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NOTES

Identification of *Bdellovibrio bacteriovorus* HD100 Bd0714 as a Nudix dGTPase▽†

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Bdellovibrio bacteriovorus **bacteria are predatory organisms that attack other gram-negative bacteria. Here, we report that Bd0714 is a Nudix dGTPase from *B. bacteriovorus* HD100 with a substrate specificity similar to that of *Escherichia coli* MutT and complements an *E. coli mutT*-deficient strain. We observed different transcription levels of the gene throughout the predator life cycle.**

Bdellovibrio bacteriovorus bacteria are gram-negative *Deltaproteobacteria* and obligate predators of a variety of gram-negative bacteria, including some pathogens (22). They have a biphasic life cycle consisting of an extracellular attack phase and a replicative phase that occurs within an intact prey cell (Fig. 1) (14, 17, 23). Despite the fascinating predatory life cycle that involves two major lytic events, prey cell invasion and release of the progeny by lysis of the prey cell, the numerous hydrolytic enzymes present in their genome have not been characterized (14).

Nudix hydrolases are a superfamily of phosphoanhydrases that catalyze cation-dependent hydrolysis of a wide range of *nu*cleoside *di*phosphates linked to another moiety *X* (Nudix) to yield NMP plus P-X (2). These enzymes are characterized by the presence of a highly conserved sequence, $GX_5EX_7REUXEEXGU$ (where U is Ile, Leu, or Val) (2, 13). Nudix substrates include nucleoside triphosphates, such as 8-oxo-dGTP (11), biosynthetic metabolites such as dihydroneopterin triphosphate (7), 5′-triphosphate-RNA (5), capped mRNA (19), and other nucleotide diphosphate compounds. Since the concentrations of these substrate molecules must be stringently regulated, Nudix hydrolases can be described as "house-cleaning" enzymes (2, 12).

In silico sequence analysis of the *B. bacteriovorus* HD100 genome showed the presence of six open reading frames containing the Nudix consensus sequence. One of them, Bd0714, has the highest sequence identity with *Escherichia coli* MutT (3). Here, we report the characterization of Bd0714 by genetic analysis and complementation assays, as well as with the in vitro kinetic analysis of its product, indicating that it can function as the mutator enzyme in *B. bacteriovorus* HD100.

Nudix sequence identification. Amino acid sequence searches for a functional homologue of MutT in *Bdellovibrio bacteriovorus* HD100 (gi:42494925) identified five putative Nudix hydrolases with canonical Nudix signature sequence (NP_967680, NP_969942, NP_969058, NP_967241, and NP_969548) and one with a modified signature sequence (NP_967621) (Fig. 2). One of these sequences, NP_967680 (Bd0714), shares 43 identical residues of the total 153 (28.1% identity) with *E. coli* MutT.

Substrate specificities and kinetics of Bd0714 and Bd0714-E70Q. The open reading frame corresponding to Bd0714 was cloned into pET24a (pBd0714; bacterial strains and plasmids are listed in Table S1 in the supplemental material). The Bd0714 protein that was overexpressed and purified to a >95% homogeneity (see the supplemental material) was shown to be a dimer by gel filtration chromatography. Purified enzyme hydrolyzed the eight canonical nucleoside triphosphates tested with a strong preference for dGTP (Fig. 3). The wild-type enzyme was optimally active with Mg^{2+} as the divalent cation and less or nonactive with Mn^{2+}, depending on the substrate tested (data not shown). Bd0714 hydrolyzes pyrophosphates but lacks (exo) phosphatase activity, since no release of phosphate is observed when inorganic pyrophosphatase is left out of the reaction (Fig. 3A). This indicates that, as in the case of MutT, Bd0714 cleaves the α-β pyrophosphate bond of dGTP.

The K_m value of wild-type Bd0714 for dGTP is 268 μM, and the k_{cat} value is 6.03 s^{-1} (Fig. 4A and 4B). The k_{cat} and K_m values for dGTP are very close to those determined for *E. coli* MutT. As with MutT from *E. coli*, the Bd0714 protein prefers dGTP but is able to cleave other deoxynucleoside triphosphate and nucleoside triphosphate substrates to some degree, in particular dUTP (4) (Fig. 3). This biochemical evidence points to the identification of Bd0714 as a MutT equivalent.

