THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 21, Issue of May 23, pp. 18754–18760, 2003
Printed in U.S.A.

A Novel H-NS-like Protein from an Antarctic Psychrophilic Bacterium Reveals a Crucial Role for the N-terminal Domain in Thermal Stability*

Received for publication, November 19, 2002, and in revised form, February 24, 2003
Published, JBC Papers in Press, March 13, 2003, DOI 10.1074/jbc.M211766200

Christian Tendeng‡§, Evelyne Krin‡, Olga A. Soutourina¶, Antoine Marin‖, Antoine Danchin‡, and Philippe N. Bertin‡‡§§**

*From the ‡Unité de Génétique des Génomes Bactériens, Institut Pasteur, 75724 Paris Cedex 15, France, ¶Laboratoire de Biochimie, Ecole Polytechnique, 91128 Palaiseau Cedex, France, ‖Atelier de Bio-informatique, Université Paris 6, 12 rue Cuvier, 75005 Paris, France, **HKU-Pasteur Research Centre, Dexter HC Man Building, 8 Sassoon Road, Pokfulam, Hong Kong, China, and ‡‡Laboratoire de Dynamique, Evolution et Expression de Génomes de Microorganismes, FRE 2326, Université Louis Pasteur/CNRS, 67083 Strasbourg Cedex, France*

We describe here new members of the H-NS protein family identified in a psychrotrophic *Acinetobacter* spp. bacterium collected in Siberia and in a psychrophilic *Psychrobacter* spp. bacterium collected in Antarctica. Both are phylogenetically closely related to the HvrA and SPB *Rhodobacter* transcriptional regulators. Their amino acid sequence shares 40% identity, and their predicted secondary structure displays a structural and functional organization in two modules similar to that of H-NS in *Escherichia coli*. Remarkably, the *Acinetobacter* protein fully restores to the wild-type H-NS-dependent phenotypes, whereas the *Psychrobacter* protein is no longer able to reverse the effects of H-NS deficiency in an *E. coli* mutant strain above 30 °C. Moreover, *in vitro* experiments demonstrate that the ability of the *Psychrobacter* H-NS protein to bind curved DNA and to form dimers is altered at 37 °C. The construction of hybrid proteins containing the N- or the C-terminal part of *E. coli* H-NS fused to the C- or N-terminal part of the *Psychrobacter* protein demonstrates the role of the N-terminal domain in this process. Finally, circular dichroism analysis of purified H-NS proteins suggests that, as compared with the *E. coli* and *Acinetobacter* proteins, the α-helical domain displays weaker intermolecular interactions in the *Psychrobacter* protein, which may account for the low thermal stability observed at 37 °C.

Life in cold habitats imposes numerous constraints on bacterial metabolism. These conditions require appropriate adaptation of the structure and the physiology of psychrophilic or psychrotolerant bacteria (1, 2). For instance, the mechanisms allowing these organisms to adapt to low temperature include enhancement of membrane fluidity, which can be obtained through a relative increase in polyunsaturated fatty acids (1, 3). Furthermore, to circumvent the limitations imposed by a reduced thermal energy, enzymatic proteins with a high specific activity are produced (4). At the molecular level, all proteins from psychrotrophic organisms studied so far have shown a decrease in their intramolecular interactions, usually associated with both higher flexibility and lower thermal stability as compared with their mesophilic or thermophilic counterparts (5). Most of the data about the molecular adaptation of proteins to low temperature concern psychrophilic enzymes (4). In contrast, little is known about regulators of gene expression (6, 7), including nucleoid-associated proteins.

The increasing number of sequencing projects has recently revealed the existence of several H-NS-related proteins in Gram-negative bacteria with different life style such as the human pathogenic bacteria *Yersinia pestis* (8) and *Pasteurella multocida* (9) and the plant pathogenic bacteria *Xylella fastidiosa* (10) and *Ralstonia solanacearum* (11). Some of these proteins have been studied in detail, *e.g.* those from *Vibrio cholerae* (12) and *Bordetella pertussis* (13). All H-NS proteins share the same structural and functional organization in two modules (14, 15). In enterobacteria, the N-terminal domain of H-NS has been recently shown to contain three α-helices (16), whereas the C-terminal three-dimensional structure of the protein resolved by NMR consists of a mix of α-β structures (17). The H-NS protein forms oligomers via its N-terminal part and is able to bind curved and AT-rich DNA fragments via its C-terminal domain (18), both essential properties of H-NS and related proteins (19). Nevertheless, the role of most proteins of the H-NS family in bacterial physiology remains unknown (15). In contrast, in *Escherichia coli* and *Salmonella typhimurium* H-NS seem to be involved in bacterial nucleoid organization and in the regulation of various genes involved in adaptation to environmental challenges (20, 21). A variety of phenotypes has been associated with a mutation in *hns*, in particular an increase in pH resistance (21, 22) and a loss of motility (23, 24). Finally, in enterobacteria and related micro-organisms, H-NS proteins are cold shock proteins (12, 25), which could explain the susceptibility to low temperature of *E. coli hns* mutant (26).

To further investigate the structure-function-evolution relationship of H-NS-related proteins, we identified and characterized orthologous proteins in bacteria isolated from extreme environments, *i.e.* a psychrotrophic bacterium *Acinetobacter* spp. isolated from Lake Baikal in Siberia[1] and a psychrophilic bacterium *Psychrobacter* spp. collected from Antarctica (27). Like the protein of *E. coli*, H-NS-like proteins of *Psychrobacter*

* This work was supported by the Institut Pasteur and CNRS Grants URA1129 and URA 2171. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by Ministère de l'Enseignement Supérieur et de la Recherche grants.

