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Cloning and Sequence Analysis of Putative Histidine Protein Kinases Isolated from *Lactococcus lactis* MG1363

MARY O'CONNELL-MOTHERWAY,[1,2] GERALD F. FITZGERALD,[1] AND DOUWE VAN SINDEREN[1,2]*

Department of Microbiology[1] and National Food Biotechnology Centre,[2] University College, Cork, Ireland

Received 16 December 1996/Accepted 25 March 1997

Eight recombinant plasmids harboring chromosomal fragments of *Lactococcus lactis* MG1363 were shown to phenotypically suppress a histidine protein kinase (HPK) deficiency in either of two different *E. coli* strains. Sequence analysis of the plasmid inserts revealed five different complete or partial open reading frames (ORFs) specifying proteins with high similarity to HPKs. One of the plasmids also harbored an additional ORF, unrelated to HPKs, with suppressing activity.

The capacity to adapt to (changing) environmental conditions increases a cell's chances of survival and/or maximizes its reproductive capacity. The sensory devices gathering the environmental information often contain communication modules consisting of proteins with specific receiver and transmitter motifs. The so-called two-component regulatory system is a very common and simple example of such a communicating module and has been identified in both eukaryotes (8, 13) and prokaryotes (for reviews see references 15, 20, 22, and 27).

A typical two-component system consists of a histidine protein kinase (HPK), usually a membrane protein capable of sensing a specific environmental signal, and a cytoplasmic response regulator (RR), which translates the incoming signal directly or indirectly into a cellular adaptive response. The general molecular mechanism underlying the signal reception and transmission of a two-component system is based on transfer of a phosphoryl group. The transmitter domain of the HPK possesses an autokinase activity which enables autophosphorylation of a conserved histidine residue. This autophosphorylating activity is mediated by sensory stimuli specific for the input domain of the HPK. Subsequently, the phosphoryl group is transferred to an aspartate residue of the receiver domain of the cognate RR. The latter phosphorylation event mediates the activity of the output domain of the RR which, in many cases, functions as a transcription factor.

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant properties	Source or reference
Strains		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*(Tc^r)]	Stratagene, Ltd., Cambridge, United Kingdom
E. coli ANCC22	*phoR* and *creC* mutations	17
E. coli ANCL1	PhoB$^-$	17
E. coli VJS3051	Δ*narQ251*::Tn*10*d (Tc^r) Δ*narX242 zch-2084*::Ω-Cm^r Φ(*fdnG-lacZ*)	24
E. coli VJS3081	Δ(*lac-argF*)*U169* λΦ(*fdnG-lacZ*) *narL215*::Tn*10*	25
L. lactis MG1363	Plasmid-free derivative of *L. lactis* subsp. *cremoris* NCDO712	7
Plasmids		
pBluescript SK$^-$	Ap^r α*lacZ*	Stratagene, Ltd.
pUC19	Ap^r α*lacZ*	34
pLlkinA	927-bp *Taq*I lactococcal chromosomal fragment[a]	This work
pLlkinB	2.28-kb *Alu*I lactococcal chromosomal fragment[b]	This work
pLlkinC-1	1.53-kb *Ssp*I lactococcal chromosomal fragment[b]	This work
pLlkinC-2	2.2-kb *Rsa*I lactococcal chromosomal fragment[b]	This work
pLlkinD-1	2.05-kb *Dra*I lactococcal chromosomal fragment[b]	This work
pLlkinD-2	1.88-kb *Ssp*I lactococcal chromosomal fragment[b]	This work
pLlkinD-3	1.45-kb *Acs*I lactococcal chromosomal fragment[c]	This work
pLlkinE-1	1-kb *Alu*I lactococcal chromosomal fragment[b]	This work
pLlkinE-2	670-bp lactococcal chromosomal fragment translationally fused to α*lacZ* of pBluescript SK$^-$	This work

[a] Cloned in the *Acc*I site of pUC19.
[b] Cloned in the *Eco*RV site of pBluescript SK$^-$.
[c] Cloned in the *Eco*RI site of pBluescript SK$^-$.

