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Identification of Mutations Involved in the Requirement of Potassium for Growth of Typical *Melissococcus plutonius* Strains

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***Melissococcus plutonius* is a fastidious honeybee pathogen, and the addition of KH_2PO_4 to culture medium is required for its growth. Using genome sequences and a newly developed vector, we showed that mutations in genes encoding Na^+/H^+ antiporter and cation-transporting ATPase are involved in the potassium requirement for growth.**

European foulbrood is an important bacterial disease of honeybee larvae. The causative agent, *Melissococcus plutonius*, is a fastidious organism, requiring microaerophilic to anaerobic conditions and carbon dioxide for growth. In addition, the Na/K ratio required for growth is described to be 1 or less (1), and thus, the addition of KH_2PO_4 to culture medium is required for the growth of typical *M. plutonius* strains.

Although *M. plutonius* had been thought to be remarkably homogeneous (2–4), Arai et al. (5) recently reported atypical *M. plutonius* strains and demonstrated that *M. plutonius* is a more heterogeneous species than previously believed. The atypical *M. plutonius* was not fastidious, and the addition of KH_2PO_4 was not required for its normal growth. Moreover, unlike typical *M. plutonius*, it was positive for β-glucosidase activity, hydrolyzed esculin, and produced acid from L-arabinose, D-cellobiose, and salicin (5). Furthermore, although typical *M. plutonius* is known to lose its virulence quickly when subcultured *in vitro*, atypical *M. plutonius* maintained virulence even after repeated subculture (5). Because of these interesting phenotypic differences, comparative analysis of typical and atypical *M. plutonius* is expected to be a major breakthrough for research on the physiology and pathogenesis of *M. plutonius*. For such analysis, genome sequencing of *M. plutonius* type strain ATCC 35311 (typical) and strain DAT561 (atypical) has recently been completed (6, 7). However, the genetic factors responsible for the phenotypic differences between them have yet to be investigated.

In our studies on *M. plutonius*, we noted that culture variants arise at low frequencies (4.62×10^{-7} to 3.52×10^{-6}) from ATCC 35311. Unlike ATCC 35311, the variants grew well even on media not supplemented with KH_2PO_4 (medium 6 and brain heart infusion [BHI] agar; see Table S1 in the supplemental material), implying that, in these variants, mutations have occurred in genes related to the cultural characteristics. Therefore, in this study, to identify genes involved in the potassium requirement, we determined the draft genome sequence of a culture variant (DAT628) and compared the data with that of the parental strain, ATCC 35311.

Selection of candidate genes involved in the potassium requirement for growth. Chromosomal DNA of DAT628 was extracted as described previously (5), and draft genome sequencing was performed at Hokkaido System Science (Sapporo, Japan), using an Illumina GA II sequencing system. Obtained sequences were 2,051,268 kb (991.6-fold coverage) and were assembled into 40 contigs. Sequencher version 4.8 (Hitachi Software Engineering, Yokohama, Japan) and the BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST) were used for the sequence analysis.

Comparative analysis of the draft genome sequence and reported genome sequences (DDBJ/EMBL/GenBank accession numbers AP012200 and AP012201) revealed that, in DAT628, putative Na^+/H^+ antiporter genes (MPTP_0420 and MPTP_0421) of ATCC 35311 were fused to a single open reading frame (ORF) (MPTP_0420-0421) due to a single nucleotide insertion in MPTP_0421 (Fig. 1A) (accession number AB778545). Interestingly, the same insertion also occurred in other variants independently obtained from ATCC 35311, while such an insertion was not present in all typical *M. plutonius* strains used in a previous study (5) (data not shown). Comparison of the deduced amino acid sequences with the homologues of other bacteria by BLASTp suggested that the fused gene (designated *napA* according to the name of the homologues of other species) is intact and functional, whereas MPTP_0420 and MPTP_0421 of ATCC 35311 are disrupted. In *Enterococcus hirae*, activities of sodium pumps, including Na^+/H^+ antiporter and Na^+-ATPase, are necessary for normal growth in Na^+-rich medium, whereas K^+-rich medium is required for normal growth of a mutant lacking these sodium pumps (8). Therefore, disruption of *napA* might cause the inability of typical *M. plutonius* to grow in Na^+-rich medium, while restoration of the gene might give DAT628 the ability to grow without the addition of KH_2PO_4.