§§ To whom correspondence should be addressed: Laboratoire de Dynamique, Evolution et Expression de Génomes de Microorganismes, Université Louis Pasteur, 28 rue Goethe, 67000 Strasbourg, France. Tel.: 33-3-19-24-20-08; Fax: 33-3-19-24-20-28; E-mail: bertin@gem.u-strasbg.fr.

[1] L. Denissova, unpublished data.

and *Acinetobacter* were both able to complement H-NS-related phenotypes in *E. coli* at 30 °C. Surprisingly, the *Psychrobacter* H-NS protein was no longer able to reverse the effects of H-NS deficiency at 37 °C. *In vivo* and *in vitro* experiments demonstrated the crucial role of the N-terminal part on the thermal stability of this unusual H-NS protein and give new insight concerning the structural and functional organization of the proteins of this family.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Plasmids—The *Psychrobacter* TAD1 strain was grown at various temperatures (from 4 to 25 °C) in Luria-Bertani (LB) medium. The *Acinetobacter* bacterial strain 20 was grown from 4 to 37 °C in LB medium. *E. coli* FB8 strain (28) and BE1410, its *hns-1001* derivative (29), were used in this study. This H-NS-deficient strain contains a Tn*5seq1* transposon insertion located in the 20th codon of the *hns* gene (30). *E. coli* cells were grown at various temperatures (from 20 to 37 °C) in LB medium or in M63 medium (31) supplemented with 40 μg/ml serine, 1 mM isopropyl-1-thio-β-D-galactopyranoside, and 0.4% glucose as a carbon source.

Metabolism of β-glucosides was tested on MacConkey agar indicator plates with 1% salicin as a carbon source. Tryptone swarm plates containing 1% Bacto-tryptone, 0.5% NaCl, and 0.3% Bacto-agar were used to test bacterial motility as previously described (23). When required, ampicillin or chloramphenicol was added at 100 μg/ml and 20 μg/ml, respectively. Ultracompetent XL1-Blue (Stratagene) cells were used to construct the genomic library of *Acinetobacter* 20 and *Psychrobacter* TAD1 strains. All experiments were performed in accordance with the European requirements for the contained use of genetically modified organisms of Group-I (agreement number 2735) and Group-II (agreement 2736 CAI).

Plasmid pDIA572 was isolated from a *Psychrobacter* TAD1 genomic library (see below) and carries a DNA fragment of 1330 bp. The insert nucleotide sequence was determined on both strands. The DNA fragment of pDIA572 contains the *hns* gene of *Psychrobacter* TAD1 and its flanking regions (accession number AJ310993). Plasmid pDIA585 carries a 2673-bp DNA fragment containing the *hns* gene of *Acinetobacter* strain 20 and its flanking regions (accession number AJ458445). To overproduce the H-NS-His6 proteins of *E. coli*, *Acinetobacter* 20, and *Psychrobacter* TAD1 strains, their structural gene was amplified from genomic DNA using primers 5′-GGAGGTTCATATGAGCGAAGCACTTAAAAT-3′ and 5′-CCGCTCGAGTTGCTTGATCAGGAAATCGT-3′, primers 5′-GGAGGTTCATATGCCAGATATTAGTAATTTATCTG-3′ and 5′-CCGCTCGAGGATGAGGAAGTCTTCCAGTTTCGCACC-3′, and primers 5′-GGAGGTTCATATGACTAATAACACTACTAT-3′ and 5′-CCGCTCGAGTACAGTAAAACTTTCTAGGT-3′, respectively. These pairs of primers introduced a *Nde*I cloning site and a *Xho*I cloning site at 5′- and 3′-end, respectively. The PCR products were inserted into the *Nde*I and *Xho*I sites of the pET-22b vector (Novagen), giving rise to plasmids pDIA569 (which contains the *hns* gene of *E. coli*), pDIA588 (which contains the orthologous gene of *Acinetobacter* strain 20), and pDIA568 (which contains the orthologous gene of *Psychrobacter* TAD1).

The genes coding for the H-NS chimeric proteins of *E. coli* and *Psychrobacter* TAD1 were constructed as follows. The promoter region and the 5′-end of the *hns* gene of *E. coli* were amplified from plasmid pDIA547 (19) using primers 5′-GTTTTTCCCAGTCACGAC-3′ and 5′-AGATTTAACGGCAGCAAGGC-3′ and its 3′-end using primers 5′-TAGAAGAGATTTTGAAGGCTGGCACCAAAGCTAAACGTGC-3′ and 5′-AGCGGATAACAATTTCACACAGGA-3′; the 5′-end of the *hns* gene of *Psychrobacter* spp. was amplified from plasmid pDIA580 using primers 5′-ATGACTAATAACACTAC-3′ and 5′-AGCCTTCAAAATCTCTTCTA-3′ and its 3′-end using primers 5′-GCCTTGCTGCCGTTAAATCTGGTGAAAGCCTAGAGAAAAAACG-3′ and 5′-CCCAAGCTTGGGTTATACAGTAAAACTTTCTAGG-3′. All constructs were inserted into the *Hin*dIII and *EcoR*I restriction sites of plasmid pDIA547 and gave rise to plasmids pDIA580, pDIA581, and pDIA582 (Table III).

