Relationship between bacterial virulence and nucleotide metabolism: a mutation in the adenylate kinase gene renders *Yersinia pestis* avirulent

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Nucleoside monophosphate kinases (NMPKs) are essential catalysts for bacterial growth and multiplication. These enzymes display high primary sequence identities among members of the family Enterobacteriaceae. *Yersinia pestis*, the causative agent of plague, belongs to this family. However, it was previously shown that its thymidylate kinase (TMPKyp) exhibits biochemical properties significantly different from those of its *Escherichia coli* counterpart [Chenal-Francisque, Tourneux, Carniel, Christova, Li de la Sierra, Barzu and Gilles (1999) Eur. J. Biochem. **265**, 112–119]. In this work, the adenylate kinase (AK) of *Y. pestis* (AKyp) was characterized. As with TMPKyp, AKyp displayed a lower thermodynamic stability than other studied AKs. Two mutations in AK ($Ser^{129} \rightarrow Phe$ and $Pro^{87} \rightarrow Ser$), previously shown to induce a thermosensitive growth defect in *E. coli*, were introduced into AKyp. The recombinant variants had a lower stability than wild-type AKyp and a higher susceptibility to proteolytic digestion. When the $Pro^{87} \rightarrow Ser$ substitution was introduced into the chromosomal *adk* gene of *Y. pestis*, growth of the mutant strain was altered at the non-permissive temperature of 37 °C. In virulence testings, less than 50 colony forming units (CFU) of wild-type *Y. pestis* killed 100 % of the mice upon subcutaneous infection, whereas bacterial loads as high as 1.5×10^4 CFU of the *adk* mutant were unable to kill any animals.

Key words: adenylate kinase, bacterial virulence, mutated enzyme, *Y. pestis*.

INTRODUCTION

Nucleotides are metabolic intermediates essential for growth and division of all living cells. Among the enzymes involved in their synthesis are the five nucleoside monophosphate kinases (NMPKs) specific for AMP/dAMP (adenylate kinase, AK), GMP/dGMP (guanylate kinase, GMPK), CMP/dCMP (cytidylate kinase, CMPK), UMP (uridylate kinase, UMPK) and dTMP (thymidylate kinase, TMPK) [1]. These enzymes share a common architecture and belong to the P-loop kinases in the Rossman-like family [2]. NMPKs from Enterobacteriaceae are also well conserved in their primary structure. However, despite high sequence identities, some NMPKs from this family could have notably different properties. This is the case for the TMPK of *Yersinia pestis* (TMPKyp), the causative agent of bubonic and pneumonic plague [3]. Although TMPKyp exhibits 70 % amino acid identity with its *Escherichia coli* counterpart, significant differences in stability and substrate specificity were observed [4]. These differences are indicative of subtle variations in the three-dimensional structure of this catalyst between the two organisms. These peculiar properties of TMPKyp prompted us to study another NMPK in this bacterial species, namely AKyp.

Although the biochemical properties of the isolated NMPKs have been extensively studied [5–9], their role (other than merely catalytic) in the living cell is difficult to assess because, with a single exception [10], disruption of their genes is lethal for the organism [11,12]. In the case of the AK from *E. coli* (AKec), substitution of two amino acid residues [P87S ($Pro^{87} \rightarrow Ser$ mutation) or S129F ($Ser^{129} \rightarrow Phe$)] rendered the bacteria thermosensitive [13–15]. These mutations resulted in a complex phenotype where synthesis of ATP, phospholipids and RNA was impaired and bacterial growth was inhibited at non-permissive temperatures. Whether these mutations would have the same effect in other bacterial AKs has never been determined.

The importance of NMPKs in bacterial growth and dissemination *in vivo* has been poorly documented until now. *Y. pestis* is a good candidate to test this effect because it is one of the most pathogenic organisms which exists. Bubonic plague is lethal in 50–70 % of the patients in less than a week if an appropriate treatment is not administered rapidly. This lethality rate reaches 100 % in the pneumonic form of plague. Mutagenesis of the *adk* locus in *Y. pestis* gave a unique opportunity to evaluate the importance of AK in bacteria multiplying in a mammalian host (at a non-permissive temperature of 37 °C).

In this study, we produced the wild-type AKyp and two modified variants exhibiting the P87S and S129F substitutions and compared their biochemical and physicochemical properties. The P87S substitution was introduced into the chromosomal *adk* gene of a virulent strain of *Y. pestis* and the impact of this mutation on bacterial growth *in vitro* was investigated.

MATERIALS AND METHODS

Strains and growth conditions

The characteristics of the *Y. pestis* and *E. coli* strains used in this study are listed in Table 1. NM554 *E. coli* strain was used for DNA purification and sequencing. BL21(DE3)/pDIA17 *E. coli* strain [16] was used for production of recombinant and

Abbreviations used: AK(ec/yp), adenylate kinase (from *Escherichia coli* or *Yersinia pestis*); Ap_5A, P^1,P^5-di(adenosine-5′)-pentaphosphate; CFU, colony forming units; CHEF, contour-clamped homogeneous electric fields; ESI-MS, electrospray ionization MS; LB, Luria-Bertani; LD_{50}, median lethal dose; NMPKs, nucleoside monophosphate kinases; P87S etc, $Pro^{87} \rightarrow Ser$ mutation etc; T_m, mid temperature; TMPK(yp), thymidylate kinase (from *Yersinia pestis*).

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Table 1 Strains and plasmids

