities is required for the complete catabolism of NMP, plasmid constructs containing the wt *Alicyclophilus* sp. strain BQ1 *nmpAB* (pBBR1::P_A*nmpAB*) or *nmpABCD* (pBBR1::P_A*nmpABCD*) genes were transformed into *E. coli* K-12 MG1655, and the growth and NMP consumption of the transformants were evaluated in MM-NMP/LB30. The results showed that the strain with the empty vector and the clone transformed with the pBBR1::P_A*nmpAB* construct were unable to grow in MM-NMP/LB30 and did not consume NMP, but the clone transformed with pBBR1::P_A*nmpABCD* was able to grow in MM-NMP/LB30 medium and to consume NMP almost as well as *Alicyclophilus* sp. strain BQ1 (Fig. 6). These results confirm that the *nmpA* and *nmpB* genes, by themselves, are not sufficient to confer the ability to consume NMP as a carbon source and that the products from the *nmpCD* genes are also needed for NMP degradation.

Double the amount of SSA, but not GABA, is detected in cytosolic extracts of *Alicyclophilus* sp. strain BQ1 growing in MM-NMP. In order to obtain evidence of whether the reaction performed by the AAO (NMPC) would produce GABA as an intermediate in the NMP degradation pathway, we measured intracellular GABA levels in cultures of wt *Alicyclophilus* sp. strain BQ1 grown in MM-NMP and in LB (as a control) at the same growth stage (OD₆₆₀ = 1.5). For this purpose, an enzymatic GABase assay

FIG 6 Heterologous complementation of *E. coli* with the *Alicyclophilus* sp. strain BQ1 *nmpABCD* genes allows it to consume NMP. The graph shows growth (solid lines, open symbols) and NMP consumption (dashed lines, black symbols) of *E. coli* MG1655 harboring the empty pBBR1MCS-5 vector (circles), pBBR1::P_A*nmpAB* (squares), or pBBR1::P_A*nmpABCD* (diamonds) and cultured in MM-NMP/LB30.

that quantifies NADP reduction in a coupled enzymatic reaction mixture containing GABA-AT and SSDH was used. The GABase assay allows discrimination of the amounts of GABA and SSA that are present in samples by running simultaneous assays with and without the GABA-AT inhibitor ethanolamine-*O*-sulfate (EOS). The results showed similar amounts of NADPH in the absence and presence of EOS (1.72 ± 0.22 and 1.91 ± 0.18 μmol per OD₆₆₀ unit, respectively, for cells grown in MM-NMP and 0.71 ± 0.13 and 0.79 ± 0.11 μmol per OD₆₆₀ unit, respectively, for cells grown in LB), indicating that SSA, but not GABA, was synthesized in both types of cultures. The fact that intracellular SSA levels in cells grown in MM-NMP (1.91 ± 0.11 μmol per OD₆₆₀ unit) were more than double the levels observed in cells grown in LB (0.79 ± 0.18 μmol per OD₆₆₀ unit) indicates that SSA, but not GABA, is synthesized when NMP is used as a carbon source. Although the possibility that GABA was not detected because it is a quickly metabolized intermediate cannot be excluded, the fact that SSA, which is also an intermediate in the proposed NMP degradation pathway, was present in MM-NMP at double the amount in LB indicates that this pathway is more active in the presence of NMP. The detection of SSA but not GABA in cells grown in MM-NMP supports the possibility that γ -*N*-methylaminobutyric acid may be converted directly to SSA, without GABA formation. This evidence implies that NMPC may perform a deamination rather than a demethylation reaction, similar to the deamination reaction performed by the AO in *A. nicotinovorans* pAO1 (40). Moreover, on searching the BQ1 genome for genes encoding proteins homologous to MABO or AO, we did not find any with close sequence similarity, suggesting that there is no other enzyme that may perform one of these activities on γ -*N*-methylaminobutyric acid. If a deamination reaction is performed by ORFC, GABA will not be synthesized, and hence GABA-AT activity is not required for NMP catabolism.