In some Nudix hydrolases, such as *E. coli* ADPRase, the glutamate after the arginine in the Nudix signature sequence is a bifurcated ligand of the required catalytic metal (6). Mutational analysis showed that this residue is the catalytic base in *E. coli* MutT (9). Mutation of this residue (E70Q) results in a reduction of the catalytic activity in Bd0714-E70Q (Fig. 3B and 4B). However, whereas in MutT this mutation leads to a

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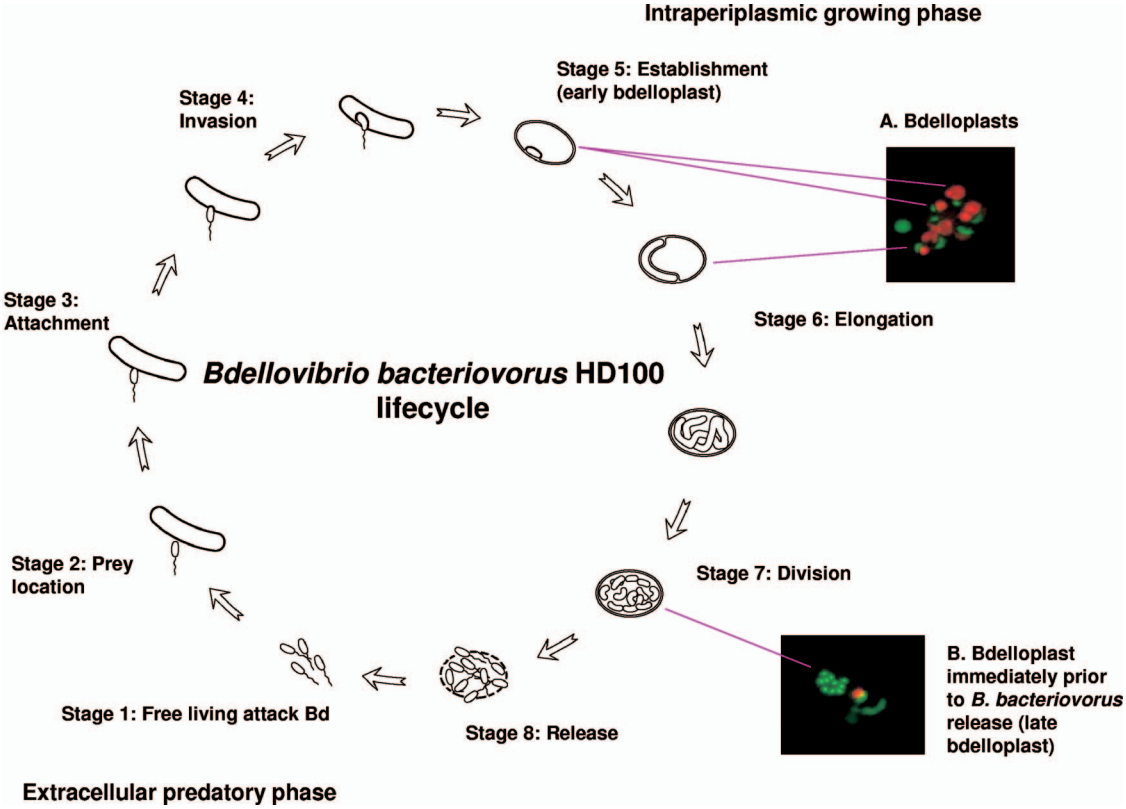

FIG. 1. *Bdellovibrio bacteriovorus* life cycle depicted in eight stages. In the extracellular predatory phase (stage 1, free-living attack *Bdellovibrio* [Bd]), the predators search for (stage 2, prey location) and attach to (stage 3, attachment) prey cells. They enter the prey cell wall initiating the intraperiplasmic or replicative phase (stage 4, invasion) (24–26, 28). In the periplasm, they feed upon the cytoplasmic contents of the prey (stage 5, establishment or early bdelloplast) (10) and form what is called the bdelloplast. They elongate (stage 6, elongation or bdelloplast) and segmentate into approximately 6 to 10 smaller components (stage 7, division or late bdelloplast) (1, 15, 16), finally releasing the progeny by lysis of the prey (stage 8, release) (14, 20). Interestingly, some strains of *Bdellovibrio* can generate host-independent mutants that grow axenically (18). The inserts show fluorescence staining of bdelloplasts that distinguishes live cells from dead cells; BacLight-stained live bacteria with intact membranes are green (*Bdellovibrio*), and dead cells with disrupted membranes are red (prey, *E. coli*) in the indicated examples.