On the basis of these results, we supposed that atypical *M. plutonius* possesses the intact *napA* gene. However, the genome sequence data of atypical strain DAT561 (accession numbers

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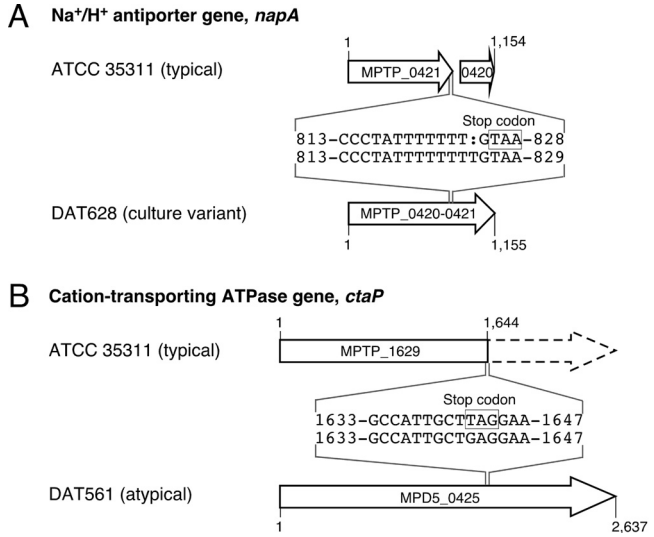

FIG 1 Genetic maps of *napA* (MPTP_0420 and MPTP_0421) and *ctaP* (MPTP_1629) regions of *M. plutonius* ATCC 35311 (typical *M. plutonius*) and the corresponding regions of DAT628 (culture variant of ATCC 35311) and DAT561 (atypical *M. plutonius*). The numbers indicate nucleotide positions in the ORFs. MPTP_0420 and MPTP_0421 of ATCC 35311 were fused to a single ORF in DAT628 due to a single nucleotide insertion in MPTP_0421. MPTP_1629 of ATCC 35311 was truncated due to a nonsense mutation, whereas the corresponding gene of DAT561 (MPD5_0425) was expected to be intact.

AP012282 and AP012283) showed that DAT561 lacks genes corresponding to *napA* (see Fig. S1A in the supplemental material). Genomic Southern hybridization analysis confirmed the absence of the *napA* homologue in all atypical strains tested (see Fig. S1B). Therefore, to identify genes involved in the growth of atypical *M. plutonius* in Na^+-rich medium such as medium 6 and BHI (see Table S1), we selected three additional genes of ATCC 35311 (MPTP_1078, MPTP_1579, and MPTP_1629) and their corresponding genes in DAT561 (MPD5_0870, MPD5_0470, and MPD5_0425, respectively) from genome sequence data of the strains (Table 1). MPTP_1078/MPD5_0870 is annotated as Na^+/H^+ antiporter, whereas MPTP_1579/MPD5_0470 and MPTP_1629/MPD5_0425 are annotated as cation transport and cation-transporting ATPase, respectively. Although there were several differences in deduced amino acid sequences of MPTP_1078/MPD5_0870 (designated *nhaP* according to the name of the homologues of other species) and MPTP_1579/MPD5_0470 (designated *ctaM* for putative cation transport ATPase of *Melissococcus*) between ATCC 35311 and DAT561, these genes seemed to be intact in both strains. On the other hand, although the cation-transporting ATPase gene (MPD5_0425) of DAT561 seemed to be intact, the corresponding gene (MPTP_1629) of ATCC 35311 had a nonsense mutation (Fig. 1B). The same nonsense mutation was also present in all typical *M. plutonius* strains and the culture variant DAT628, whereas such mutation was not present in all atypical *M. plutonius* strains used in our previous study (5) (data not shown), implying that the mutation in MPTP_1629 is another factor that makes typical *M. plutonius* fastidious. Therefore, we designated MPTP_1629/MPD5_0425 *ctaP* (for putative cation-transporting ATPase involved in potassium requirement).