Construction of Genomic DNA Libraries—Genomic DNA was isolated from *Acinetobacter* 20 and *Psychrobacter* TAD1 bacterial strains. The *Psychrobacter* genomic library was constructed in plasmid pcDNA 2.1 (Invitrogen) as previously described (15), whereas the *Acinetobacter* genomic library was constructed in plasmid pDIA561 (21). About 60,000 clones were selected on LB plates supplemented with 100 μg/ml ampicillin or 20 μg/ml chloramphenicol and pooled. Large scale plasmid DNA isolation was carried out using the JETstar kit (GENOMED).

Protein Purification—The recombinant H-NS-His6 proteins of *E. coli* and *Psychrobacter* TAD1 strains were purified from *E. coli* BL21 (DE3) (Stratagene) carrying pDIA17 and pDIA569 or pDIA568 using $NiSO_4$ chelation columns (Qiagen), as previously described (19).

TABLE I
Amino acid composition of E. coli, Acinetobacter spp., and Psychrobacter spp.

Shown are H-NS proteins using the ProtParam software on the ExPASy web site (www.expasy.org/tools/protparam.html). Modifications in the content of amino acids usually associated with a low protein thermal stability are shown in bold.

	Amino acid composition of H-NS proteins		
	E. coli	*Acinetobacter* spp.	*Psychrobacter* spp.
Ala (A)	15 (11.0%)	9 (8.5%)	9 (8.5%)
Arg (R)	11 (8.1%)	7 (6.6%)	6 (5.7%)
Asn (N)	**6 (4.4%)**	**3 (2.8%)**	**10 (9.4%)**
Asp (D)	**6 (4.4%)**	**6 (5.7%)**	**3 (2.8%)**
Cys (C)	1 (0.7%)	0 (0.0%)	1 (0.9%)
Gln (Q)	7 (5.1%)	8 (7.5%)	6 (5.7%)
Glu (E)	19 (14.0%)	11 (10.4%)	11 (10.4%)
Gly (G)	6 (4.4%)	5 (4.7%)	5 (4.7%)
His (H)	0 (0.0%)	0 (0.0%)	2 (1.9%)
Ile (I)	6 (4.4%)	7 (6.6%)	8 (7.5%)
Leu (L)	14 (10.3%)	11 (10.4%)	10 (9.4%)
Lys (K)	12 (8.8%)	10 (9.4%)	9 (8.5%)
Met (M)	3 (2.2%)	0 (0.0%)	0 (0.0%)
Phe (F)	1 (0.7%)	2 (1.9%)	2 (1.9%)
Pro (P)	**3 (2.2%)**	**4 (3.8%)**	**1 (0.9%)**
Ser (S)	6 (4.4%)	9 (8.5%)	6 (5.7%)
Thr (T)	**9 (6.6%)**	**4 (3.8%)**	**10 (9.4%)**
Trp (W)	1 (0.7%)	2 (1.9%)	2 (1.9%)
Tyr (Y)	3 (2.2%)	2 (1.9%)	2 (1.9%)
Val (V)	**7 (5.1%)**	**6 (5.7%)**	**3 (2.8%)**

Gel Retardation Experiments—Gel retardation experiments were performed as previously described (23), with H-NS-purified protein of *Psychrobacter* TAD1 either at 4 or 37 °C. Restriction fragments derived from plasmid pDIA525 that contain *flhDC* or *bla* DNA fragments of *E. coli* were used as competitors.

Protein-Protein Cross-linking—Cross-linking experiments were performed as previously described (12) with 25 μM H-NS of *Psychrobacter* TAD1 used in each reaction. After adding cross-linking reagents, *i.e.* 200 mM 1-ethyl-3(3-dimethylaminopropyl)carbodiimide and 50 mM *N*-hydroxysuccinimide, the reaction mixtures were incubated for 1 h either at 4 °C or at 37 °C, loaded onto a SDS-14% Prosieve acrylamide gel, and silver-stained.

Circular Dichroism (CD) Spectroscopy—CD spectra were obtained with a Jobin-Yvon CD6 dichrograph equipped with a thermostatted cell holder. The CD spectra were recorded between 190 and 260 nm from 4 to 70 °C (after 10 min of temperature equilibration before recording the data). The results are the mean values of two successive spectra. CD spectra of purified proteins were determined in pure water with a protein concentration of 14 μM.

In Silico Sequence Analysis—The ProtParam software on the ExPASy web site (www.expasy.org/tools/protparam.html) was used to determine the amino acid composition of proteins. The CLUSTALw method (32) was used for sequence alignments. Secondary structure prediction was performed using the PREDATOR method (33), available on the web site pbil.univ-lyon1.fr. The fold recognition method FROST (Fold Recognition Oriented Search Tool), available on the web site www-mig.jouy.inra.fr/mig/index.html, was used for fold assignments to H-NS protein sequences (34).

Nucleotide Sequence Accession Numbers—The nucleotide sequences of 16 S rRNA and *hns* genes from *Psychrobacter* TAD1 bacterial strain have been assigned EMBL nucleotide sequence data base accession numbers AJ310992 and AJ310993, respectively. The 1379 nucleotide sequence of 16 S rRNA gene of *Acinetobacter* strain 20 was in accordance with the partial sequence in databases under accession number AJ222834. The *Acinetobacter* spp. *hns* gene and its product have been assigned EMBL accession number AJ458445.