50,000-fold drop in k_{cat} (Fig. 4A) (9), in *B. bacteriovorus* it results in only a 13.5-fold drop in k_{cat} with no effect on K_m. Furthermore, in contrast to its effect in MutT, this mutation does not flatten or shift the pH profile of the enzyme, indicating that Glu70 may not be the catalytic base, but only a ligand of the catalytic metal (Fig. 4B). Nevertheless, the kinetic evidence still points to Bd0714 as having MutT type activity, but likely with an altered mechanism in which Glu70 is not the catalytic base. This may suggest that Bd0714 has an additional role in *B. bacteriovorus* in addition to its role as a mutator enzyme.

Complementation of the mutator phenotype. To obtain direct evidence that the Bd0714 enzyme is a functional homologue of MutT, the Bd0714 gene was expressed in an *E. coli* strain in which the *mutT* gene was inactivated (SB3 *mutT* mutant) (see Table S1 in the supplemental material) (3). The SB3 *mutT* mutant was transformed with pTrc99a, pTrc99mutT, or pTrc99Bd0714 (see Table S1 in the supplemental material) and challenged with media supplemented with either streptomycin or nalidixic acid, and the mutation frequencies per 10^9 cells were evaluated. The SB3 mutant harboring pTrc99Bd0714 had a much lower mutation frequency than did the SB3 mutant harboring pTrc99A in both media (Table 1). This rate is even lower than that observed in the SB3 mutant transformed with pTrc99mutT, which had a threefold-higher mutational frequency. This analysis showed that Bd0714 can efficiently complement MutT function in *E. coli* SB3. The com-

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                .         .         .         .         .         .         .
Bd2220    30  PDQSGAGFWEFPGGKVE.AGEAPEQALAREITEELALNI...RVHDLIGEVDFAY
Bd0714    42  ENNSLAGQWEFPGGKIE.NGETPEEALARELNEELGIEA...EVGELKLACTHSY
Bd2755    39  YNP....AWILPGGTVE.AEESPSEALQRELKEELGLNI...QAGSLLAVDYVSN
Bd0654    66  DSDRWSGQLAFPGGKRE.DSDKTDLDAALRETLEEVGIDL...TNPELLGRLNDIQ
Bd3179    64  RHAVKKVFLEFPAGKRD.HNEETLLTAKRELLEETGYEA...KDWKFLTTIHPVI
Bd0236   185  DPRAQVPYFFLPGGAVE.KGESPATTAARECLEETGYKVRILEDSAFERVYDFPW
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FIG. 2. Nudix sequences of the *Bdellovibrio bacteriovorus* genome. Bd0714 (NP_967680), Bd3179 (NP_969942), Bd2220 (NP_969058), Bd0236 (NP_967241), Bd2755 (NP_969548), and one modified signature sequence in Bd0654 (NP_967621). The black background indicates identity in a proline residue and in the Nudix signature sequence. Bd0654, the open reading frame with the modified signature sequence, has a lysine residue instead of the typical first glutamate in $GX_5EX_7REUXEEXGU$. The boxes show homologous residues. The alignment was done with ClustalW and the figure with ESPript (8).