Development of a novel gene expression system for *M. plutonius*. To investigate the effect of the mutations found in ATCC 35311 on the cultural characteristics, complementation analyses using gene expression systems are necessary. However, because no one has ever performed gene manipulations in *M. plutonius*, we constructed a novel gene expression vector on the basis of *Streptococcus suis* gene expression vector pMX1 (9). pMX1 consists of the broad-host-range replication origin, *S. suis malX* promoter, multiple cloning sites, and spectinomycin resistance gene. However, because *M. plutonius* strains were relatively resistant to spectinomycin (MIC, 128 to 512 μg/ml on KSBHI agar) but susceptible to chloramphenicol (MIC, 2 to 4 μg/ml on KSBHI agar) (D. Takamatsu, unpublished data), we replaced the spectinomycin resistance gene with the chloramphenicol resistance gene (*cat*). A fragment containing the replication origin, *malX* promoter, and multiple cloning sites of pMX1 and the *cat* gene of pSET6s (10) was amplified by the primers listed in Table S2 in the supplemental material. Amplified fragments were digested with XhoI, ligated to each other using a DNA ligation kit (TaKaRa Bio, Otsu, Japan), and introduced into *Escherichia coli* MC1061 by electroporation using standard procedures (11). Transformants were then selected on Luria-Bertani agar (Becton, Dickinson, Sparks, MD) containing 15 μg/ml chloramphenicol, and one of the candidate plasmids, the structure of which was confirmed by sequencing, was designated pMX2 (Fig. 2A).

TABLE 1 State of genes that might be involved in the potassium requirement of *M. plutonius*

Gene	Locus tag(s) in ATCC 35311 (accession no.)[a]	Annotation in ATCC 35311	Locus tag in DAT561 (accession no.)[a]	State		
				ATCC 35311/DAT584 (typical)	DAT628 (variant of ATCC 35311)	DAT561 (atypical)
napA	MPTP_0420 and MPTP_0421 (AB778538)	Na^+/H^+ antiporter		Truncated due to a frameshift mutation	Present[b]	Absent
nhaP	MPTP_1078 (AB778539)	Na^+/H^+ antiporter	MPD5_0870[c] (AB778542)	Present[d]	Present[d]	Present[d]
ctaM	MPTP_1579 (AB778540)	Cation transport ATPase	MPD5_0470 (AB778543)	Present[e]	Present[e]	Present[e]
ctaP	MPTP_1629 (AB778541)	Cation-transporting ATPase, E1-E2 family	MPD5_0425 (AB778544)	Truncated due to a nonsense mutation	Truncated due to a nonsense mutation	Present

[a] Selected genes were resequenced with the primers listed in Table S2 in the supplemental material by the Sanger method.
[b] MPTP_0420 and MPTP_0421 of ATCC 35311 were fused to a single open reading frame due to a single nucleotide insertion in MPTP_0421 (Fig. 1A).
[c] Although MPD5_0870 of DAT561 is annotated as a pseudogene in the genome sequence data (accession number AP012282), resequencing of the region by the Sanger method revealed that there is a single nucleotide sequence error in MPD5_0870, and so this gene was considered an intact gene.
[d] Amino acid sequence identity: 100% between ATCC 35311/DAT584 and DAT628 and 99.4% between ATCC 35311/DAT584 and DAT561.
[e] Amino acid sequence identity: 100% between ATCC 35311/DAT584 and DAT628 and 99.6% between ATCC 35311/DAT584 and DAT561.