RESULTS

Characterization of Natural Bacterial Isolates from Siberia and Antarctica—Two Gram-negative bacteria were isolated from lake Baikal in Siberia and from frozen water in Antarctica. The first strain was isolated from samples collected from the central basin at about 1,000 m below the surface of the lake (35). The selected strain was able to grow under a wide range of

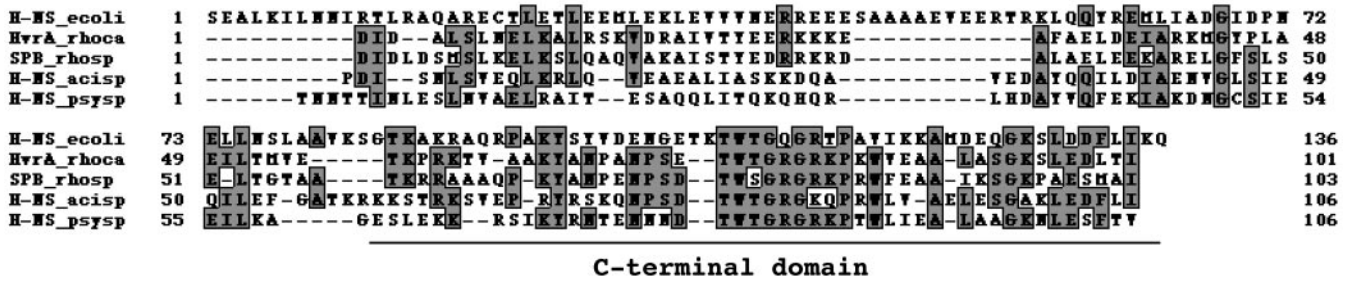

FIG. 1. **Structurally based alignment of H-NS proteins of *E. coli* (*ecoli*), *Rhodobacter capsulatus* (*rhoca*), *Rhodobacter sphaeroides* (*rhosp*), *Acinetobacter* spp. (*acisp*), and *Psychrobacter* spp (*psysp*).** The alignment was achieved using CLUSTALw (32) and refined manually. Residues conserved in at least three sequences are in *gray boxes*, and the C-terminal domain of H-NS and related proteins (19) is *underlined*.

temperatures extending from 4 to 37 °C but with an optimum growth temperature of 25 °C. Morphological, biochemical, and phenotypic characterization, *e.g.* rods occurring in pairs, nonmotile bacterium, oxidase-negative and catalase-positive, suggest that this strain belongs to the *Acinetobacter* genus. Its taxonomic position was further investigated by determination of 16 S rRNA gene sequence (accession number AJ222834) and comparative analysis with different DNA sequences present in databases. The construction of a phylogenetic tree further supports the phylogenetic position of this strain, largely in accordance with morphological characterizations, and suggests that *Acinetobacter* spp. was closely related to *Acinetobacter lwoffii* A382 and *Acinetobacter johnsonii* ATCC 17979 (data not shown).

The second strain was collected from frozen continental water in Terre Adélie in Antarctica by C. Gerday, as previously mentioned (27). The morphological and biochemical characteristics of this strain, *e.g.* coccoid occurring in pairs, oxidase- and catalase-positive, nonmotile bacterium, and growth at low temperatures from 0 to 25 °C with an optimum growth temperature close to 15 °C, indicate that it could be classified either in *Moraxella* or *Psychrobacter* groups in agreement with recent data (36). Sequence analysis of the 16 S rDNA (accession number AJ310992) together with other characteristics of TAD1 bacterial strain such as a tolerance to 8% NaCl and a susceptibility to bile salt allowed us to refine the classification of this bacterium within the *Psychrobacter* genus and indicated that this strain could be related to *Psychrobacter* phenotypic group 2 (phenon 2), which is represented by *Psychrobacter uratovorans* (37).

Isolation and Characterization of Cold-adapted H-NS-like Proteins—To isolate a putative *hns*-like gene from both bacterial strains, we took advantage of the serine susceptibility of *hns* mutants in *E. coli* (38). A genomic library was constructed for both strains (see "Materials and Methods"), and each of them was introduced into the *hns E. coli* strain BE1410. The selection was performed on minimal medium supplemented with serine, as previously described (12, 15). Several clones were screened at 20 °C for 3 additional phenotypes, *i.e.* swarming on semi-solid medium, β-glucoside metabolism on MacConkey agar plate, and mucoidy on rich medium.