Is GABA-AT activity required for NMP catabolism? GABA-AT catalyzes the conversion of GABA to SSA, by which the GABA amino group is donated to α -ketoglutarate. In *E. coli*, GABA-AT is encoded by two genes: *gabT* encodes an enzyme involved in the GABA shunt (41), and *puuE* encodes a secondary GABA-AT that acts in putrescine metabolism, in the absence of *gabT* expression, when arginine, ornithine, agmatine, and putrescine are used as nitrogen sources (42). Since GABA was not detected in BQ1 cells grown in MM-NMP, a GABA-AT activity seems not to be required for NMP metabolism, which is in accordance with the absence of a gene encoding this enzyme in the *nmpABCDE* cluster. To demonstrate that GABA-AT activity is not required for NMP degradation, and since there is no technique to knock down genes in *Alicyclophilus* sp. strain BQ1, two single mutants (Δ *puuE* and Δ *gabT*) and a double mutant (Δ *puuE* Δ *gabT*) were generated by homologous recombination in *E. coli* DY329 (Fig. 7A), transformed with the pBBR1::P_A*nmpABCD* construct, and analyzed in MM-NMP/LB30 medium. When it was transformed with the empty vector, *E. coli* DY329 showed a maximum OD₆₆₀ of 1.5 (Fig. 7C), similar to the behavior previously observed for *E. coli* MG1655 (Fig. 6). However, when it was transformed with the pBBR1::P_A*nmpABCD*

FIG 7 Construction of *E. coli* DY329 GABA-AT deletion mutants and complementation with the wt *Alicyclophilus* sp. strain BQ1 *nmpABCD* genes. (A) For construction of the single mutant DY329 $\Delta puuE$, the *puuE* gene was exchanged for a kanamycin resistance cassette by homologous recombination, and then the resistance was eliminated by FRT site-specific recombination; primers used to detect the deletion are indicated. For construction of the single mutant DY329 $\Delta gabT::Km^r$, the *gabT* gene was exchanged for a kanamycin resistance cassette by homologous recombination; primers used to detect the deletion are indicated. For construction of the double mutant DY329 $\Delta puuE \Delta gabT::Km^r$, both the *puuE* and *gabT* genes were deleted (the exchange of the *gabT* gene for a kanamycin resistance cassette was made in the DY329 $\Delta puuE$ strain). In wt DY329, the *puuE* and *gabT* genes are intact. (B) PCR products amplified with primers *puchkF* and *puchkR* for *puuE* and primers *gtchkF* and *gtchkBR* for *gabT*. The gel shows the amplicons detected for strain DY329 $\Delta puuE$, where instead of the *puuE* gene an FRT scar is left and the *gabT* gene remains intact; the amplicons detected for strain DY329 $\Delta gabT::Km^r$, in which the *gabT* gene was exchanged for a kanamycin resistance cassette and the *puuE* gene remains intact; the amplicons detected for DY329 $\Delta puuE \Delta gabT::Km^r$, in which both genes are deleted; and the amplicons detected for strain DY329, in which both the *puuE* and *gabT* genes are intact. (C) Growth in MM-NMP/LB30 of *E. coli* DY329 strains bearing the empty pBBR1MCS-5 vector (open) or the pBBR1::P_A*nmpABCD* construct (closed) transformed into different genetic backgrounds, as follows: diamonds, wt; triangles, $\Delta puuE::Kan^r$; circles, $\Delta gabT::Kan^r$; and squares, $\Delta puuE \Delta gabT::Kan^r$. Error bars indicate standard deviations for three independent experiments.

construct, it showed increased growth, similar to that of *E. coli* MG1655 transformed with the same construct (Fig. 6) and that of wt *Alicyclophilus* sp. strain BQ1 (Fig. 4B). Interestingly, the three deletion mutants transformed with pBBR1::P_A*nmpABCD* were able to grow in MM-NMP/LB30 similarly to wt *E. coli* DY329 transformed with the *nmpABCD* genes (Fig. 7C), indicating that they could utilize NMP as a carbon source and that GABA-AT activity was not required for NMP degradation. These results strongly support the hypothesis that deamination of γ -N-methylaminobutyric acid is the mechanism used by the amino acid oxidase (NMPC) to produce SSA. Whether this protein might perform both demethylation and deamination reactions will have to be explored in future work.