Strain/Plasmid	Relevant characteristics	Source or reference
Strain		
E. coli NM554	F- *ara*D139 Δ(*ara-leu*)7696 *gal*E15 *gal*K16 Δ(*lac*)X74 *rps*L(Str^r) *hsd*R2 (r_K^- m_K^+) *mcr*A *mcr*B1 *rec*A13	[38], but see [38a]
E. coli BL21(DE3)	F- *omp*T *gal* [*dcm*] [*lon*] *hsd*S_B (r_B^- m_B^-) with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
E. coli CR341 T28	F- *thr leu ile thy lac*Y *met adk*^{ts}	[13]
E. coli RZ1032	HfrKL16 P0/45 [*lys*A(61–62)] *dut*1 *ung*1 *thi*1 *rel*A1 Zbd-279::Tn10 *sup*E44	[39]
E. coli SM10 λ pir	*thi thr leu ton*A *lac*Y *sup*E *rec*A::RP4-2-Tc::Mu λ pir Km	[40]
Y. pestis 6/69c	Natural isolate of biotype Orientalis, cured of the virulence plasmid	[25]
Y. pestis 371	Spontaneous Nal^R derivative of 6/69c	This study
Y. pestis 70-8 (5)	Derivative of 371 containing a mutated *adk* gene obtained by a double crossover	This study
Plasmid		
pUC19	ColE1 Ap^R cloning vector	GenBank® accession no. X02514
pET22b	ColE1 Ap^R cloning/expression vector	Novagen
pCVD442	Ap^R suicide vector requiring λ *pir* for replication	[41]
pHL70-1	pUC19 derivative (2 kb chromosomal *Y. pestis* insert containing the *adk* gene)	This study
pHL70-2	pHL70-1 derivative (*Age*I-*Sma*I deletion)	This study
pHL70-3	pET22b derivative (amplification of the *adk* gene by PCR using pHL70-1 as template)	This study
pHL70-4	pHL70-3 derivative (CCG → TCG alteration corresponding to the P87S amino acid substitution)	This study
pHL70-5	pHL70-3 derivative (TCA → TTC alteration corresponding to the S129F amino acid substitution)	This study
pHL70-6	pHL70-3 derivative (CCG → TCG and TCA → TTC alteration corresponding to the double P87S S129F amino acid substitutions)	This study
pHL70-7	*Y. pestis* *Ase*I-*Sac*I insert of pHL70-1 transferred in pCVD442	This study
pHL70-8	pHL70-7 derivative (CCG → TCG alteration corresponding to the P87S amino acid substitution)	This study

modified AKyp proteins. Cultures were grown in 2YT medium [17] supplemented with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol. Synthesis of recombinant protein was induced with isopropyl β-D-thiogalactoside (final concentration 1 mM) when cultures reached $D = 1.5$ at 600 nm (where D is attenuance). Bacteria were harvested by centrifugation 3 h after induction. *Y. pestis* strains were grown on Luria-Bertani (LB) agar plates with 30 μg/ml nalidixic acid and 2.5 % (w/v) hemin for 48 h at 28 °C, or in LB medium containing 30 μg/ml nalidixic acid for 6–48 h with shaking at 28 °C or 37 °C.

Cloning and sequencing of *Y. pestis adk* gene

Y. pestis 6/69c genomic DNA was partially cleaved by *Sau*3A and ligated to pUC19 DNA linearized by *Bam*HI. *E. coli* strain CR341 T28 was electroporated with the ligation mix and plated at a ratio of 1:10 at 37 °C, or at a ratio of 9:10 at 42 °C on LB plates containing 100 μg/ml ampicillin and 0.01 % thymine. Out of 90000 putative clones, six were capable of growing at 42 °C. After re-plating on solid medium at 42 °C, or on liquid medium at 37 °C, only two clones retained their ability to grow. One clone, harbouring plasmid pHL70-1 and displaying the highest AK activity, was selected for DNA sequencing using the double-stranded dideoxynucleotide sequencing technique [18]. Deletion of the *Age*I (in the insert)–*Sma*I (in pUC19) fragment leading to plasmid pHL70-2 resulted in loss of AKyp activity and enabled us to precisely locate the *adk* gene in the insert. Six synthetic oligonucleotides were used to cover the entire *adk* gene.

The *adk* gene was amplified by PCR using pHL70-1 as template and the following two synthetic oligonucleotides: GGGGCATATGCGTATCATTCTGCTGGGCGCTC (F2 primer) and CCCCAAGCTTTTAACCGAGTATAGTCGCCAGTTCAG-CACTG (R2 primer). The amplified DNA fragment (approx. 660 bp) was digested with *Nde*I and *Hin*dIII, two restriction enzymes whose sites were included at the 5′ end of primers F2 and R2 respectively (underlined in the oligonucleotide sequence), and ligated into the pET22b vector digested by the same restriction enzymes. The resulting plasmid was named pHL70-3.

Site-directed mutagenesis

Site-directed mutagenesis was performed on the single-stranded form of plasmid pHL70-3 in the RZ1032 *E. coli* strain in the presence of the helper phage M13KO7 [19]. Sequences of oligonucleotides used for mutagenesis were: 5′-AATGGTAC-GCGAGAACCCGTCTAA-3′ for the P87S substituted AKyp (corresponding plasmid: pHL70-4) and 5′-AACACGGCCG-AAAGCAGCATGTAC-3′ for the S129F substituted AKyp (corresponding plasmid: pHL70-5). A mixture of these two oligonucleotides was used to obtain the double mutant (corresponding plasmid: pHL70-6). For each mutagenesis, the molecular mass of the recombinant protein was determined by electrospray ionization MS (ESI-MS) to check for the absence of any other mutation.

Purification and activity assay of AKyp

The wild-type AKyp was purified by a two-step procedure involving chromatography on Blue Sepharose (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and Ultrogel AcA54 (Réactifs IBF, Villeneuve la Garenne, France) [20]. The S129F modified protein was purified following the same procedure, with slight modifications allowing separation of endogenous AKec from the recombinant AKyp (see Results section). The two other modified variants of AKyp (P87S and P87S/S129F) were found in the pellet after cell breakage and centrifugation. The proteins were solubilized with 7 M urea in 50 mM Tris/HCl, pH 7.4, and refolded by dilution in the assay medium. AK activity was determined at 30 °C using the coupled spectrophotometric assay at 334 nm (0.5 ml final vol.) on an Eppendorf ECOM 6122 photometer [21]. One unit of enzyme activity corresponds to 1 μmole of the product formed in 1 min at 30 °C and pH 7.4.