Analysis of the nucleotide sequence of different plasmid DNA inserts revealed the presence of a coding sequence of 321 bp, coding for a 107-amino acid protein with a predicted molecular mass of about 12 kDa and a pI of about 8 in both organisms. The analysis of flanking regions suggests that both genes are not part of a polycistronic operon. As compared with the *E. coli* H-NS, the modification in the amino acid composition, *e.g.* the proline content, of the *Psychrobacter* spp. orthologous protein (Table I) seemed to be similar to that commonly observed from mesophilic to psychrophilic proteins (39). Multiple alignment with various H-NS-related proteins revealed that both cold-adapted proteins share 40% amino acid identity in common; they also showed more than 30% identity with the H-NS-related proteins of *Rhodobacter* species, *i.e.* HvrA and SPB, and less than 20% with the *E. coli* H-NS amino acid sequence (Fig. 1). Despite this, the N-terminal part of these two new proteins was predicted to adopt an α-helical structure, like H-NS in *E. coli* (data not shown). Moreover, the *Acinetobacter* H-NS protein displayed the H-NS consensus motif, *i.e.* YX_6(G/S)-(E/D)$X_{(0/2)}$TW(T/S)G(Q/R)G(R/K)$XPX_{(4/5)}AX_{(3/4)}$G (0/2, 4/5, and 3/4 indicate 0 or 2, 4 or 5, and 3 or 4 residue(s), respectively) (15), whereas the (G/S) residue was replaced by an asparagine in the *Psychrobacter* protein (Fig. 1). Finally, using FROST, both C-terminal domains were clearly predicted to share a similar three-dimensional structure with the *E. coli* H-NS protein, with an error rate lower than 1% as indicated by the normalized distances obtained, *i.e.* 7.5 and 8.1, respectively, for the *Acinetobacter* and *Psychrobacter* proteins (34).

TABLE II
Effect of temperature on the in vivo complementation of H-NS deficiency in an E. coli mutant strain by hns genes of Acinetobacter spp. or Psychrobacter spp.

E. coli strain (relevant genotype)	β-Glucoside utilization[a]/mucoidy[b]		
	22 °C	30 °C	37 °C
FB8 (wild-type)	W/−	W/−	W/−
BE1410 (*hns-1001*)	R/+	R/+	R/+
BE1410/pDIA585 (*Acinetobacter hns*)	W/−	W/−	W/−
BE1410/pDIA572 (*Psychrobacter hns*)	W/−	P/+	R/+

[a] R, β-glucoside metabolism revealed by the appearance of red colonies on MacConkey-salicin agar plates; W, no metabolism by white colonies; P, intermediate metabolism by pink colonies.
[b] +, mucoid phenotype observed on MacConkey agar plates; −, no mucoid phenotype.

Effect of a Moderate Temperature Increase on in Vivo Biological Activity and in Vitro Biochemical Properties of the Psychrobacter H-NS Protein—Because of the low growth temperature optimum of both *Acinetobacter* spp. and *Psychrobacter* spp. strains, the ability of their H-NS-like proteins to complement phenotypes of *E. coli hns* mutant was evaluated at various temperatures. Remarkably, the overexpression of *Acinetobacter* protein fully restored, like the *E. coli* H-NS protein, a wild-type phenotype with regard to β-glucoside utilization and mucoidy at all temperatures tested, whereas the *Psychrobacter* H-NS protein was no longer able to reverse the effects of H-NS deficiency in an *E. coli* mutant strain above 30 °C (Table II). This loss of *in vivo* complementation observed above 30 °C did not result from the proteolysis of the protein. Indeed, the overexpressed H-NS protein of *Psychrobacter* was visualized on a polyacrylamide gel after extraction from *E. coli hns* cells grown at 37 °C (data not shown). This observation prompted us to

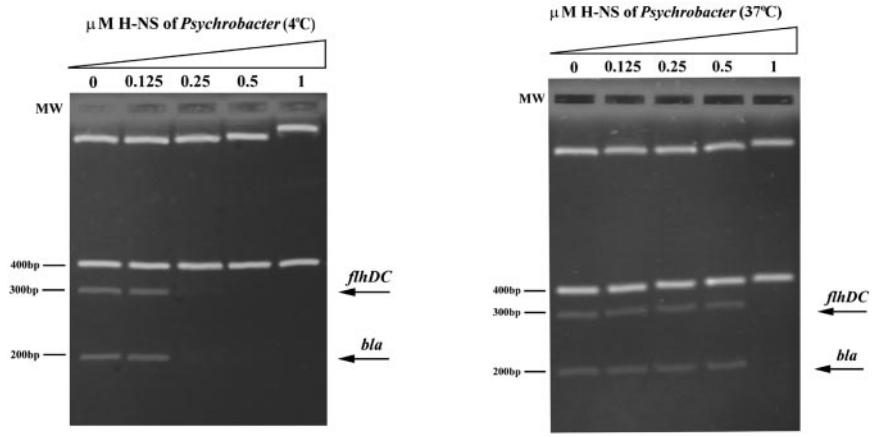

FIG. 2. **Effect of temperature on DNA binding evaluated by competitive gel retardation assay with the *Psychrobacter* H-NS His-6 protein and restriction fragments derived from plasmid pDIA525, which contains *flhDC* and *bla* promoter regions.** These DNA fragments were incubated with the indicated protein concentration at 4 and 37 °C.

further examine the biochemical properties of purified *Psychrobacter* H-NS protein at various temperatures.

One of the most important functions of H-NS-like proteins is their ability to bind curved DNA (18). Therefore, the binding properties of a recombinant His-6 *Psychrobacter* H-NS protein was analyzed at 4 and 37 °C in gel retardation experiments. As compared with the 400-bp DNA fragment used as a control, a full retardation in the electrophoretic mobility of both *flhDC* and *bla* curved DNA fragments was observed at 4 °C at a protein concentration of 0.25 μM (Fig. 2). No such retardation was detected at 37 °C at the same concentration (Fig. 2). Nevertheless, an increase in the concentration of the protein up to 1 μM resulted in a specific retardation of both curved DNA fragments at 37 °C (Fig. 2).