In this study, we identified and characterized the six-gene cluster *nmpABCDEF*, involved in NMP degradation, from the betaproteobacterium *Alicyclophilus* sp. strain BQ1. Genetic analyses, functional homologous and heterologous complementation, and measurements of NMP consumption and of the intracellular levels of some of the proposed intermediates in the pathway allowed us to propose the functions of some of the *nmpABCDEF*-encoded enzymes, as well as a novel NMP degradative pathway. In

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference
Strains		
<i>Alicyclophilus</i> strains		
BQ1	Wild-type strain able to consume NMP	8
BQ1 Nal ^r	Spontaneous nalidixic acid-resistant mutant	This work
BQ1 NMP ⁻ A.IV.74	BQ1 mutant with mini-Tn5::Km ^r inserted by transposition; unable to grow in MM-NMP	This work
<i>A. denitrificans</i> BC	Benzene-degrading environmental strain	5
<i>A. denitrificans</i> K601 ^T	Cyclohexanol-degrading environmental strain	6
<i>E. coli</i> K-12 strains		
DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r _K ⁻ m _K ⁻) phoA supE44 λ ⁻ thi-1 gyrA96	53
S17-1	recA F ⁻ hsdR	54
HB101	F ⁻ mcrB mrr hsdS20(r _B ⁻ m _B ⁻) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 (Sm ^r) glnV44 λ ⁻	55
MG1655	F ⁻ λ ⁻ ilvG rfb-50 rph-1	56
DY329	W3110 Δ lacU169 nadA::Tn10gal490 pgl Δ 8 [λ cl857 Δ cro bio A]	57
DY329 Δ puuE::Kan ^r		This work
DY329 Δ gabT::Kan ^r		This work
DY329 Δ puuE		This work
DY329 Δ puuE Δ gabT::Kan ^r		This work
Plasmids		
pUT/mini-Tn5 Km ^r	Tn5-based delivery plasmid with Km ^r and Ap ^r cassettes	18
pRK2013	Conjugation helper plasmid with Tra, ColE1 replicon, and Km ^r cassette	55
pBR1MCS-5	Broad-host-range cloning vector with lacZ and Gm ^r cassette	46
pBR1::P _A nmpAB	pBBR1MCS-5 with 4.1-kb HindIII-XbaI fragment with nmpA and nmpB genes and Gm ^r cassette	This work
pBR1::P _A nmpABCD	pBBR1MCS-5 with 5.9-kb HindIII-XbaI fragment with nmpA, nmpB, nmpC, and nmpD genes and Gm ^r cassette	This work
pCP20	FLP recombinase expression; Ap ^r Cm ^r	58
pBSFRTNeo	Harbors kanamycin transcriptional unit flanked by FRT sites	59

^aKm^r, Ap^r, Nal^r, Gm^r, and Cm^r indicate kanamycin, ampicillin, nalidixic acid, gentamicin, and chloramphenicol resistances, respectively.

this pathway, the NMP ring is hydrolytically cleaved in the C-N bond, and successive deamination and oxidation reactions produce succinate, which is channeled to the Krebs cycle. Previously, a NMP degradation pathway was proposed for *Paracoccus* sp. strain NMD-4, an alphaproteobacterium, in which it was suggested that the NMP ring is oxidized prior to a double cleavage of the two C-N bonds to produce methylamine and succinaldehyde, which is oxidized again to produce succinic acid (17). It will be interesting to define whether the two types of catalytic mechanisms to degrade NMP exist in different classes of bacteria.

MATERIALS AND METHODS

Strains, growth conditions, and reagents. A complete list of strains and plasmids used in this study is included in Table 1. *Alicyclophilus* sp. strain BQ1 was isolated from deteriorated PU foam pieces collected from an open-air dump site (Mexico City, México) in 2005, as previously described (8). *Alicyclophilus denitrificans* BC (DS18852) was generously donated by M. J. Oosterkamp and Alfons J. M. Stams (Wageningen University, Wageningen, The Netherlands), whereas *A. denitrificans* K601^T (DSM14773) was purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Strains were cultured in Luria-Bertani medium (LB) (43) or in minimal medium (8) with 25 mM *N*-methylpyrrolidone (Sigma-Aldrich) (MM-NMP) or 10 mM malate as the carbon source. Solid media were prepared by adding 1.5% agar. Previously, other simple carbon compounds (2, 5, and 10 mM glucose, 1, 5, and 10 mM acetate, 10 and 40 mM citrate, and 5, 10, 20, and 40 mM glycerol) were tested, but they poorly sustained or did not sustain *Alicyclophilus* sp. strain BQ1 growth. Incubation conditions were 37°C with shaking at 200 rpm. When needed, kanamycin (30 μ g ml⁻¹), gentamicin (20 μ g ml⁻¹), or nalidixic acid (20 μ g ml⁻¹) was included. Growth was monitored by measuring the optical density at 660 nm (OD₆₆₀) by use of a UV-visible spectrophotometer (Ultrospec 2000; Pharmacia Biotech). Cloning was performed by standard protocols. Antibiotics, restriction enzymes, T4 DNA ligase, Phusion high-fidelity and Taq DNA polymerases, and PCR and cloning reagents were purchased from Thermo Scientific Inc. Primers were synthesized in the DNA Synthesis and Sequencing Facility at the Biotechnology Institute, Universidad Nacional Autónoma de México (DSSF-IBT-UNAM), or by Sigma-Aldrich or Macrogen (South Korea). Sequences of the primers are presented in Table 2.