* Corresponding author. Mailing address: Department of Microbiology, University College, Cork, Ireland. Phone: 353 21 902811. Fax: 353 21 903101. E-mail: douwe@ucc.ie.

Members of two-component signal transduction systems have been identified in several lactic acid bacteria, all of which are involved in regulating the production of a particular bacteriocin (5, 6, 10, 23, 31). In this study we report the cloning and sequence analysis of five different putative HPK-encoding genes from *Lactococcus lactis* MG1363 by a complementation method. This method is based on the well-documented fact that an RR is capable of acquiring a phosphoryl group not only from its cognate HPK but, under certain circumstances (e.g., when expressed from a high-copy-number vector or when parts of the input domain have been removed), also from a heterologous HPK through "cross-talk" (27, 32, 33).

Microorganisms and culture conditions. Strains and plasmids used in this study are listed in Table 1. *L. lactis* was cultivated at 30°C in M17 broth (29) containing 0.5% (wt/vol) glucose. *E. coli* strains were grown in Luria-Bertani (LB) broth (3) at 37°C with agitation. Ampicillin, chloramphenicol, and tetracycline were used where necessary at final concentrations of 100, 20, and 12.5 μg/ml, respectively.

Phenotypic suppression of HPK-deficient *E. coli* mutants. In order to identify HPK-encoding genes from *L. lactis* MG1363, the following complementation strategy using two *E. coli* strains was employed. Strain ANCC22 exhibits a PhoA$^-$ (alkaline phosphatase [AP]-negative) phenotype due to mutational lesions of both the *phoR* and *creC* genes. The latter two genes encode the HPKs responsible for the activation of their cognate RR, PhoB, and for the consequent expression of the phosphate regulon, which includes the structural gene for AP, *phoA* (17). Complementation of this mutant through cross-talk of a cloned HPK will lead to activation of PhoB and therefore the expression of *phoA*, resulting in increased AP activity. The second strain, VJS3051, is a double mutant of the HPK-encoding genes *narX* and *narQ* of the anaerobic respiration system of *E. coli* and carries in addition an *fdnG-lacZ* fusion on the chromosome (24–26). Cross-talk complementation of this mutant will lead to the activation of the cognate RR, NarL, which in turn induces transcription of the *fdnG-lacZ* fusion and thus results in increased β-galactosidase (β-Gal) activity (24).

Total chromosomal DNA isolated from *L. lactis* MG1363 (12) was partially digested with various restriction enzymes. DNA fragments ranging from 1 to 6 kb were isolated from agarose gels, ligated into pBluescript SK$^-$ or pUC19, and used to transform *E. coli* ANCC22 and VJS3051 by electroporation. Transformants were selected on LB agar containing ampicillin and an indicator of AP activity (5-bromo-4-chloro-3-indolylphosphate; 40 μg/ml) or of β-Gal activity (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml), respectively. Among the Ap^r transformants a number of positive (blue) colonies were obtained; these were selected for further study.

Phenotypic complementation of some transformants is caused by cloned lactococcal chromosomal fragments. The phenotypic suppression observed on the indicator plates was quantified by measuring the AP activity (1) in the ANCC22 transformants and the β-Gal activity (14) in the VJS3051 transformants. Of 16 *E. coli* ANCC22 transformants which exhibited phenotypic suppression on the indicator plates, only 5 gave increased AP activity (Fig. 1A). In the case of *E. coli* VJS3051, three of four transformants which displayed phenotypic suppression on plates exhibited increased β-Gal activity (Fig. 1B). Only those transformants which exhibited increased enzymatic activity were selected for further analysis.