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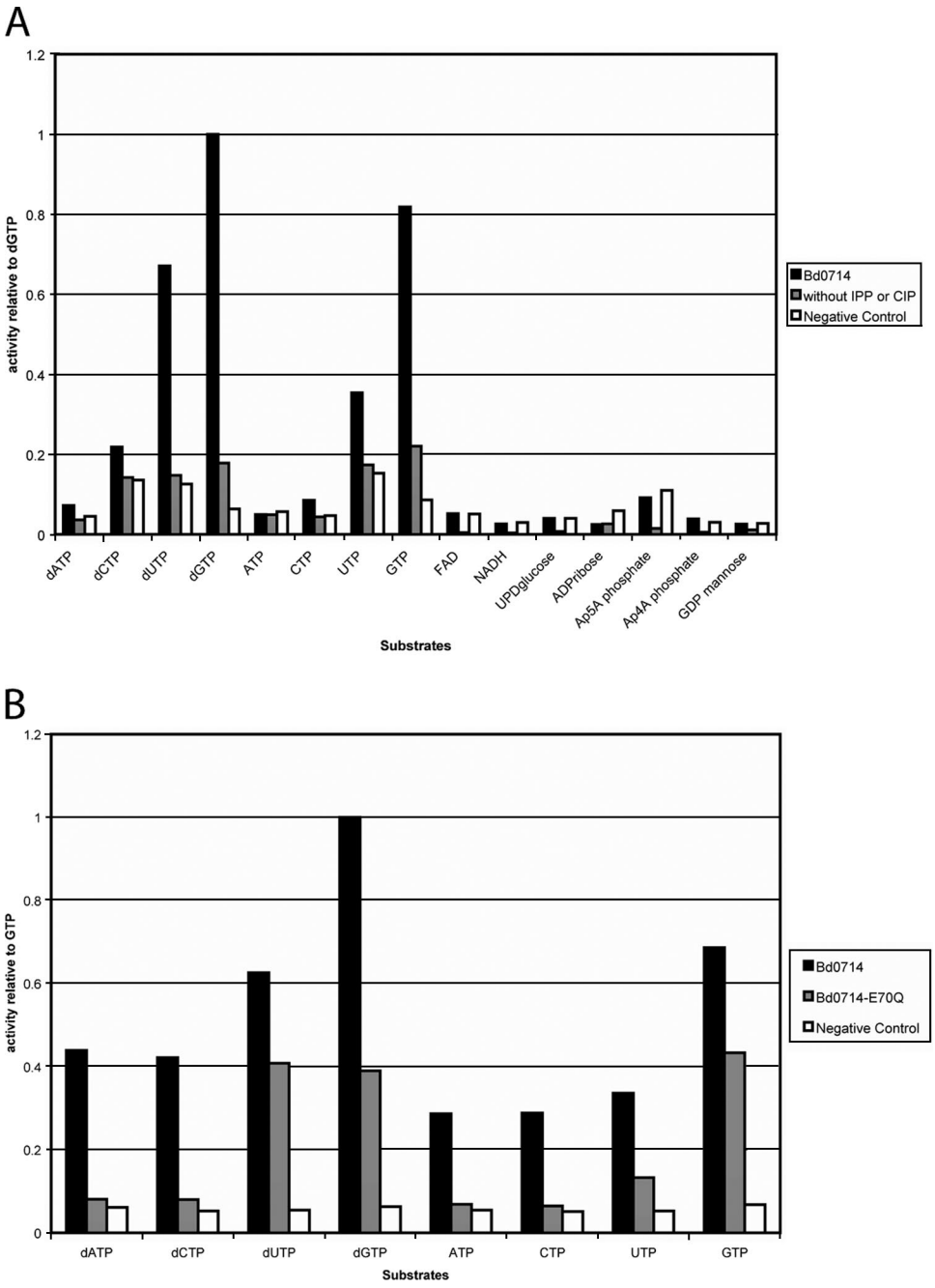

FIG. 3. (A) Relative activity of the Bd0714 for possible nucleotide substrates; dGTPase activity was normalized to 100%. Black bars correspond to the colorimetric assay with added enzyme, gray bars correspond to all components of the assay except for inorganic pyrophosphatase (IPP) or calf intestinal phosphatase enzyme (CIP), and white bars correspond to the reaction carried out without the Bd0714 enzyme. (B) Relative activity of Bd0714 versus Bd0714-E70Q for deoxynucleoside triphosphates, with dGTP normalized to 100% activity.

parable kinetic and biochemical profiles, along with the complementation analysis, indicate that Bd0714 can function as a mutator enzyme in *B. bacteriovorus*.

***B. bacteriovorus* HD100 Bd0714 deletion mutant.** A deficiency of the *mutT* gene in *E. coli* has been shown to increase the level of spontaneous mutations compared to that in wild-type *E. coli* (27). To evaluate the functional role of Bd0714 in *B. bacteriovorus*, an in-frame deletion mutant was constructed by allelic exchange (21). An extremely low frequency of knockout mutants was found during the construction, compared with the results of previous work (21); only one deletion excisant was recovered on a total of 123 plaques screened by PCR.