Transposon mutagenesis and screening. A transposon insertional mutagenesis protocol for *Alicyclophilus* sp. strain BQ1 was developed in our laboratory by mobilizing the suicide plasmid pUT

TABLE 2 Sequences of primers used in this study

Primer name	Sequence (5' to 3')
Tn50	GGCCGCACTTGTGTATAAG
Tn51	GGCCAGATCTGATCAAGAG
Tn50 rev	CTTATACACAAGTGCGGCC
Tn51 rev	CTCTTGATCAGATCTGGCC
gyrAF	CTGAGCCATACGGGCTACAT
gyrAR	TCCAGTCGTCTTCCTTGTC
HydAF	CCGATCTGGACAGGATCAAC
HydAR	CGGATCAATTCGATCTCGTT
NMHydF	GGTGAAAAAGGCCATGAAGA
NMHydR	CCATCGGTATCGGCATAGTC
AaOXiF	GGCACGCCAACATTCTCTAT
AaOXiR	ACTGGGTCCATTATCCTTG
CupF	CCTCAGGCCGAATACAGCTA
CupR	GCGGCAAAGTAAATGGAGTC
PucF	GAACACCGATATCCCCAGA
PucR	ATGGAAACGGCAAAGACATC
SSDHf	TTCTTCGAGCCCACCATACT
SSDHR	AATCCGTATTCCGTGTCGTT
NMHbF	CCCAAGCTTATTCCATGGATTGGCTATGC
NMHaR	ATTCTAGAAATGGTCTTTCGGCGTATGAG
AaOXR	ATTCTAGAGAATCGGCGTGTCTATGGAT
nmpABR	GAACGTGATGGGATCGATGT
nmpBCF	CCTGGACGAGTACATCAGCA
nmpBCR	ACTGGATCGTTCGATCTTC
nmpCDR	GACATGCCATTCCCCTTG
nmpEFF	GGCGCACACCAAGGATATT
nmpEFR	TGTAGGCATGCTGCTTGAAC
nmpDER	AGTTCGGCATCTGGGTGAT
pukmF	GGGCGTTTAATTTCCGATTAACCGTGAAGAGTCAAAAGGTGTGAA ACATGTCTGCAAACCTATGCTACTCCGTCG
pukmR	AGCGCATCAGACATTATTGCGTTGGGCTATTAATCGCTCAGCGCA TCCTGTCCCGCGGATTTGCTCTAC
gtkmF	CTTAGAAATCAAATATATGTGCATCGGTCTTTAACTGGAGAATGC GAATGTCTGCAAACCTATGCTACTCCGTCG
gtkmR	GCAGCGTCGCCTCCGGCATAGGAGCGGCGCTACTGCTTCGCCTC ATCAAATCCCGCGGATTTGCTCTAC
gtchkF	TCGGGTCTGGGTCGTGAAG
gtchkBR	TGTGTCACTTGGGAGAGCG
puchkF	GTGTGCCGTTTACCGCATG
puchkR	CAGACATTATTGCGTTGGGC