In order to exclude the possibility that the phenotypic suppression of the *E. coli* strains ANCC22 and VJS3051 was caused by either the inadvertent cloning of an *L. lactis* phosphatase gene (in the case of ANCC22), a silent (MG1363 is phenotypically β-Gal negative) β-Gal gene (in the case of VJS3051), or a transcriptional activator for *phoA* (in the case of ANCC22) or the *fdnG-lacZ* fusion (in the case of VJS3051), plasmid DNA from each of the ANCC22- or VJS3051-complementing transformants was isolated (4) and used to transform either strain ANCL1 (lacking *phoB*) or VJS3081 (*fdnG-lacZ narL*::Tn*10*), respectively. *E. coli* ANCL1 lacks the RR of the Pho regulon, whereas *E. coli* VJS3081 contains a knockout mutation in the RR gene, *narL*, of the anaerobic respiration system. If the phenotypic suppression of ANCC22 or VJS3051 was due to cross-talk, rather than to any of the above-mentioned alternative possibilities, introduction of the complementing plasmids into strain ANCL1 or VJS3081 would not lead to increased AP or β-Gal activity, respectively. Since complementation was not observed with any of these transformations (data not shown), it was assumed that the cloned DNA

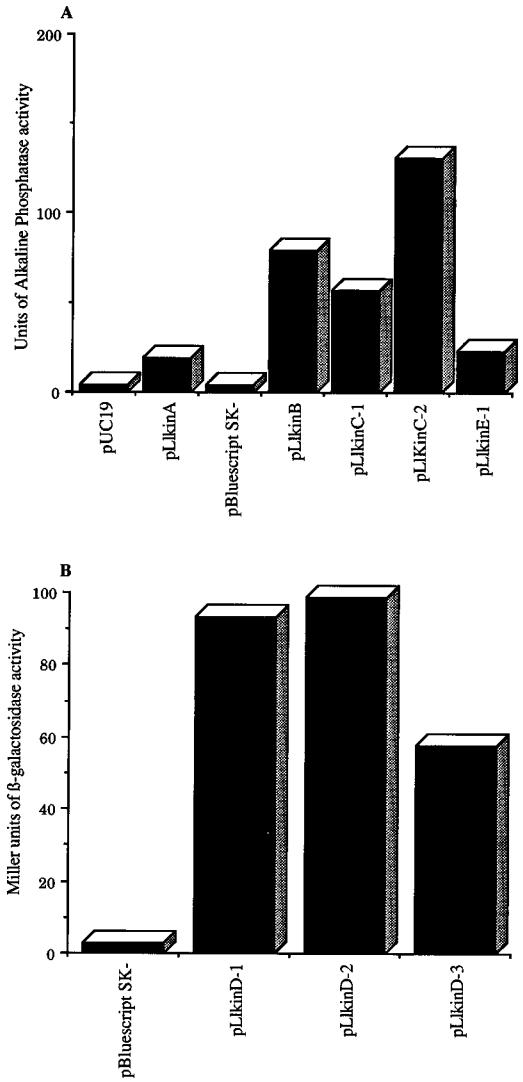

FIG. 1. Phenotypic suppression of the *phoR creC* (A) and *narX narQ* (B) mutations by the pLlkin plasmids (see Fig. 2 for description of plasmids). *E. coli* ANCC22 and VJS3051 cells carrying the indicated plasmids were used to determine AP or β-Gal activity, respectively. Plasmids pUC19 and pBluescript SK$^-$ were used as negative controls to determine the background level of each of the enzymatic tests.