Construction of an *adk* mutant of *Y. pestis* 371

Plasmid pHL70-1 was digested by *Ase*I (a site located at the 3′-end of the insert), treated by the Klenow fragment, and then digested with *Sac*I (a site located in pUC18). The suicide

plasmid pCVD442 was digested by *Xba*I, treated with the Klenow fragment, and digested with *Sac*I. After agarose gel extraction according to the NucleoSpin Extract protocol (Macherey Nagel), fragments of approx. 1.1 kb (insert from pHL70-1) and 6.2 kb (linear pCVD442) were ligated, leading to plasmid pHL70-7. To introduce the P87S substitution in the *adk* gene, the *Bsm*I–*Age*I fragment from pHL70-7 was exchanged with the *Bsm*I–*Age*I fragment from pHL70-4, yielding plasmid pHL70-8. The exchange was checked by *Nla*IV digestion of the resulting plasmid (introduction of the mutation leads to the loss of a *Nla*IV site compared with the wild-type *adk* gene). The suicide plasmid pHL70-8 was introduced into the recipient *Y. pestis* strain 371 by conjugation with the donor *E. coli* SM10 λ pir/pHL70-8. For this purpose, 100 μl each of diluted overnight culture of the *E. coli* donor strain (10^9 bacteria/ml) and of the *Y. pestis* recipient strain (10^8 bacteria/ml) were mixed and incubated at 28 °C for 2 h without selection. The mating mixture was inoculated into BHI (brain heart infusion) medium, and incubated with shaking for 48 h at 28 °C. *Y. pestis* transconjugants were selected on LB-agar plates containing ampicillin, nalidixic acid and hemin. Proper integration of the plasmid into the chromosomal *adk* gene by a single crossing-over event was verified by Southern hybridization with the *adk* and pCVD442 probes. The second recombination event that left the mutated copy of the *adk* gene on the *Y. pestis* chromosome, after excision of pCVD442, was obtained by subculturing selected ampicillin–nalidixic acid-resistant transconjugants in LB broth without ampicillin selection, and plating on LB medium (without NaCl) containing 5 % (w/v) sucrose. Sucrose-resistant colonies that were sensitive (indicative of the loss of the suicide vector) were tested for the presence of the mutated *adk* gene by amplification of *adk* with the F2/R2 pair of primers and digestion of the PCR product with *Nla*IV. The mutation in the *adk* gene was confirmed by sequencing. One mutant strain [70-8(5)] was selected and further analysed. Presence of the *hms* locus, which confers the pigmentation phenotype to *Y. pestis*, was determined on Congo Red agar plates after 4 days of growth at 28 °C, as described previously [22]. Extraction of the three *Y. pestis* endogenous plasmids was based on the alkaline lysis method of Birnboim and Doly [23]. The *Eco*RV restriction profiles were compared with that of the parental strain. Absence of macroscopic chromosomal rearrangements in the mutant was checked by pulse-field gel electrophoresis.

Southern hybridization

Total genomic DNA was isolated with an IsoQuick nucleic acid extraction kit (Microprobe). Approx. 10 μg of DNA was digested with *Eco*RI, separated on a 0.7 % agarose gel, and transferred to nylon membranes using a vacuum blotting system (Hybaid). Plasmid pCVD442 and the *adk* gene were labelled with Digoxigenin-11-dUTP using the random primed labelling system, using a DIG DNA labelling kit (Roche) and nested PCR [24] with the pairs of T7 promoter/T7 terminator and F2/R2 primer pairs respectively. Hybridizations were performed overnight at 65 °C.

Pulse-field gel electrophoresis

Genomic DNA was prepared in agarose plugs as described previously [25]. After digestion with *Spe*I, macro-restriction fragments were resolved by contour-clamped homogeneous electric fields (CHEF) electrophoresis, using a CHEF-DRIII apparatus (Bio-Rad Laboratories), an electric field of 6 V/cm, and an angle of 120°. Initial and final pulse times were 0.1 s and 6 s respectively. Migration of DNA fragments was performed in 0.5 × Tris/Borate/EDTA buffer in a 0.9 % agarose gel at 14 °C for 18 h.

Animal infection and estimation of the median lethal dose (LD_{50})

To compare the LD_{50} of the parental strain *Y. pestis* 371 with the isogenic *adk* mutant 70-8(5), 0.5 ml or 0.1 ml aliquots of 10-fold serial dilutions of bacterial suspensions were inoculated intravenously or subcutaneously respectively, into 5-week-old CD1 female mice (Charles River, France). Groups of five mice were used for each dilution, and three (parental strain) or six (*adk* derivative) dilutions were used. The number of deaths was recorded daily for 3 weeks, and the LD_{50} was calculated according to the method of Reed and Muench [26].

Analytical procedures

Protein concentration was determined according to Bradford [27] using a Bio-Rad kit or by amino acid analysis. SDS/PAGE was performed as described by Laemmli [28] and gels were stained with Coomassie Blue. CD spectra were recorded on a JASCO 700 instrument equipped with a thermoelectric cell holder using a 1 mm quartz cuvette. The far-UV spectra were obtained as an average of five runs and were corrected for contribution of the buffer (10 mM Tris/HCl, pH 7.4). Urea denaturation was monitored by changes in ellipticity at 222 nm. Aliquots of concentrated solutions of protein were added to urea solutions (0–8 M) and samples were incubated until equilibrium was reached at 20 °C. The thermodynamic parameters of denaturation were estimated as described previously [29]. Thermal denaturation curves were monitored at a fixed wavelength (222 nm). The sample was heated at a rate of 1 °C/min and cooled down rapidly after the denaturation scan to determine reversibility of the unfolding–folding transition by measuring ellipticities before and after heating. The data were evaluated using JASCO program package. Ion-spray mass spectra were recorded on a quadrupole mass spectrometer API-365 (PerkinElmer) equipped with an ion-spray (nebulizer-assisted electrospray) source.

RESULTS

Molecular cloning of the *adk* gene from *Y. pestis* and overexpression of the recombinant enzyme in *E. coli*

Owing to a thermolabile AK, *E. coli* CR341 T28 strain is a temperature-sensitive mutant. We cloned the *Y. pestis adk* gene by complementation of the thermosensitive growth phenotype using a library of *Y. pestis* 6/69c genomic DNA inserted into pUC19 vector. Two clones growing at 42 °C were isolated and one was further analysed because of its higher AKyp activity. A region of 1097 bp (GenBank® accession no. AF65382) which contained the complete *Y. pestis* 6/69 *adk* gene was sequenced. The 3′-terminal region of the *htp*G gene was identified upstream of the *adk* gene, and the 5′-terminal region of the *hem*H gene was found downstream of the *adk* gene. This sequence [which contained two silent errors, i.e. errors with no changes in protein sequence, at position 783 (T instead of C) and position 954 (T instead of A)] was found to be 100 % identical at the nucleotide level with the *adk* gene from strains CO92 [30] and KIM5 (http://www.genome.wisc.edu/html/pestis.html) that were subsequently sequenced. Alignment of AKyp with three other AKs from enterobacteria (*Yersinia enterocolitica*, *E. coli* and *Salmonella typhimurium*) revealed over 85 % amino acid identity (Figure 1). To increase the expression of the *adk* gene, which is