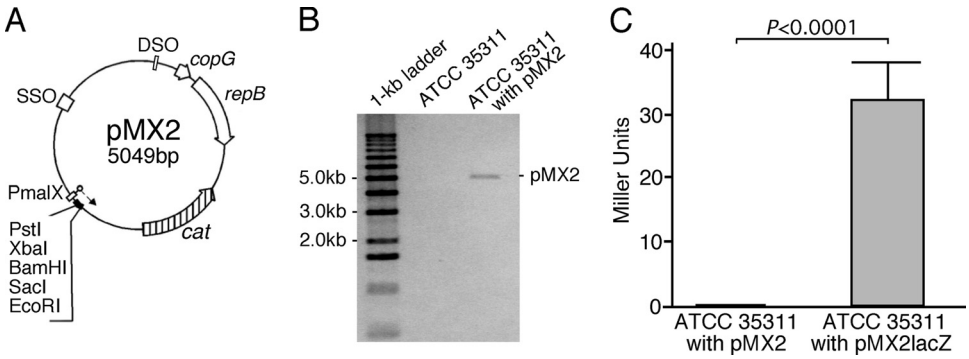

FIG 2 (A) Physical map of pMX2. The unique restriction cleavage sites are indicated in the map. SSO, single-strand origin; DSO, double-strand origin; *copG*, transcriptional repressor protein gene; *repB*, replication initiation-termination protein gene; *cat*, chloramphenicol resistance gene; PmalX, *malX* promoter of *S. suis*. (B) Genomic Southern hybridization analysis to confirm introduction of pMX2 into *M. plutonius*. Genomic DNAs of ATCC 35311 and ATCC 35311 transformed with pMX2 were digested with EcoRI, separated by agarose gel electrophoresis, and probed with the *cat* probe. (C) β-Galactosidase activity of ATCC 35311 transformed with pMX2 and pMX2lacZ. In pMX2lacZ, the ribosomal binding site of the *B. subtilis spoVG* gene and the promoterless *lacZ* reporter gene of *E. coli* are located downstream of PmalX. Data were collected from six independent experiments. β-Galactosidase activity is expressed as Miller units (mean ± standard deviation). Differences in β-galactosidase activity were compared by the unpaired *t* test, using the Welch modification.

We then designed an electroporation protocol for *M. plutonius* (Table 2) on the basis of the protocol for *S. suis* (12) and the results of our preliminary experiments (data not shown). Transformation of *M. plutonius* ATCC 35311 with pMX2 by this method yielded chloramphenicol-resistant colonies at an average efficiency of 1.76×10^3 colonies/μg DNA in five independent trials. The colonies were positive for *M. plutonius*-specific PCR (13). In addition, genomic Southern hybridization performed as described previously (14) using the *cat* probe amplified from pMX2 by primers catIF and catIR (see Table S2 in the supplemental material) confirmed the presence of pMX2 in the *M. plutonius* cells (Fig. 2B); that is, *M. plutonius* was successfully transformed by this method.

To investigate if the *malX* promoter in pMX2 could work in *M. plutonius*, the ribosomal binding site (RBS) of the *Bacillus subtilis spoVG* gene and the promoterless β-galactosidase gene (*lacZ*) of *E. coli* were amplified from pEVP3 (15) by primers LacZ1 and LacZ2.2 (see Table S2 in the supplemental material) and cloned into BamHI and EcoRI sites of pMX2. The resultant plasmid pMX2lacZ was introduced into ATCC 35311, and production of β-galactosidase was assessed according to the method of Miller (16) after growing the transformant for 3 days at 37°C on KSBHI agar containing 6 μg/ml chloramphenicol. As shown in Fig. 2C, β-galactosidase activity of ATCC 35311 transformed with pMX2lacZ was significantly higher than that with pMX2, confirming that the *malX* promoter can work in *M. plutonius*. These results suggest that the gene expression system developed in this study can be used for complementation of *M. plutonius*.

Complementation analysis. We then constructed Na^+/H^+ antiporter and cation-transporting ATPase gene expression vectors using pMX2. For construction of the *napA* expression vector (pDAT628NapA), a DNA fragment containing the coding region and RBS of *napA* was amplified from DAT628 by the primers listed in Table S2 in the supplemental material, cloned into pCR2.1 (Invitrogen, Carlsbad, CA), and then subcloned into PstI and EcoRI sites of pMX2. For construction of *ctaP*, *ctaM*, and *nhaP* expression vectors (pDAT561CtaP, pDAT561CtaM, and pDAT561NhaP, respectively), fragments containing the coding regions and RBSs were amplified from DAT561 by the primers