mini-Tn5::Km^r (18) from *E. coli* S17-1, by triparental mating, into an *Alicyclophilus* sp. strain BQ1 Nal^r strain. The *Alicyclophilus* sp. strain BQ1 Nal^r strain was generated by plating the wt BQ1 strain on LB plus nalidixic acid (20 μ g ml⁻¹) until some colonies started to grow. One colony was selected and grown for several rounds with the same antibiotic, and then its ability to grow in NMP was tested; the strain showed the same growth as that of the wild type (Fig. 1C; Table 1). Donor and recipient strains along with the helper strain *E. coli* HB101, carrying the self-transmissible plasmid pRK2013, were grown overnight in LB broth at 37°C with shaking. Subsequently, 0.5 ml of each culture was used to independently inoculate 24.5 ml of fresh LB broth. Cultures were incubated again at 37°C to exponential phase ($OD_{660} = 1.0$), and cells were harvested by centrifugation. Pellets were washed twice and resuspended in 1 ml of MgSO₄ (10 mM). Aliquots (100 μ l) of the donor strain and corresponding amounts of helper and recipient strains (1:1:3) were mixed, spread onto LB agar plates with no antibiotic, and incubated for 24 h at 30°C. Exconjugants were collected by adding 1 ml of MgSO₄ (10 mM), and 300- μ l aliquots were spread onto agar plates with kanamycin and nalidixic acid and incubated for 48 h at 37°C. After several conjugation experiments, more than 7×10^3 kanamycin- and nalidixic acid-resistant mutants were recovered, stored at -70°C in 30% glycerol, and tested for the ability to grow on MM-NMP (25 mM) and MM-malate (10 mM) agar plates.

NMP and malate growth drop test. The wt BQ1 and NMP-A.IV.74 strains were grown overnight in LB, their OD_{660} values determined, and “stock suspensions” with an OD_{660} of 1.5 (10^9) prepared. Serial dilutions (10^{-4} to 10^{-7}) in MgSO₄ (10 mM) were done, and aliquots (10 μ l) of each dilution were spotted onto MM-NMP and MM-malate agar plates; after 2 days of incubation at 37°C, the plates were photographed.

NMP quantification. NMP was extracted by mixing cell-free supernatants of MM-NMP or MM-NMP/LB30 cultures (2 ml) and chloroform (1 ml) (8). The organic phase was recovered and quantified by gas chromatography coupled to mass spectrometry detection (GC-MS) (Agilent 7890B and Agilent 5977A, respectively) with two 5% phenyl-methylpolysiloxane columns (15 m $\times$ 250 μ m $\times$ 0.25 μ m). The oven was heated from 50°C to 100°C at 20°C/min. Helium was used as a carrier gas, at a flow rate of 1 ml/min,

and 1 μ l of sample was injected. The NMP concentration was obtained based on a standard curve for pure NMP (0.25 to 3 mg/ml). Measurements were performed in triplicate.

Identification of the transposon-interrupted gene. To identify the region affected by the transposon insertion, PstI restriction fragments of genomic DNA (gDNA) isolated from the NMP-A.IV.74 mutant were self-circularized in a ligation reaction mixture with T4 DNA ligase (Thermo Scientific) and used as the template for inverse PCR amplification with *Taq* DNA polymerase (Thermo Scientific) and the transposon-specific primers Tn50 and Tn51 (44). PCR was carried out in a Veriti 96-well thermal cycler (Applied Biosystems), with an initial denaturation cycle at 95°C for 1 min; 35 cycles of denaturation at 95°C for 30 s, alignment at 60°C for 30 s, and extension at 72°C for 1 min; and a final extension cycle at 72°C for 7 min. An approximately 300-bp amplicon was synthesized, purified by use of a GeneJet gel extraction kit (Thermo Scientific), and sequenced with primer Tn51 in an ABI 3130 xl sequencer (Applied Biosystems) at DSSF-IBT-UNAM. The sequence adjacent to the transposon was identified in the *Alicyclophilus* sp. strain BQ1 genome (GenBank accession number [NZ_NKDB000000000.1](#)).

Bioinformatic analysis. Homology searches and conserved domain analysis were conducted against the GenBank database by using the BLASTX algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the sequence editor ApE (<http://biologylabs.utah.edu/jorgensen/wayned/ape/>). Multiple protein sequences were aligned using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Putative promoters were predicted by use of BPROM (<http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>) and neural network promoter prediction (http://www.fruitfly.org/seq_tools/promoter.html), whereas Rho-independent transcription terminators were identified using Arnold Finding Terminators (<http://rna.igmors.u-psud.fr/toolbox/arnold/>). All programs used in this study are freely available online.