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Y.p.  MRIILLGAPG AGKGTQAQFI MEKYGIPQIS TGDMLRAAVK AGSELGLKAK EIMDAGKLVT    60
Y.e.  MRIILLGAPG AGKGTQAQFI MEKYGIPQMC TGDMLRAAVK AGSELGLKAK EIMDAGKLVT    60
E.c.  MRIILLGAPG AGKGTQAQFI MEKYGIPQIS TGDMLRAAVK SGSELGKQAK DIMDAGKLVT    60
S.t.  MRIILLGAPG AGKGTQAQFI MEKYGIPQIS TGDMLRAAVK SGSELGKQAK DIMDAGKLVT    60

                                        ↓
Y.p.  DELVIALVKE RITQEDCRDG FLLDGFPRTI PQADAMKEAG IKVDYVLEFD VPDELIVERI   120
Y.e.  HELVIALVKE RITQDDCRDG FLLHGFPRTI PQADAMKEAG IKVDYVLEFD VPDELIVDRI   120
E.c.  DELVIALVKE RIAQEDCRNG FLLDGFPRTI PQADAMKEAG INVDYVLEFD VPDELIVDRI   120
S.t.  DELVIALVKE RIAQEDCRNG FLLDGFPRTI PQADAMKEAG IVVDYVLEFD VPDELIVDRI   120

              ↓
Y.p.  VGRRVHAASG RVYHVKFNPP KVEDKDDVTG EELTIRKDDQ EATVRKRLIE YHQQTAPLVS   180
Y.e.  VGRRVHAASG RVYHIKFNPP KVEDKDDVTG EELTIRKDDQ EATVRKRLVE YHQQTAPLVS   180
E.c.  VGRRVHAPSG RVYHVKFNPP KVEGKDDVTG EELTTRKDDQ EETVRKRLVE YHQMTAPLIG   180
S.t.  VGRRVHAASG RVYHVKFNPP KVEGKDDVTG EDLTTRKDDQ EETVRKRLVE YHQMTAPLIG   180


Y.p.  YYHKEADAGN TQYFKLDGTR NVAEVSAELA TILG   214
Y.e.  YYRKEADAGN TQYFKLDGTR KVAEVSAELA TILG   214
E.c.  YYSKEAEAGN TKYAKVDGTK PVAEVRADLE KILG   214
S.t.  YYQKEAEAGN TKYAKVDGTQ AVADVRAALE KILG   214
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Figure 1 Alignment of the amino-acid sequences of AKyp (Y.p., SwissProt accession no. O69172) with AKs from *Yersinia enterocolitica* (Y.e., SwissProt accession no. P43412), *E. coli* (E.c., SwissProt accession no. P05082) and *Salmonella typhimurium* (S.t., SwissProt accession no. P37407)

Identical residues are shown in bold. Arrows indicate the position of P87 and S129 residues of AKyp.

Table 2 Comparative kinetic parameters of wild-type AKec, wild-type AKyp and two modified forms of AKyp

The reaction medium (0.5 ml final vol. at 30 °C) contained either 50 mM Tris/HCl, 50 mM KCl, 1 mM glucose, 0.4 mM $NADP^+$, 2 mM $MgCl_2$, different concentrations of ADP and 2 units each of hexokinase and glucose-6-phosphate dehydrogenase (reverse), or 50 mM Tris/HCl, pH 7.4, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 50 mM KCl, 2 mM $MgCl_2$ and 2 units each of lactate dehydrogenase and pyruvate kinase (forward). The reaction was started with enzymes diluted at 7–70 μg/ml in 50 mM Tris/HCl, pH 7.4. K_m (ADP) and V_{max} (ADP) were determined from plots of 1/*v* versus $1/ADP^2$, which assumes that the two molecules of ADP bind to the enzyme with the same affinity. For the reverse reaction, reaction velocities were measured at different fixed ATP concentrations (40, 60 and 100 μM) over a range of AMP concentrations (20–150 μM). The different parameters were calculated assuming that the reaction follows a random-ordered Bi Bi mechanism. The results are mean values from two or three separate experiments and the standard errors of the means are given in parentheses. The kinetic parameters of AKec are from Munier-Lehmann et al. [32]. WT, wild-type.

Reaction	AKec (WT)	AKyp (WT)	AKyp (P87S)	AKyp (S129F)
Forward				
K_m^{ATP} (μM)	51	16.1 (1)	270 (21.8)	53.5 (7.7)
K_m^{AMP} (μM)	38	13.4 (1.4)	320 (13.9)	68 (5.6)
k_{cat} (s^{-1})	403	274 (3.5)	207 (19.3)	11 (0.9)
Reverse				
K_m^{ADP} (μM)	92	44.5 (1.5)	110 (7.6)	105.5 (7.8)
k_{cat} (s^{-1})	239	243 (19)	55 (2.1)	24.7 (1)

Table 3 Molecular masses (Da) of various forms of AKyp measured by electrospray ionization mass spectrometry in the positive-ion mode

The solvent was 20 % (v/v) acetonitrile and 0.1 % formic acid in water. The molecular mass was calculated from the multiply charged ion envelope.

Enzyme	Calculated from sequence	Measured
Wild-type	23672.14	23672.10 ± 1.18
P87S	23662.10	23661.85 ± 0.60
S129F	23732.23	23732.21 ± 0.81
P87S; S129F	23722.20	23721.81 ± 1.10

under its own promoter in plasmid pHL70-1, the *adk* gene was amplified by PCR and cloned into the pET22b vector under the control of the T7 promoter. This construct (plasmid pHL70-3) was introduced into the BL21(DE3)/pDIA17 *E. coli* strain [16]. The recombinant AKyp represented over 40 % of total bacterial proteins in this strain.

Purification and characterization of AKyp

Like other AKs, AKyp interacted strongly with the Blue-Sepharose matrix, and was eluted with a mixture of 1 mM ATP and 1 mM AMP, yielding over 90 % pure protein. Gel permeation chromatography served to remove the low molecular mass contaminants, also indicating that AKyp is monomeric (results not shown). Under these conditions the recombinant enzyme co-eluted with the chromosome-encoded AKec. From the specific activity of the two proteins (Table 2) and the relative abundance of the chromosome-encoded AKec (about 0.15 % of total *E. coli* proteins), we estimated that AKec represents, at most, 0.5 % of the purified AKyp. The molecular mass of AKyp, measured by ESI-MS was identical to that calculated from the deduced sequence (Table 3). Microsequencing of the first 40 amino acid residues of AKyp isolated from soluble extracts of *Y. pestis* cultures demonstrated that the recombinant and native AKs are identical in this region.