Both the *B. bacteriovorus* wild-type and ΔBd0714 deletion strains were grown in liquid coculture along with *E. coli* SB3 prey and plated with *E. coli* SB3Nal prey in media supplemented with nalidixic acid to determine the difference in the number of spontaneous mutants obtained between the wild-type and Bd0714 mutant strains. No mutants were found for

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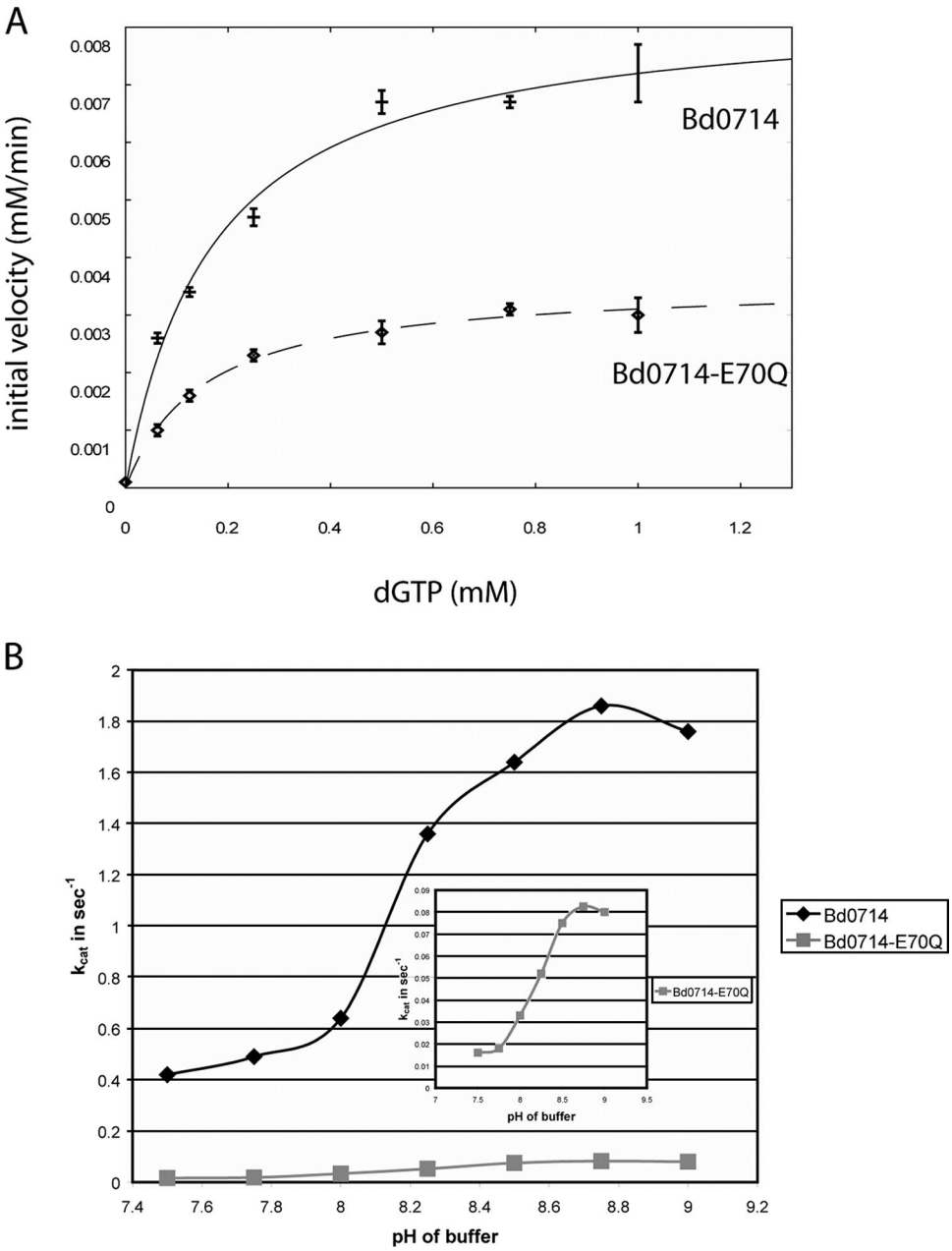

FIG. 4. Kinetic data for Bd0714 and Bd0714-E70Q. (A) Initial velocities as a function of dGTP concentration. The continuous curves correspond to nonlinear least-square fits of the data to the Michaelis-Menten equation. (B) Plot of the k_{cat} values of pH in the range of 7.5 to 9 for Bd0714 and Bd0714-E70Q.