The H-NS activity also relies on its propensity to form oligomers (40). Therefore, the ability of the *Psychrobacter* H-NS protein to oligomerize *in vitro* was analyzed by cross-linking experiments at 4 and 37 °C (Fig. 3). After a 60-min incubation, the protein was able to form oligomers at 4 °C in the presence of linking reagents (Fig. 3). In contrast, only a residual amount of protein oligomerized as dimers at 37 °C. In addition, no higher order form was observed as compared with those visualized at 4 °C (Fig. 3).

The Low Thermal Stability of the Psychrobacter H-NS Protein Depends on the N-terminal Domain—The loss of *Psychrobacter* H-NS protein activity *in vivo* could result from an altered conformation during a moderate temperature increase. To evaluate the role of the different domains of the *Psychrobacter* protein in its thermosensitivity, we constructed different chimeric proteins with either the N- or the C-terminal part of *E. coli* H-NS fused to the C- or N-terminal part of the *Psychrobacter* protein (see "Materials and Methods"). To rule out any effect of temperature above 30 °C on *Psychrobacter hns* gene expression, all constructs were placed under the same transcriptional control of the *E. coli hns* promoter, giving rise to three plasmids (Table III) pDIA580 (producing the *Psychrobacter* wild-type H-NS protein), pDIA581 (producing a chimeric protein containing the N-terminal part of the *E. coli* H-NS and the C-terminal domain of the *Psychrobacter* protein), and pDIA582 (producing a chimeric protein containing the N-terminal part of the *Psychrobacter* protein and the C-terminal domain of the *E. coli* H-NS).

These plasmids were used in *in vivo* complementation experiments at two different temperatures, *i.e.* 25 and 37 °C. The synthesis of a chimeric protein containing the N-terminal part of the *Psychrobacter* H-NS protein (*i.e.* from plasmid pDIA582) in the *hns E. coli* mutant strain restored the wild-type phenotypes at 25 °C but not at 37 °C, as observed with the wild-type *Psychrobacter* protein from plasmid pDIA580 (Tables II and III). In contrast, the synthesis of a chimeric protein containing

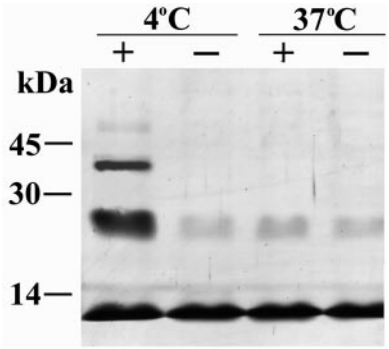

FIG. 3. **Effect of temperature on protein oligomerization evaluated by *in vitro* chemical cross-linking experiments using *Psychrobacter* His-6 protein.** After 1 h of incubation at 4 and 37 °C with (+) or without (−) 1-ethyl-3(3-dimethylaminopropyl)carbodiimide-*N*-hydroxysuccinimide cross-linking reagents, proteins were loaded onto a SDS, 14% acrylamide gel and silver-stained.

TABLE III

Effect of temperature on the in vivo complementation of H-NS deficiency in an E. coli hns strain by wild-type and hybrid hns genes

○, *E. coli hns* promoter; ■, *hns* gene of *E. coli*; □, *hns* gene of *Psychrobacter* spp.; ◧, gene encoding the N-terminal part of *E. coli* H-NS and the C-terminal part of *Psychrobacter* H-NS; ◨, gene encoding the N-terminal part of *Psychrobacter* H-NS and the C-terminal part of *E. coli* H-NS; R, β-glucoside metabolism revealed by the appearance of red colonies on MacConkey-salicin agar plates; W, no metabolism by white colonies.

		E. coli hns strain / plasmid			
		pDIA547	pDIA580	pDIA581	pDIA582
β-glucoside utilization	25°C	W	W	W	W
	37°C	W	R	W	R

the N-terminal part of the *E. coli* H-NS protein fused to the *Psychrobacter* C-terminal domain (*i.e.* from plasmid pDIA581) fully reversed the effects of H-NS deficiency in an *E. coli hns* mutant also at 37 °C (Table III).

Biophysical Properties of the Psychrobacter H-NS, a Thermosensitive Protein—The influence of an increase in temperature on the conformation of the *Psychrobacter* H-NS protein was evaluated by CD spectroscopy (41). Below 25 °C, the spectra of the protein revealed the presence of 1 positive maximum band at 190 nm and of 2 negative maxima bands at 208 and 222 nm, indicating the existence of a large proportion of α-helix structures (Fig. 4*A*). As a control, CD analyses of the *E. coli* H-NS

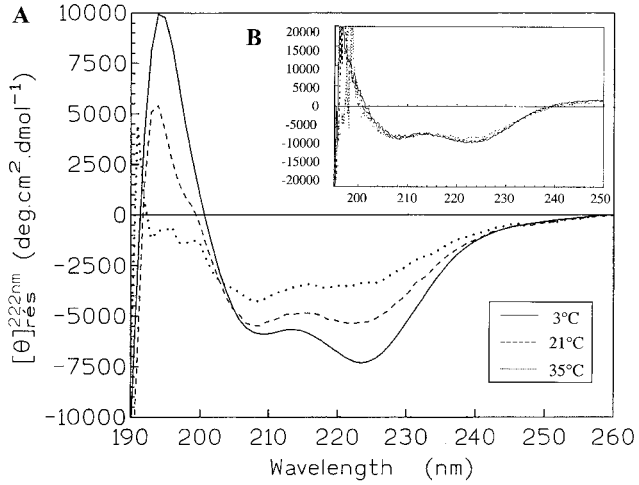

FIG. 4. **Effect of temperature on secondary structure of H-NS proteins visualized by CD spectroscopy.** *A*, CD spectra of the *Psychrobacter* H-NS obtained with 14 μM protein at different temperatures, *i.e.* 3 °C (*solid line*), 21 °C (*broken line*), and 35 °C (*dotted line*). *B*, given in the *inset* are the CD spectra at 3 °C (*solid line*), 21 °C (*broken line*), and 35 °C (*dotted line*) of the *Acinetobacter* H-NS protein.