Nucleic acid purification and RT-PCR. gDNA was extracted from overnight cultures growing in LB by use of an adapted protocol (45) in which the first incubation step, at 37°C for 60 min with lysozyme (1.7 μ g/ μ l) and RNase (1 μ g/ μ l), was incorporated to improve cell lysis and remove RNA contamination. Subsequent steps were performed as described previously, except for a longer incubation in cetyltrimethylammonium bromide (CTAB)-NaCl (30 min) and an additional chloroform extraction after the phenol-chloroform treatment, as well as suspension of gDNA samples in sterile water. Plasmid DNA was isolated using a GeneJET plasmid miniprep kit (Thermo Scientific) following the manufacturer's protocol. For RT-PCR, total RNA was isolated from log-phase cultures in MM-NMP (25 mM) by use of RNeasy RT (Sigma-Aldrich) and treated with RNase-free DNase I (Thermo Scientific) according to the manufacturers' instructions. Before the synthesis of cDNA, the absence of DNA from the DNase-treated RNA preparation was confirmed by a PCR using the gyrAF-gyrAR primer set, and no amplicon was produced. cDNA was synthesized from total RNA by use of a RevertAid First Strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer's recommendations, using total RNA (2 μ g) and a gene-specific primer mix (HydAR, NMHydR, AaOXiR, CupR, PucR, SSDHR, and gyrAR) (20 pmol). All the PCRs were performed using *Taq* DNA polymerase (Thermo Scientific). To perform intragenic or intergenic amplifications, gDNA (90 ng) and cDNA (150 ng) were used as templates for PCR and RT-PCR, respectively, using different primer pairs (Table 2; Fig. 3). Amplification conditions were as follows: initial denaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 30 s, alignment at 62°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min.

Genetic complementation. Two constructs, containing the wild-type *nmpAB* and *nmpABCD* genes and the putative regulatory region P_A from *Alicyclophilus* sp. strain BQ1, were generated in the mobilizable vector pBBR1MCS-5 Gm^r (46). The wild-type 4.1-kb *nmpAB* and 5.6-kb *nmpABCD* sequences, flanked by HindIII and XbaI recognition sites, were obtained by PCR amplification using gDNA from *Alicyclophilus* sp. strain BQ1 as the template, Phusion high-fidelity DNA polymerase (Thermo Scientific), and the primer pairs NMHbF-NMHaR and NMHbF-AaOXR, respectively (Table 2). PCR conditions were as follows: initial denaturation at 98°C for 30 s; 40 cycles of denaturation at 98°C for 10 s, alignment at 60°C for 20 s, and extension at 72°C for 3 min; and a final extension at 72°C for 5 min. The resulting products were cloned into the HindIII-XbaI sites of pBBR1MCS-5 Gm^r, yielding the constructs pBBR1:: P_A *nmpAB* and pBBR1:: P_A *nmpABCD*. The constructs were transformed by thermal shock into *E. coli* DH5 α competent cells, the gentamicin-resistant transformants were selected, and their plasmids were analyzed by HindIII/XbaI double digestion. The cloned inserts, P_A *nmpAB* and P_A *nmpABCD*, were sequenced in order to confirm that no mutations were introduced during PCRs. To confirm the role of the mutated gene in NMP metabolism, the NMP-A.IV.74 mutant was complemented with pBBR1:: P_A *nmpAB* or pBBR1:: P_A *nmpABCD* by conjugation, as previously described, and transconjugants were selected on agar plates with gentamicin and nalidixic acid. The growth and NMP consumption of the NMP-A.IV.74 mutant bearing the empty vector pBBR1MCS-5 or the constructs in MM-NMP with 30% LB (MM-NMP/LB30) were quantified. In order to determine if the proteins encoded by the *nmpABCD* genes would be sufficient to metabolize NMP, the pBBR1:: P_A *nmpAB* and pBBR1:: P_A *nmpABCD* constructs were transformed into *E. coli* K-12 MG1655. In addition, to determine if GABA-AT activity is involved in NMP metabolism, *E. coli* K-12 DY329 wt, Δ *puuE*::Kan^r, Δ *gabT*::Kan^r, and double mutant Δ *puuE* Δ *gabT*::Kan^r strains were transformed with pBBR1:: P_A *nmpABCD*. The abilities of the *E. coli* strains bearing the constructs or the empty vector pBBR1MCS-5 to grow in MM-NMP/LB30 and to consume NMP were determined.