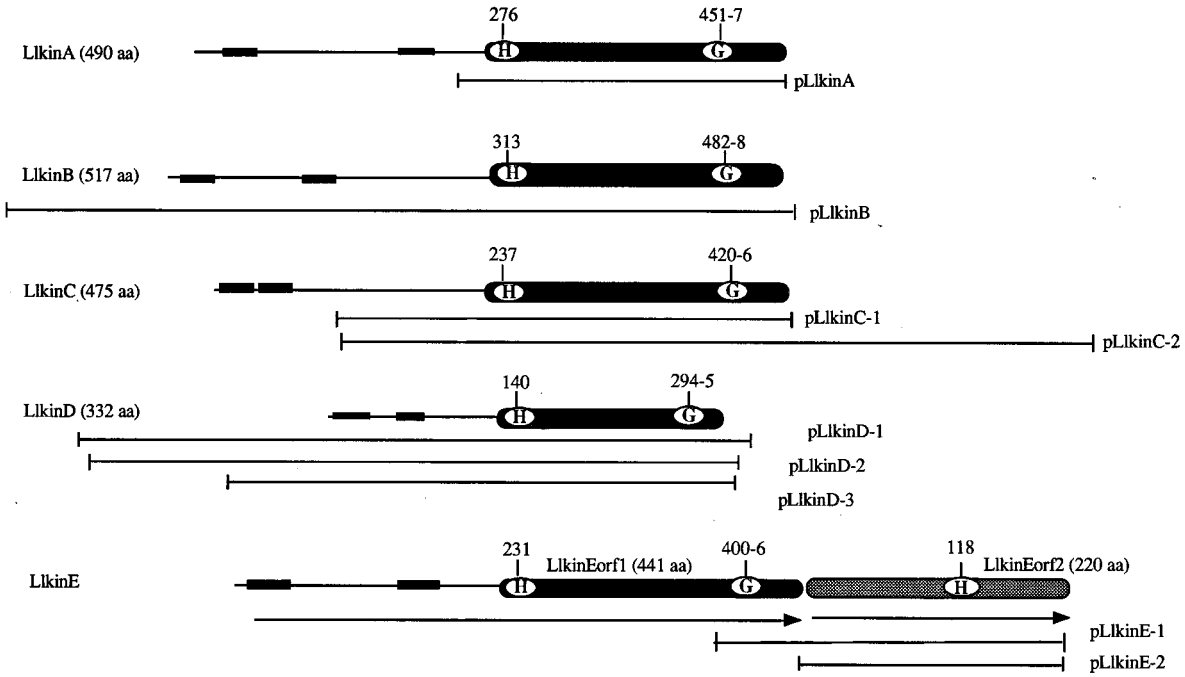

FIG. 2. Schematic representation of the five putative HPKs from *L. lactis*. The positions of the conserved histidine residue (H) and G2 box (G) are shown. Putative transmembrane regions are indicated by a solid rectangles. The extent of the corresponding chromosomal DNA fragment cloned in each of the original complementing plasmids is delineated as a horizontal line ending in short perpendicular dashes, together with the name of the plasmid.

fragments specify (part of) a protein that exhibits cross-talking HPK activity.

To determine if the recombinant plasmids causing phenotypic suppression in one mutant strain were also capable of phenotypic suppression in the other strain, plasmid DNA from each of the complementing *E. coli* VJS3051 transformants was introduced into *E. coli* ANCC22 and vice versa. None of the resulting transformants complemented the HPK-deficient mutant, which indicated that the observed cross-talk was specific for each system.

Sequence analysis of the DNA inserts of complementing plasmids reveals five different putative HPKs. Analysis of the plasmid content of the eight complementing transformants indicated that DNA fragments ranging between 900 bp and 2.5 kb had been cloned. DNA sequencing of the inserts from the eight complementing plasmids (the names of which are given in Fig. 2) revealed the presence of five different open reading frames (ORFs), designated *llkinA* through *-E*, each of which specifies a protein with significant similarity (BLAST searches gave similarities with *P* values of $<10^{-8}$ [2]) to HPKs (Fig. 3). The complete putative HPK-encoding ORF appeared to be cloned in the cases of plasmids pLlkinB, pLlkinD-1, pLlkinD-2, and pLlkinD-3. The latter plasmids harbored additional DNA sequences, which, upon further examination, were shown not to be responsible for the complementing phenotype (data not shown). The inserts of plasmids pLlkinA, pLlkinC-1, and pLlkinE were shown to harbor only the 3′ portions of the respective putative HPKs (except in the case of *llkinE*; see below), encompassing the genetic information specifying the complete conserved C-terminal transmitter domain of HPKs (Fig. 2). The finding that HPKs lacking their N-terminal input domains are still capable of complementation has been reported previously (17) and may even be a requirement for cross-talk to occur because of increased phosphorylating aspecificity or activity of the truncated HPK. Based on the nucleotide sequences, primers were designed in order to obtain the 5′ portions of *llkinA*, *llkinC*, and *llkinE* by inverse PCR, essentially as described by Ochman et al. (18).