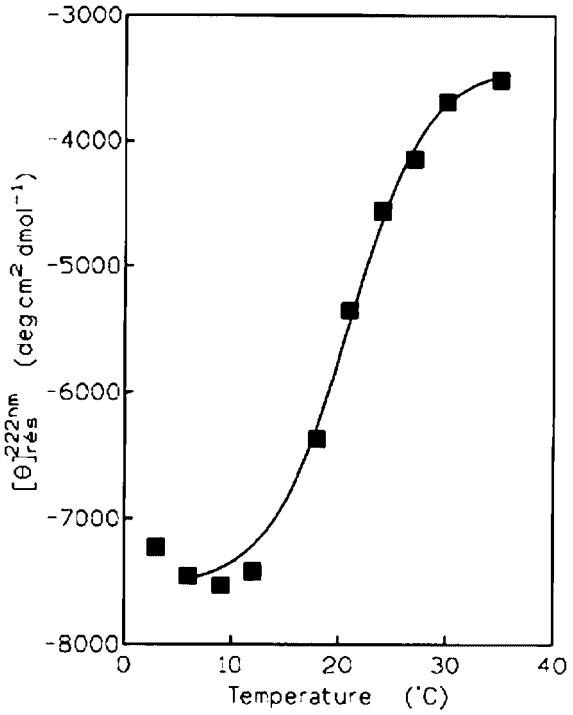

FIG. 5. **Sigmoid curve of the *Psychrobacter* H-NS resulting from CD spectra between 6 and 35 °C.** The curve allowed the determination of a T_m of 21 °C.

protein also revealed the existence of a large portion of α-helix structures (data not shown), which mainly reflect the secondary structure of its N-terminal part, in agreement with recent results (40). In contrast, unlike both the *E. coli* (data not shown) and the *Acinetobacter* (Fig. 4*B*) H-NS proteins, a moderate increase in temperature led to a complete change in CD profile of the *Psychrobacter* H-NS protein. The presence of an isodichroic point at 202 nm indicated a transition from the α-helix structures toward random coils (Fig. 4*A*). In addition, the CD profile did not revert to the native spectrum after a temperature down-shift from 50 to 20 °C, and after cooling the CD profile remained similar to that observed above 30 °C (Fig. 4*A*), providing evidence that, unlike *E. coli* H-NS (40, 42) and *Acinetobacter* H-NS (Fig. 4*B*), the perturbation observed was irreversible. Finally, from the sigmoidal shape of the denaturation curve resulting from the CD spectra, we could determine a melting temperature (T_m) of 21 °C, which further supports the low thermal stability of the *Psychrobacter* H-NS protein (Fig. 5).

DISCUSSION

Many adaptations in the structure and the physiology of organisms are required to sustain growth in cold environments (3). Although the properties of psychrophilic enzymes have been well studied at the molecular level (4), almost no information is available about nucleoid-associated proteins from psychrophilic organisms. In this work, we isolated and characterized two *hns* genes from two cold-adapted bacteria, *i.e. Acinetobacter* spp. and *Psychrobacter* spp. Each gene codes for a 107-amino acid protein with more than 30% identity with the H-NS-like proteins of *Rhodobacter* species, *i.e.* HvrA and SPB, and less than 20% with the *E. coli* H-NS amino acid sequence (Fig. 1). Nevertheless, *in silico* analysis suggests that both *Acinetobacter* and *Psychrobacter* proteins display the typical structural and functional organization in two modules observed in other H-NS-related proteins (14, 15, 19). Indeed, the N-terminal part is predicted to mainly adopt an α-helix conformation, whereas as predicted by the fold recognition method FROST, the C-terminal domain shares a similar three-dimensional structure with the *E. coli* H-NS protein resolved by NMR (see "Results") (17). These predictions were substantiated by the ability of both proteins to complement *hns* phenotypes in a *hns*-defective *E. coli* mutant (Table II), including a restoration of motility although *Acinetobacter* spp. and *Psychrobacter* spp. strains are non-motile (data not shown). These results provide evidence that the two proteins identified in this work belong to the H-NS family and are the first members of this family isolated from cold-adapted bacteria. In contrast, no H-NS-related protein has been so far identified in hyperthermophiles bacteria whose genome has been recently sequenced (igweb.integratedgenomics.com/GOLD).