The thermal stability of AKyp was determined by recording the ellipticity at 222 nm (Figure 2). The denaturation curve exhibited two co-operative transitions separated by a plateau. A comparable amount of secondary structure was unfolded in each step. The mid-temperature (T_m) of the co-operative transition for the first event was 47.7 °C. The second transition (T_m of 76 °C) was less co-operative. The stability of AKyp was further studied with urea as denaturant. A single sigmoidal transition was observed (Figure 3). Denaturation by urea was reversible and confirmed by the recovery of over 90 % of initial catalytic activity upon removal of denaturing agent. Curve fitting using a two-state model enabled us to calculate the thermodynamic parameters characterizing

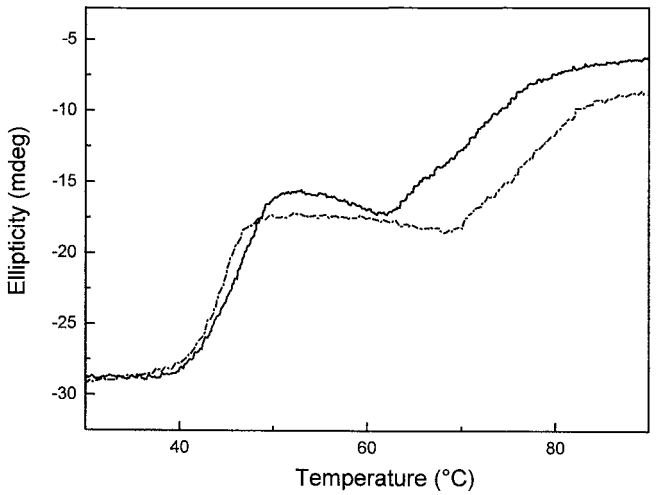

Figure 2 Thermal unfolding of wild-type (–) and S129F (- -) AKyp monitored by circular dichroism

A protein concentration of 10 μM was used.

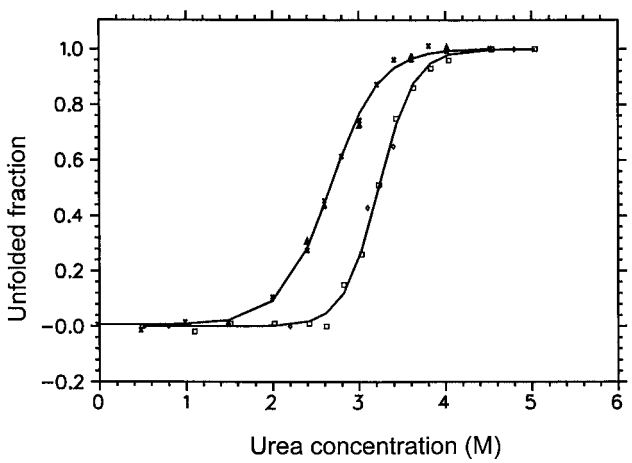

Figure 3 Urea denaturation and renaturation curves of recombinant wild-type and S129F AKyp

Urea denaturation and renaturation curves of recombinant wild-type (□, ◇) and S129F (✖, △) AKyp were measured by CD-spectroscopy, as described in the Materials and methods section. The protein concentration was 10 μM.

the stability of AKyp (Table 4). These results indicated lower thermodynamic stability for Akyp, as compared with AKec.

The catalytic properties of AKyp were also studied. The enzyme had a broad specificity for nucleoside triphosphates, with ATP and dATP acting as the best donors. Even pyrimidine nucleoside triphosphates could replace ATP as donor, although with significantly lower reaction rates. Among nucleoside monophosphates other than AMP: 2′dAMP, 3′dAMP and AraAMP acted as good acceptors (results not shown). Table 2 summarizes the kinetic parameters of AKyp and AKec in both forward and reverse reactions. Enzyme activity determined at various temperatures between 20 °C and 40 °C showed linear Arrhenius plots. The calculated ΔH^* value (12.6 kcal/mol) for AKyp was close to that of AKec (11 kcal/mol) [31].

Table 4 Parameters of the thermal and urea unfolding for AKyp and AKec

WT, wild-type.

Protein	AKyp (WT)	AKyp (S129F)	AKyp (P87S)	AKec (WT)
Thermal unfolding, T_m (°C)*	47.7 (0.2)	44.7 (0.2)	33.5 (0.2)	52.5 (0.2)
Urea unfolding†				
C_m (M)‡	3.2 (0.1)	2.7 (0.1)		3.4 (0.2)
ΔG (H_2O) (kcal/mM)	9.2 (0.4)	5.5 (0.2)		9.8 (0.9)
m§ (kcal/mol/M)	2.9 (0.1)	2.0 (0.1)		2.9 (0.3)

* T_m, the mid-temperature of the first unfolding step is the mean of two experiments. The estimated error is given in parentheses.

† The parameters were obtained with non-linear least squares to fit the entire unfolding curve to a two-state model; the standard deviations are given in parentheses.

‡ Urea concentration at which half of the molecules are unfolded.

§ The coefficient of linear dependence between the free energy of unfolding and urea concentration.

Site-directed mutagenesis experiments and characterization of modified variants of AKyp

Three modified variants of AKyp were obtained by site-directed mutagenesis: P87S, S129F and P87S/S129F (Table 1). They were overexpressed in *E. coli* at the same level as the wild-type enzyme. The molecular mass of these variants determined by ESI-MS corresponded to the mass calculated from the sequence (Table 3).

The S129F variant, like the wild-type AKyp, was soluble and was purified by chromatography on Blue-Sepharose. However, equimolecular mixtures (1 mM) of ATP and AMP, or 0.5 M NaCl did not elute the protein from the matrix. Only 1 M NaCl in Tris/HCl buffer was capable of desorbing the S129F protein from Blue-Sepharose. This property allowed complete separation of modified AKyp from the chromosome-encoded AKec.

The recombinant P87S protein formed inclusion bodies when bacteria carrying the plasmid pHL70-4 were grown at 37 °C. The enzyme was solubilized with 7 M urea in 50 mM Tris/HCl (pH 7.4), then re-activated by dilution. The highest yield was obtained by dilution at 25 °C in 50 mM Tris/HCl (pH 7.4) supplemented with 1 mM P^1,P^5-di(adenosine-5′)-pentaphosphate (Ap_5A) and at a protein concentration below 1 mg/ml. The specific activity of the enzyme in the reverse reaction with 1 mM ADP as substrate was 70 units/mg of protein. When growth and induction of recombinant *E. coli* strain was performed at 20 °C, between 50 and 75 % of the P87S form of AKyp was found to be soluble. The soluble P87S mutant was purified by Blue-Sepharose chromatography, exactly as described for the wild-type protein. This form of P87S mutant (specific activity in the reverse reaction of 100 units/mg of protein) was further analysed.