TABLE 2 Electroporation protocol for *M. plutonius* developed in this study

Step no.	Protocol
1	Grow *M. plutonius* strains on KSBHI agar at 35°C for 3 days under anaerobic conditions
2	Harvest cells, suspend them in sterilized deionized distilled water to an OD_{600}[e] of 0.8, and inoculate 2 ml of the bacterial suspension into 500 ml[a] of KSBHI broth containing 40 mM DL-threonine
3	Grow *M. plutonius* cells at 35°C under anaerobic conditions
4	Stop culturing *M. plutonius* at early logarithmic phase (OD_{600} = 0.05 to 0.1)
5	Chill the culture on ice for 15 to 30 min and collect cells by centrifugation at 12,000 × *g* for 15 min at 4°C[b]
6	Wash the cells once with 1/50 to 1/100 culture vol of ice-cold CTB (55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, and 10 mM PIPES, pH 6.7), resuspend the cells in 1/50 to 1/100 culture vol of ice-cold CTB, and incubate the suspension on ice for 30 min
7	Collect the cells by centrifugation at 12,000 × *g* for 5 to 15 min at 4°C
8	Wash the cells three times with 1/50 to 1/100 culture vol of ice-cold EB (2 mM potassium phosphate containing 10% sucrose, pH 8.4) and once with 1/X[c] culture vol of ice-cold EB containing 15% glycerol and resuspend the final cell pellet in 1/X[c] culture vol of ice-cold EB containing 15% glycerol[d]
9	Add plasmid DNA (1 μg) to an 80-μl aliquot of ice-cold cells and mix gently
10	Pulse the cell-DNA mixture (20.0 kV/cm, 200 Ω, and 25 μF) in a cold 0.1-cm-gap electroporation cuvette, add 920 μl of room-temp KSBHI broth containing 10% glucose, and disperse the cells with gentle pipetting
11	Plate on KSBHI agar containing 4 μg/ml chloramphenicol immediately and incubate at 35°C for 4 to 5 days under anaerobic conditions

[a] Scale up or scale down, depending on the application.
[b] It is important to keep the cells at 4°C or on ice for the remainder of the procedure. The cells, and any bottles or solutions that they come in contact with, must be prechilled on ice.
[c] X = $50/OD_{600}$ of *M. plutonius* culture at step 4.
[d] Although cells are suspended in the buffer containing glycerol, freeze-thaw procedures drastically reduce the transformation efficiency.
[e] OD_{600}, optical density at 600 nm.