TABLE 1. Mutation frequencies of the *E.coli* SB3 *mutT* mutants transformed with different plasmids

Plasmid	No. of indicated mutants per 10^9 cells	
	Streptomycin resistant	Nalidixic acid resistant
pTrc99A	491 ± 206	1,123 ± 257
pTrc99mutT	50 ± 26	79 ± 75
pTrc99Bd0714	7 ± 2	23 ± 2

either strain. Similar phenotypic analysis of the mutant strain could not be performed using streptomycin since the wild-type *B. bacteriovorus* is already streptomycin resistant (21). The *B. bacteriovorus* wild-type and ΔBd0714 strains were synchronically cultured and monitored by microscopy to determine the effect of the deletion on their life cycle; no substantial difference was found.

Transcription of the Bd0714 gene during the *B. bacteriovorus* life cycle. To better understand the role of Bd0714 during the *B. bacteriovorus* life cycle, transcription of the Bd0714 gene at various stages in the life cycle was monitored by reverse tran-

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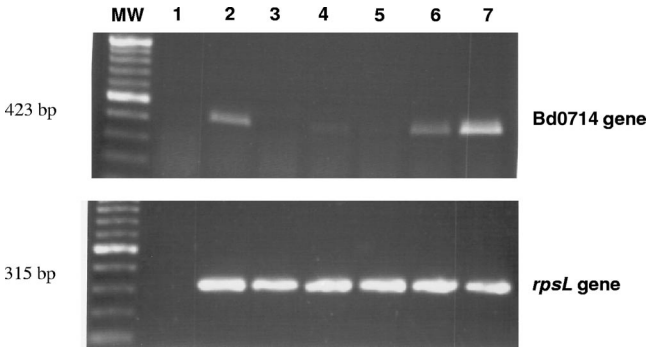

FIG. 5. RT-PCR analysis of the Bd0714 gene in *B. bacteriovorus* HD100, the ΔBd0714 mutant, and host-independent *B. bacteriovorus*. Total RNA was extracted from *E. coli* ML35 prey (lane 1), host-independent *B. bacteriovorus* (lane 2), the attack-phase ΔBd0714 mutant (lane 3), and wild-type *B. bacteriovorus* HD100 (lane 4, free-living attack phase; lane 5, early bdelloplast; lane 6, bdelloplast; and lane 7, late bdelloplast) throughout the life cycle and used in RT-PCRs with primers specific for the Bd0714 gene. The *rpsL* gene was used as a load control for *B. bacteriovorus* RNA. The absence of an RT-PCR product in lane 1 indicates that the product seen for both the Bd0714 and *rpsL* genes corresponds to *B. bacteriovorus* RNA rather than to any possible contaminating *E. coli* RNA. MW, molecular weight marker.

scription-PCR (RT-PCR). RNA was extracted from *B. bacteriovorus* at stages 1, 5, 6, and 7 of the life cycle (Fig. 1). RNA was also obtained from cultures of the attack phase (stage 1) of the *B. bacteriovorus* HD100 ΔBd0714 mutant and from the host-independent *B. bacteriovorus* HI100. Transcription of the Bd0714 gene was observed during the free-swimming attack phase, but barely perceptible in stage 5 (establishment or early bdelloplast) (Fig. 5). However, increasing transcription was seen throughout the bdelloplast stages, reaching a maximum in stage 7 (late bdelloplast or prior to release). Similar levels of transcription were found in the host-independent strain and wild-type *B. bacteriovorus* in stage 6 (elongation). As further confirmation of the deletion, RT-PCR product was not observed in the extracellular attack phase for the HD100 ΔBd0714 deletion mutant, even though product was observed for the wild-type strain in the same stage in the life cycle.

Biochemical and molecular evidence indicates that Bd0714 is a mutator enzyme in *B. bacteriovorus*. However, while most of the evidence points to Bd0714 as an *E. coli* MutT orthologue, kinetic analysis of the Glu70 mutant enzyme indicates that, in contrast to MutT, in Bd0714, this residue is not the catalytic base. These results suggest that Bd0714 and MutT may use different catalytic mechanisms, possibly reflecting the fact that Bd0714 may have additional physiological functions.

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