The double P87S/S129F modified protein, recovered as inclusion bodies irrespective of the growth temperature, was isolated by a procedure similar to that employed for the insoluble P87S variant. The specific activity of the double mutant after the denaturation/renaturation procedure was estimated to be around 2 units/mg of protein in the reverse reaction.

Thermodynamic analysis of the proteins modified by site-directed mutagenesis indicated a decreased stability as compared with the wild-type AKyp, the most dramatic changes being observed with the P87S variant. In the case of the S129F enzyme, the denaturation curve exhibited two co-operative transitions separated by a plateau, as for the wild-type enzyme; the T_m for the first event was 44.7 °C (Figure 2 and Table 4). The thermal denaturation curve of P87S protein exhibited a single transition, with a T_m of 33.5 °C (Table 4) in agreement with the temperature of half-inactivation. Limited proteolysis was also used as a test of

Table 5 Lethality of the parental *Y. pestis* strain (371) and the *adk* mutant derivative [70-8(5)] in mice injected either intravenously (IV) or subcutaneously (SC)

CFU represents the number of bacteria injected into each mouse and lethality represents the number of dead animals out of five injected.

Route of injection	*Y. pestis* strain							
IV	371	CFU	0	3.6	36			
		Lethality	0	1	5			
	70-8(5)	CFU	0	1.5	15	150	1500	15 000
		Lethality	0	0	0	0	0	0
SC	371	CFU	0	4.3	43			
		Lethality	0	0	5			
	70-8(5)	CFU	0	3.5	35	350	3500	35 000
		Lethality	0	0	0	0	0	0

conformational changes in AKyp due to various amino acid substitutions. The first-order rate constant of inactivation of wild-type AKyp by TCPK-trypsin (at a ratio of 500:1) at pH 7.4 and 30 °C (0.2×10^{-3} s^{-1}) was in agreement with the decrease in absorption of the AK band scanned after SDS/PAGE and Coomassie Blue staining. Under identical conditions, P87S and S129F modified enzymes were proteolysed approx. 20–30 times faster ($k_1 = 6.2 \times 10^{-3}$ s^{-1} for P87S protein and 4.4×10^{-3} s^{-1} for the S129F protein). Ap_5A at 1 mM exerted a considerable protective effect on the P87S variant by decreasing the k_1 value to 0.1×10^{-3} s^{-1}. This protective effect of Ap_5A was significantly weaker on the S129F variant.

Construction of a mutated *adk* gene corresponding to the P87S variant of AK in *Y. pestis* and effect on bacterial growth and pathogenesis

The P87S substitution was chosen to mutate the *adk* gene of *Y. pestis* 371 because it was responsible for the most dramatic changes in the thermostability of the enzyme. This mutant was obtained in two steps, as described in the Materials and methods section. To study the effect of the mutation on the thermosensitivity of *Y. pestis*, the parental strain and its *adk* derivative were grown at 28 °C and 37 °C in LB medium supplemented with nalidixic acid (results not shown). Growth of the *adk* mutant strain was delayed at 28 °C compared with the parental strain, but reached an D_{600} value close to that of the parental strain after 48 h. In contrast, growth of the *adk* mutant was severely impaired at 37 °C.

The effect of the mutation of the *adk* gene on the virulence of *Y. pestis* was determined using the mouse experimental model of infection. The presence of the virulence attributes, such as the three endogenous plasmids and the *pgm* locus, was checked in the mutant, along with the absence of genomic rearrangements, prior to infection. By the intravenous route of infection, although 36 colony forming units (CFU) of wild-type *Y. pestis* were sufficient to kill 100 % of the mice, doses as high as 1.5×10^4 CFU of the *adk* thermosensitive mutant were unable to kill even a single animal (Table 5). Similar results were obtained by the subcutaneous route of infection: 43 CFU of the wild-type killed 100 % of the animals whereas 3.5×10^4 CFU of the *adk* mutant did not kill any mice (Table 5). In terms of LD_{50}, the wild-type and *adk* mutant strains were 8.7 and $>1.5 \times 10^4$ CFU respectively by the intravenous route, and 5.9 and $>3.5 \times 10^4$ CFU respectively by the subcutaneous route. The mutation in the *adk* gene of *Y. pestis* thus induced a >10000-fold increase in the LD_{50} for mice, both after intravenous and subcutaneous injections.

DISCUSSION

Although *Y. pestis* is a species close to *E. coli*, its NMPKs may nonetheless have different biochemical and physico-chemical properties. Indeed, we have previously shown that despite a high degree of amino acid identity and the conservation of nucleotide binding and catalytic amino acids, TMPK from *Y. pestis* and *E. coli* exhibited significant differences in thermodynamic properties and rates of AZTMP phosphorylation [4]. In this study, we wanted to analyse AKyp and also to determine whether this NMPK had peculiar properties. For this purpose, the *adk* gene from *Y. pestis* was cloned and overexpressed in *E. coli*, the recombinant wild-type protein purified, and its biochemical and thermodynamic properties studied. We found that the apparent K_ms of AKyp for the natural nucleotides were similar to those of other AKs [32]. Its specific activity in the forward and reverse reactions were comparable with that of AKec. However, AKyp had a thermodynamic stability lower than that of other bacterial or eukaryotic AKs, as indicated by its thermal and urea denaturation parameters. The 90 % sequence identity between AKec and AKyp suggests that the difference in thermodynamic stability is due to local factors, rather than to a predominance of a group of amino acids over the other (an excess of arginine over lysine being considered to increase the stability of a given protein) [33].