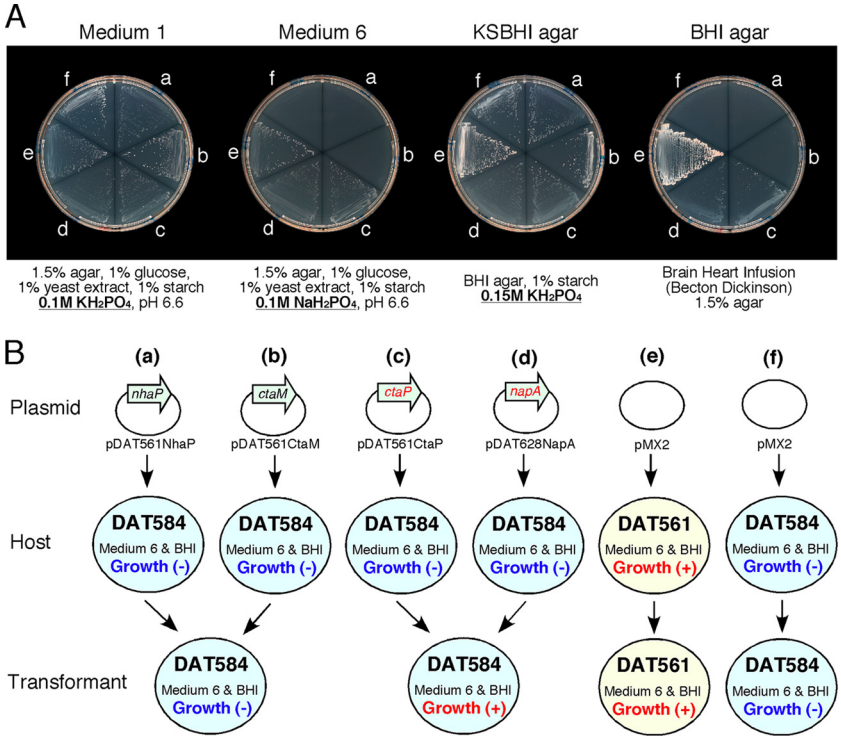

FIG 3 Complementation analysis. (A) Growth of *M. plutonius* strains transformed with various expression vectors on various agar plates. *M. plutonius* strains were cultured at 35°C for 1 week under anaerobic conditions. a, DAT584 + pDAT561NhaP (Na^+/H^+ antiporter gene expression vector); b, DAT584 + pDAT561CtaM (cation transport ATPase gene expression vector); c, DAT584 + pDAT561CtaP (cation-transporting ATPase gene expression vector); d, DAT584 + pDAT628NapA (Na^+/H^+ antiporter gene expression vector); e, DAT561 + pMX2 (control); f, DAT584 + pMX2 (control). (B) Schematic representations of the procedures and results of the complementation analysis.

listed in Table S2 and cloned into PstI and BamHI sites (*ctaP*) or the PstI site (*ctaM* and *nhaP*) of pMX2. The resultant plasmids were introduced into a typical *M. plutonius* strain, and the cultural characteristics were examined under anaerobic conditions. Because culture variants arise spontaneously from ATCC 35311, this strain is not suitable as a host for complementation. On the other hand, because such variants rarely arose from typical strain DAT584 (frequency, $<3.53 \times 10^{-9}$), DAT584 was used as the host. As controls, DAT584 and atypical strain DAT561 were also transformed with pMX2.

As expected, although DAT561 transformed with pMX2 grew under all conditions tested, DAT584 transformed with pMX2 grew only on media supplemented with KH_2PO_4 (medium 1 and KSBHI agar) (Fig. 3). However, when DAT584 was transformed with pDAT628NapA or pDAT561CtaP, the transformants grew not only on medium supplemented with KH_2PO_4 but also on medium not supplemented with KH_2PO_4 (BHI) or supplemented with NaH_2PO_4 (medium 6). On the other hand, the cultural characteristics of DAT584 were not changed by transformation with pDAT561CtaM or pDAT561NhaP (Fig. 3). Because chromosomal genes corresponding to *napA* and *ctaP* were still disrupted in the DAT584 transformants (data not shown), these results demonstrated that introduced functional *napA* or *ctaP* affected the cultural characteristics of DAT584.

Conclusion. Our results indicate that the potassium requirement for the growth of typical *M. plutonius* is associated with loss of function of a putative Na^+/H^+ antiporter gene (*napA*) and a cation-transporting ATPase gene (*ctaP*) and that imparting either functional *napA* or *ctaP* to typical *M. plutonius* is sufficient to remove the potassium requirement of the strains. Potassium is the major intracellular cation in bacteria as well as in eukaryotic cells (17), and the accumulation of K^+ is known to play a primary role in maintaining the osmotic balance of the cell in some bacteria (18). In *E. hirae*, it was speculated that sodium extrusion systems eliminate Na^+ from cytoplasm to make room for K^+ accumulation (8). Although further analysis is needed to show if products encoded by *napA* and *ctaP* really function as sodium pumps, typical *M. plutonius*, which may not be able to eliminate Na^+ from the cytoplasm, may require the addition of potassium salts to make the environmental K^+ concentration high and take K^+ in efficiently by osmotic pressure. In nature, *M. plutonius* multiplies in the larval gut of the honeybee (19). Honeybee larvae are fed with royal (or worker) jelly, honey, and pollen, and they are known to contain more potassium than sodium (20–22). Therefore, larval gut contents are considered to represent high-K^+ conditions. Because of this environment, typical *M. plutonius* may be able to multiply in larvae.

In this study, we identified mutations involved in a phenotypic difference between typical and atypical *M. plutonius* using a newly developed plasmid vector. Although *M. plutonius* was originally described a century ago (23), our understanding of its physiology and pathogenesis remains very limited. A lack of tools and genome information for molecular approaches was a factor that hampered studies of *M. plutonius*. Although there is room for improvement in the transformation efficiency, our genetic tools in combination with the genome sequences of typical and atypical *M. plutonius*

should promote future research on this important honeybee pathogen.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank database under the accession numbers AB778538 to AB778545.

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