Computer-aided analysis of the deduced sequences of the N-terminal amino acids of LlkinA through -E identified regions of hydrophobicity in each case. These stretches of hydrophobic residues may represent transmembrane regions, suggesting that each of the deduced putative HPKs is membrane associated and is involved in sensing an environmental signal (Fig. 2). Figure 3A shows the amino acid sequences of the C-terminal transmitter domains of four of the putative lactococcal HPKs. Each of these deduced proteins possesses the four motifs common to most HPKs (Fig. 3A): the autophosphorylation site (H box), at which a conserved histidine is phosphorylated upon signal detection; the phenylalanine/aspartate (F/D) and glycine (G) boxes, which are thought to be involved in nucleotide binding; and the asparagine (N) box, which is of unknown function (21, 28). The deduced gene product of *llkinD*, which complemented *E. coli* VJS3051 (Δ*narX* Δ*narQ*), is somewhat different from the other putative lactococcal HPKs and is more similar to NarX and NarQ (Fig. 3B). Similar amino acid comparisons by others had already indicated the existence of subfamilies of HPKs (24, 26). Our work suggests that these subfamilies may also be functionally different, since the plasmids containing *llkinA*, *llkinB*, *llkinC*, or *llkinEorf2* did not seem to complement the Δ*narX* Δ*narQ* mutations. Similarly, the plasmid harboring *llkinD* did not complement the mutations in *phoR* and *creC*. On the other hand, Nagasawa et al. (17) have shown that cross complementation does occur between two different *E. coli* mutants deficient in HPKs which belong to the same subfamily.