AK belongs to a family of essential enzymes, as its inactivation is not compatible with cell survival. Only conditionally lethal mutants defective in the *adk* gene might be used to decipher the role of this catalyst. A few thermosensitive mutants have been previously identified and characterized in *E. coli* [13–15]. Two sites of amino acid substitution in AKec, S129F and P87S were identified in thermosensitive strains. When these mutations were introduced in AKyp, the purified enzymes also exhibited sensitivity to temperature and proteolytic enzymes. In the case of the S129F mutation, the thermal stability of the isolated variant was not affected drastically compared with wild-type AKyp (3 °C loss in T_m). The urea unfolding parameters were also moderately altered. In AKec, S129 is part of a 28-residue-long insertion organized into a domain called LID, which forms a single distorted antiparallel β-sheet, two turns and one loop structure, exposed to the solvent and undergoing a large movement during catalysis [6]. The β-sheet is stabilized by hydrogen backbone interactions and attractive forces between a few side-chains inside the β-sheet. Four amino acids (H126, S129, D146 and T149) belong to this hydrogen binding network, while three additional amino acid side-chains (R131, E151 and Y133) stabilize the network by connecting the β-sheet segments in a sandwich-like structure, and are conserved in AKyp. The substitution of the small and polar S129 by the bulky apolar phenylalanine residue has a perturbing effect on the tertiary structure of the protein.

The P87S mutation has a more drastic effect on the thermostability of AKyp. This is the most frequently encountered mutation in *adk* genes from several thermosensitive *E. coli* strains [14,15]. P87, conserved as P96 in mammalian muscle cytosolic AK (also called AK1), is part of an invariant sequence forming a β-turn inserted between a β-strand and an 8-residue α-helix. This short segment acts as a hinge which stabilizes the whole tertiary structure, also essential for nucleotide binding. Substitution of P87 by a more flexible and polar serine residue weakens the stabilizing role of this short segment.

Growth of *Y. pestis* producing a P87S-mutated AKyp was only slightly delayed at the permissive temperature of 28 °C but was severely impaired at the non-permissive temperature of 37 °C. Together, these results indicate that the P87S mutation had the same effects on the thermosensitive growth of the two

micro-organisms. It has been suggested that AK might be involved in functions other than catalysis. Goelz and Cronan [34] presented experimental evidence for a functional interaction of AKec with phospholipid synthesis. Purified AKec was shown to stabilize mutationally altered *sn*-glycerol 3-phosphate acyltransferase in membrane preparations. The functional relationship between AKec and other bacterial enzymes was also suggested by co-purification of AK with IMP-dehydrogenase (involved in GMP synthesis) or phosphoglycerate kinase (A.-M. Gilles, personal communication). It is thus possible that relatively weak and transient interactions between these proteins are important for cell growth, by coupling energy metabolism to nucleic acid synthesis.

Introduction of a thermosensitive mutation in the AK of a pathogenic bacterium was also a unique opportunity to study the importance of this enzyme for bacterial survival and multiplication in the particular environment of a mammalian host. Since the body temperature of mammals is $\geqslant 37\,^{\circ}C$, a temperature which renders P87S-mutated AK inactive under *in vitro* conditions, it could be expected that the enzyme would also be inactive once the bacteria are inside their mammalian host. This is indeed what we observed when *Y. pestis* bearing the P87S substitution was injected into mice. While less than 50 wild-type bacteria were sufficient to kill 100% of the animals, 15×10^3 (intravenous route) to 35×10^3 (subcutaneous route) *adk* mutants were unable to kill a single animal. This demonstrates that the AK-driven maintenance of adenine nucleotide homoeostasis previously known to be essential for bacterial growth *in vitro* is also crucial for growth *in vivo* and for virulence of *Y. pestis*. Recently, it has been shown that *Pseudomonas aeruginosa* is capable of secreting AK, previously thought to be a typically cytosolic enzyme, and that this secreted form played a role in macrophage cell death [35]. Thus the possibility that, in addition to adenine nucleotide homoeostasis, AKyp exerts some pathogenic effects when the bacteria reside inside mammalian hosts cannot be excluded.

Both antibiotics and vaccines have been used to prevent *Y. pestis* infections. However, current vaccines cause severe adverse reactions [36] and do not provide protection against the pneumonic form of the disease. Furthermore, although *Y. pestis* is usually susceptible to the antibiotics active against Gram-negative bacteria, a multidrug-resistant strain was recently isolated in Madagascar from a patient with symptoms of bubonic plague [37]. The need for new vaccines and bacterial inhibitors exists for all pathogens but is even more critical for potential biological warfare agents such as *Y. pestis*. In this respect, NMPKs may represent potential candidate targets against *Y. pestis* infections and probably against most, if not all, other bacterial pathogens.