GABase assay. To determine the intracellular concentration of GABA in *Alicyclophilus* sp. strain BQ1 grown in MM-NMP (25 mM) or LB, a GABase assay (Sigma-Aldrich) was used. This assay uses GABA-AT, which converts GABA from an experimental sample to SSA in the presence of α -ketoglutarate, and SSDH, which transforms SSA to succinate in the presence of NADP⁺, which is reduced to NADPH and can be quantified by the absorption at 340 nm (47, 48). This is a stoichiometric reaction; therefore, the amount of NADPH reflects the level of GABA present in the intracellular extract. The limitation of this assay is that

if SSA is present in the samples, it will be transformed to succinate by SSDH, producing NADPH, which will result in an overestimation of the amount of GABA in the sample. To overcome this problem, a simultaneous GABase assay including the GABA-AT inhibitor EOS (60 mM) is performed using the same cytosolic extract to quantify the amount of SSA naturally present in the sample. Hence, by using a calibration curve generated with different GABA concentrations and subtracting the amount of NADPH obtained in the reaction with the inhibitor from the amount of NADPH measured without the inhibitor, the GABA concentration in the sample can be precisely determined. The effectiveness of EOS inhibition of GABA-AT was confirmed by preparing GABA solutions and measuring them with the GABase assay in the presence and absence of the inhibitor. In this control, no NADPH was detected when EOS was included in the assay mixture, demonstrating complete GABA-AT inhibition. *Alicyclophilus* sp. strain BQ1 was grown in either MM-NMP or LB to an OD₆₆₀ of 1.5. A 1-ml culture was harvested by centrifugation at $10,000 \times g$ for 10 min, resuspended in 1 ml of water, and lysed by boiling (49). An aliquot of cell lysate (40 μ l) was added to 60 μ l of GABase assay preparation containing 80 mM Tris buffer (pH 9), 750 mM sodium sulfate, 10 mM dithiothreitol, 1.4 mM NADP⁺, 2 mM α -ketoglutarate, and 0.03 U GABase. Microtiter plates containing the samples and standards were incubated for 60 min at 37°C and finally placed in an Epoch microplate spectrophotometer (Biotek) operated by Gen5 data analysis software, where the absorbance at 340 nm was measured. Three biological replicates were analyzed.

Construction of GABA-AT deletion mutants in *E. coli* DY329. In order to suppress GABA-AT activity, the *puuE* and *gabT* genes were deleted from *E. coli* DY329, which harbors a defective prophage that supplies the λ Red recombination functions (proteins γ , β , and exo) under the control of a thermosensitive cI857 repressor. To generate the single deletion strains DY329 $\Delta\text{puuE}::\text{Kan}^r$ and DY329 $\Delta\text{gabT}::\text{Kan}^r$, recombineering was performed as previously published (50), with some minor modifications. Sequences of the target genes *puuE* and *gabT* were replaced by homologous recombination with a kanamycin resistance cassette, amplified by PCR with Phusion DNA polymerase and the primer sets pukmF-pukmR (to delete *puuE*) and gtkmF-gtkmR (to delete *gabT*). Each recombining PCR-amplified cassette is flanked by the 50-bp homology regions H1 and H2 (51) and FRT recombining sites; the PCR products were then electroporated into temperature-induced (42°C, 15 min) DY329 electrocompetent cells. For the double deletion mutant, the antibiotic resistance cassette in DY329 $\Delta\text{puuE}::\text{Kan}^r$ was removed by FRT site-directed recombination with plasmid pCP20 as described before (52). A second homologous recombination was made in the resultant strain, DY329 ΔpuuE , to replace the *gabT* gene with a kanamycin resistance cassette as described before, generating a strain with the double deletion genotype DY329 $\Delta\text{puuE} \Delta\text{gabT}::\text{Kan}^r$. Specific primer pairs gtchkF-gtchkBR and puchkF-puchkR were used to verify the mutations.

Accession number(s). The complete nucleotide sequence of the *nmpABCDE* gene cluster has been deposited into the GenBank database under accession number [KY695150](#). The whole-genome shotgun sequencing project has been deposited into DDBJ/ENA/GenBank under accession number [NZ_NKDB000000.1](#).

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We have no conflicts of interest to declare.

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