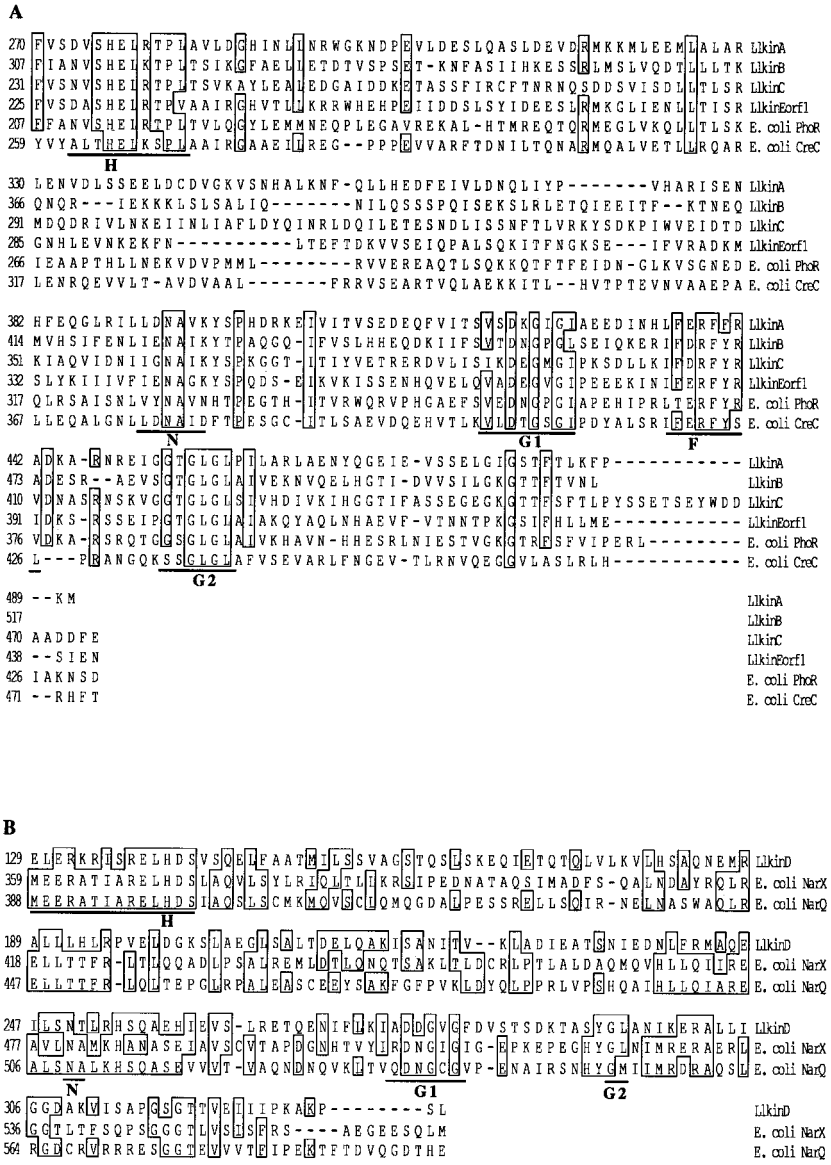

FIG. 3. Multiple sequence alignments of the deduced carboxy-terminal domains of LlkinA, LlkinB, LlkinC, and LlkinEorf1 with those of *E. coli* PhoR and CreC (A), and sequence alignments of the deduced carboxy-terminal domain of LlkinD with those of *E. coli* NarX and NarQ (B). Amino acids are boxed if at least four (A) or at least two (B) of the depicted proteins contain an identical residue at a particular position. Conserved motifs present in members of the HPK family, designated H, N, G1, F, and G2, are indicated by lines below the corresponding sequences (note that the F motif is absent in the alignment of panel B). Numbers in the left-hand margin refer to the amino acid positions in the corresponding protein indicated in the right-hand margin.

Plasmid pLlkinE-1 encodes an unusual protein kinase. During sequence analysis of the complementing insert of pLlkinE-1, it was noted that this harbored the 3′ portion of an ORF specifying a protein with high similarity to HPKs but did not include the DNA region encoding the conserved histidine residue (Fig. 2). This finding raised doubts as to the complementing capacity of this truncated protein. Upon further sequence analysis a second, complete ORF (*llkinEorf2*) was identified immediately downstream of *llkinEorf1*. This second ORF encoded a protein of 220 amino acids with no significant similarity ($P > 0.5$ [2]) to any protein in the available databases. In order to determine whether the complementation observed was due to the 3′ portion of *llkinEorf1* or to *llkinEorf2*, the DNA encompassing the latter ORF was amplified from the chromosome of *L. lactis* by PCR and cloned into pBluescript SK$^-$, generating pLlkinE-2. Transformation of *E. coli* ANCC22 with this construct resulted in phenotypic suppression of this mutant strain and increased expression of the *phoA* gene (Fig. 4). This finding indicated that the protein encoded by *llkinEorf2* is capable of phosphorylating PhoB. A similar alternative phosphorylating activity has been identified in the BarA and ArcB HPKs and has been deduced for other HPKs (9). In all these proteins, the alternative kinase activity is thought to be specified by the C-terminal portion of a composite protein which also contains a classical HPK. In the case of pLlkinE-1, this activity was shown to be specified by a separate ORF, immediately downstream of *llkinEorf1*, which encodes a protein with high similarity to HPKs. The C-terminal portions of the composite proteins mentioned above do not display similarity to each other except for a

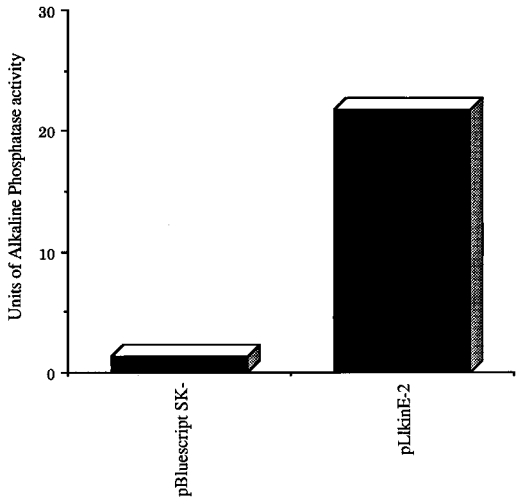

FIG. 4. AP assay illustrating phenotypic suppression of *E. coli* ANCC22 (with mutations in *phoR* and *creC*) by pLlkinE-2. Cells of *E. coli* ANCC22 carrying plasmid pLlkinE-2 were used to determine AP activity. Plasmid pBluescript SK$^-$ was used as a negative control to determine the background level of the enzymatic test.

small region of approximately 25 amino acids. This region contains a conserved histidine residue which, in the cases of ArcB and BarA, was shown to be the phosphorylation site (9). Upon close inspection of the protein specified by *llkinEorf2*, such a small region of similarity could be identified (Fig. 5), although outside of this region none of these proteins appeared to be similar.