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REFERENCES

1 Yan, H. and Tsai, M.-D. (1999) Nucleoside monophosphate kinases: Structure, mechanism, and substrate specificity. Adv. Enzymol. Related Areas Mol. Biol. **73**, 103–133
2 Cheek, S., Zhang, H. and Grishin, N. (2002) Sequence and structure classification of kinases. J. Mol. Biol. **320**, 855–881
3 Yersin, A. (1894) La peste bubonique à Hong-Kong. Ann. Inst. Pasteur **2**, 428–430
4 Chenal-Francisque, V., Tourneux, L., Carniel, E., Christova, P., Li de la Sierra, I., Barzu, O. and Gilles, A. M. (1999) The highly similar TMP kinases of *Yersinia pestis* and *Escherichia coli* differ markedly in their AZTMP phosphorylating activity. Eur. J. Biochem. **265**, 112–119
5 Dreusicke, D., Karplus, P. A. and Schulz, G. E. (1988) Refined structure of porcine cytosolic adenylate kinase at 2.1 Å resolution. J. Mol. Biol. **199**, 359–371
6 Müller, C. W. and Schulz, G. E. (1992) Structure of the complex between adenylate kinase from *Escherichia coli* and the inhibitor Ap_5A refined at 1.9 Å resolution. A model for a catalytic transition state. J. Mol. Biol. **224**, 159–177
7 Müller-Dieckmann, H.-J. and Schulz, G. E. (1994) The structure of uridylate kinase with its substrates, showing the transition state geometry. J. Mol. Biol. **236**, 361–367
8 Scheffzek, K., Kliche, W., Wiesmüller, L. and Reinstein, J. (1996) Crystal structure of the complex of UMP/CMP kinase from *Dictyostelium discoideum* and the bisubstrate inhibitor P^1-(5′-adenosyl)P^5-(5′-uridyl) pentaphosphate (UP_5A) and Mg^{2+} at 2.2 Å: implications for water-mediated specificity. Biochemistry **35**, 9716–9727
9 Lavie, A., Ostermann, N., Brundiers, R., Goody, R. S., Reinstein, J., Konrad, M. and Schlichting, I. (1998) Structural basis for efficient phosphorylation of 3′-azidothymidine monophosphate by *Escherichia coli* thymidylate kinase. Proc. Natl. Acad. Sci. U.S.A. **95**, 14045–14050
10 Fricke, J., Neuhard, J., Kelln, R. A. and Pedersen, S. (1995) The *cmk* gene encoding cytidine monophosphate kinase is located in the *rpsA* operon and is required for normal replication rate in *Escherichia coli*. J. Bacteriol. **177**, 517–523
11 Konrad, M. (1992) Cloning and expression of the essential gene for guanylate kinase from yeast. J. Biol. Chem. **267**, 25652–25655
12 Smallshaw, J. and Kelln, R. A. (1992) Cloning, nucleotide sequence and expression of the *Escherichia coli* K-12 *pyr*H gene encoding UMP kinase. Genet. (Life Sci. Adv.) **11**, 59–65
13 Kohiyama, M., Cousin, D., Ryter, A. and Jacob, F. (1966) Thermosensitive mutants of *Escherichia coli* K 12. I. Isolation and rapid characterization. Ann. Inst. Pasteur **110**, 465–486
14 Haase, G. H., Brune, M., Reinstein, J., Pai, E. F., Pingoud, A. and Wittinghofer, A. (1989) Adenylate kinases from thermosensitive *Escherichia coli* strains. J. Mol. Biol. **207**, 151–162
15 Gilles, A. M., Saint-Girons, I., Monnot, M., Fermandjian, S., Michelson, S. and Barzu, O. (1986) Substitution of a serine residue for proline-87 reduces catalytic activity and increases susceptibility to proteolysis of *Escherichia coli* adenylate kinase. Proc. Natl. Acad. Sci. U.S.A. **83**, 5798–5802
16 Munier, H., Gilles, A. M., Glaser, P., Krin, E., Danchin, A., Sarfati, R. and Barzu, O. (1991) Isolation and characterization of catalytic and calmodulin-binding domains of *Bordetella pertussis* adenylate cyclase. Eur. J. Biochem. **196**, 469–474
17 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning, a laboratory manual (2nd ed), Cold Spring Harbor Laboratory Press, NY
18 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
19 Dotto, G. P. and Zinder, N. D. (1984) Reduction of the minimal sequence for initiation of DNA synthesis by qualitative or quantitative changes of an initiator protein. Nature (London) **311**, 279–280
20 Barzu, O. and Michelson, S. (1983) Simple and fast purification of *Escherichia coli* adenylate kinase. FEBS Lett. **153**, 280–284
21 Saint Girons, I., Gilles, A. M., Margarita, D., Michelson, S., Monnot, M., Fermandjian, S., Danchin, A. and Barzu, O. (1987) Structural and catalytic characteristics of *Escherichia coli* adenylate kinase. J. Biol. Chem. **262**, 622–629
22 Surgalla, M. J. and Beesley, E. D. (1969) Congo red-agar plating medium for detecting pigmentation in *Pasteurella pestis*. Appl. Microbiol. **18**, 834–837
23 Birnboim, H. C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**, 1513–1523
24 Chenal, V. and Griffais, R. (1994) Chemiluminescent and colorimetric detection of a fluorescein-labelled probe and a digoxigenin-labelled probe after a single hybridization step. Mol. Cell. Probes **8**, 401–407
25 Guiyoule, A., Rasoamanana, B., Buchrieser, C., Michel, P., Chanteau, S. and Carniel, E. (1997) Recent emergence of new variants of *Yersinia pestis* in Madagascar. J. Clin. Microbiology **35**, 2826–2833
26 Reed, L. J. and Muench, H. (1938) A simple method on estimating fifty percent endpoints. Am. J. Hyg. **27**, 493–497
27 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. **72**, 248–254
28 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
29 Burlacu-Miron, S., Perrier, V., Gilles, A. M., Pistotnik, E. and Craescu, C. T. (1998) Structural and energetic factors of the increased thermal stability in a genetically engineered *Escherichia coli* adenylate kinase. J. Biol. Chem. **273**, 19102–19107
30 Parkhill, J., Wren, B., Thomson, N., Titball, R., Holden, M., Prentice, M., Sebaihia, M., James, K., Churcher, C., Mungall, K. et al. (2001) Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature (London) **413**, 523–527

31 Glaser, P., Presecan, E., Delepierre, M., Surewicz, W. K., Mantsch, H. H., Bârzu, O. and Gilles, A.-M. (1992) Zinc, a novel structural element found in the family of bacterial adenylate kinases. Biochem. **31**, 3038–3043
32 Munier-Lehmann, H., Burlacu-Miron, S., Craescu, C. T., Mantsch, H. H. and Schultz, C. P. (1999) A new subfamily of short bacterial adenylate kinases with the *Mycobacterium tuberculosis* enzyme as model: A predictive and experimental study. Proteins **36**, 238–248
33 Menéndez-Arias, L. and Argos, P. (1989) Engineering protein thermal stability. Sequence statistics point to residue substitutions in α-helices. J. Mol. Biol. **206**, 397–406
34 Goelz, S. E. and Cronan Jr., J. E. (1982) Adenylate kinase of *Escherichia coli*: evidence for a functional interaction in phospholipid synthesis. Biochemistry **21**, 189–195
35 Markaryan, A., Zaborina, O., Punj, V. and Chakrabarty, A. (2001) Adenylate kinase as a virulence factor of *Pseudomonas aeruginosa*. J. Bacteriol. **183**, 3345–3352
36 Marshall, Jr, J. D., Bartelloni, P. J., Cavanaugh, D. C., Kadull, P. J. and Meyer, K. F. (1974) Plague immunization. II. Relation of adverse clinical reactions to multiple immunizations with killed vaccine. J. Infect. Diseases **129**, S19–S25
37 Galimand, M., Guiyoule, A., Gerbaud, G., Rasoamanana, B., Chanteau, S., Carniel, E. and Courvalin, P. (1997) Multidrug resistance in *Yersinia pestis* mediated by a transferable plasmid. New Engl. J. Med. **337**, 677–680
38 Raleigh, E. A., Murray, N. E., Revel, H., Blumenthal, R. M., Westaway, D., Reith, A. D., Rigby, P. W., Elhai, J. and Hanahan, D. (1988) McrA and McrB restriction phenotypes of some *E. coli* strains and implications for gene cloning. Nucleic Acids Res. **16**, 1563–1575
38a Erratum (1995) Nucleic Acids Res. **23**, 3612
39 Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods in Enzymology **154**, 367–382
40 Simon, R., Priefer, U. and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1**, 784–791
41 Donnenberg, M. S. and Kaper, J. B. (1991) Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59**, 4310–4317

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