Conclusion. Several strategies have been employed to identify two-component HPK and RR-encoding genes from prokaryotic species. Osbourn et al. (19) identified two-component regulatory genes from *Xanthomonas campestris* pathovar *campestris* by Southern hybridization with degenerate oligonucleotide probes corresponding to the conserved amino acid sequence of the HPK G2 box (21, 28). Lee and Stock (11) reported the identification of a two-component system from the hyperthermophilic bacterium *Thermotoga maritima* by PCR with degenerate oligonucleotides derived from the conserved N and G2 boxes (21, 28). Complementation of HPK-deficient *E. coli* mutants has led to the identification of several two-component systems (1, 16, 17, 30), including the five putative HPKs described in this report. Although this complementation method seems to be applicable to many different bacteria, it is likely to be limited because it will only allow the identification of a certain number of HPKs of a particular bacterium. This number will depend not only on the *E. coli* strain used for complementation for the identification of members of a particular subclass of HPKs but, more importantly, on the intrinsic properties of both the (sometimes truncated) HPK and the RR, which will determine whether cross-talk is possible.

HPK and RR members are frequently organized as an operon, and it is therefore expected that sequencing the regions surrounding the putative HPK-encoding genes will reveal, at least in some cases, the gene encoding the cognate RR. Sequence comparisons with other two-component systems may give an indication as to the regulatory function of each system, but ultimately experimental proof is required to establish the regulatory role of each of the five systems identified in this study.

Nucleotide sequence accession numbers. GenBank accession numbers for the five putative HPK-encoding genes and the additional ORF sequenced in this study are as follows: *llkinA*, U81166; *llkinB*, U81485; *llkinC*, U81486; *llkinD*, U81487; *llkinEorf1*, U81488; and *llkinEorf2*, U81489.

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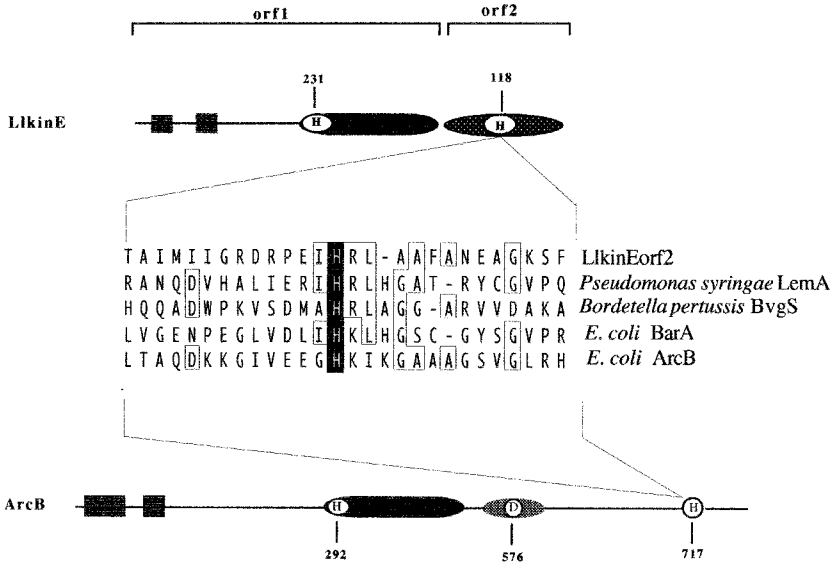

FIG. 5. Alignment of a short region of similarity between proteins belonging to the unorthodox kinase family (9) and the corresponding region of LlkinEorf2. The central histidine, which is the only completely conserved residue, is indicated by a solid box. Amino acids are boxed if at least three of the depicted protein sequences contain an identical residue at a particular position. The relative positions of the compared sequences and the organization of the corresponding genes are shown in the schematic structures of the representatives of *E. coli* ArcB and *L. lactis* LlkinEorf2. The numbers refer to the positions of the conserved histidine and aspartate residues in each of these proteins.

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