The catalytic activity of psychrophilic enzymes is known to decrease dramatically as the temperature increases (4). For instance, the activity of a α-amylase enzyme secreted by the Antarctic bacterial strain *Alteromonas haloplanctis* decreases above 25 °C because of a low thermal stability, whereas a homologous protein of pig pancreas displays an optimum activity at 60 °C (43). This low thermal stability in psychrophilic enzymes, which is often considered as a consequence of higher flexibility and catalytic activity, results from a decrease in electrostatic and hydrophobic interactions (5, 44). Usually various modifications are observed when psychrophilic proteins are compared with their mesophilic counterparts, such as a decrease of charged residues, a substitution of hydrophobic residues, *e.g.* Ala instead of Val, or a decrease in proline content (39). In this respect, as compared with the *E. coli* H-NS protein, the aspartate and valine content was decreased in the *Psychrobacter* H-NS protein sequence (Table I). Recent results show that the substitution of two alanines by two threonines and one alanine by one valine could create a psychrophilic-like subtilisin from a mesophilic enzyme (45). Interestingly, the threonine content increases in the *Psychrobacter* H-NS protein as compared with the orthologous protein in *E. coli*. In addition, the asparagine content, which is considered as a thermolabile residue (46), is increased in the *Psychrobacter* protein. No such differences were observed in the sequence of the *Acinetobacter* H-NS protein (Table I). Finally, the *Psychrobacter* H-NS protein has the lowest proline content, *i.e.* one proline residue (Fig. 1), as compared with all H-NS-related proteins identified so far. This proline residue, which is located in the C-terminal do-

main, corresponds to the Pro-115 residue in *E. coli*. In this organism, this proline has been suggested to play a crucial role in oligomer formation despite its location in the DNA binding domain (47). Although this proline residue could also be involved in the oligomerization of other H-NS proteins, its presence is not sufficient to confer a thermal stability to the H-NS protein of *Psychrobacter*. In this respect, we were not able to create a mesophilic-like protein from H-NS of *Psychrobacter* spp. by random mutagenesis experiments,[2] suggesting that more than one amino acid modification is involved in its structural adaptation to low temperature.

The *Acinetobacter* H-NS protein fully restored to the wild type H-NS-dependent phenotypes at all temperatures tested although the *Psychrobacter* H-NS protein was no longer able to reverse the effects of H-NS deficiency in an *E. coli* mutant strain above 30 °C (Table II). Moreover, the *Psychrobacter* H-NS protein could no longer bind curved DNA fragments at 37 °C excepted at a concentration of 1 μM (Fig. 2). In contrast, no oligomer form could be visualized at the same temperature even with a protein concentration of 25 μM (Fig. 3). This suggests that, like the H-NS protein of *E. coli* (48), the *Psychrobacter* protein could bind curved DNA as a monomer although with a lower efficiency. More importantly, these observations suggest that the effect of an increase in temperature results in an alteration of the conformation of the N-terminal domain. The construction of chimeric proteins between the H-NS protein of *E. coli* and the orthologous protein of *Psychrobacter* supports this hypothesis. Indeed, the protein containing the N-terminal part of the *Psychrobacter* H-NS combined with the C-terminal part of the *E. coli* H-NS restored the wild-type phenotypes at 25 °C but not at 37 °C (Table III). In contrast, the protein containing the N-terminal part of *E. coli* H-NS fused to the C-terminal part of the *Psychrobacter* protein reversed the effects of H-NS deficiency in an *hns E. coli* mutant strain at 37 °C (Table III). These results clearly demonstrated that the organization of N-terminal domain of the *Psychrobacter* protein may account for the low thermal stability of this H-NS-like protein.

In *S. typhimurium*, the N-terminal domain of H-NS has been recently shown to contain three α-helical regions (16). The authors proposed a model of global topological fold for the N-terminal domain; the third and longest α-helix forms the core of a coiled-coil configuration, whereas the two others stabilize the structure (16). Secondary structure prediction methods clearly suggest that the *Psychrobacter* H-NS protein characterized in the present study contain less than three α-helical regions (data not shown). It is, therefore, tempting to speculate that the amino acid modifications and the different α-helix organization observed in its N-terminal domain as compared with that of the orthologous *E. coli* protein reflect a structural adaptation to low temperature of the *Psychrobacter* H-NS protein. These features might, therefore, offer weaker intermolecular interactions as compared with other H-NS proteins such as those of *Acinetobacter* and *E. coli* (or *S. typhimurium*). CD analyses performed with the *Psychrobacter* H-NS protein in comparison with the *E. coli* and *Acinetobacter* H-NS proteins support this hypothesis (Fig. 4). Indeed, unlike the *E. coli* protein, which has been isolated more than 30 years ago as a heat-stable factor (49), and the *Acinetobacter* H-NS protein (Fig. 4*B*), the α-helical structure of the *Psychrobacter* protein was irreversibly denatured upon a moderate temperature increase (Fig. 4*A*). In addition, although the T_m of the *E. coli* H-NS protein was estimated around 60 °C (40, 42) and that of *Acinetobacter* H-NS around 40 °C (data not shown), we could clearly determine the T_m of the *Psychrobacter* H-NS protein, *i.e.* 21 °C (Fig. 5). This T_m value is significantly lower than that of both *E. coli* and *Acinetobacter* H-NS proteins and may explain the temperature effect observed *in vitro* and *in vivo* with the *Psychrobacter* H-NS protein. A moderate temperature increase could, therefore, easily disassociate oligomers with a subsequent and/or concomitant irreversible denaturation of the protein, as observed by CD analysis. We can hypothesize from these results that the organization of α-helix structures in the N-terminal domain is crucial for the structure and the function of H-NS-related proteins. Hence, the identification and characterization of proteins from micro-organisms phylogenetically distant and/or with very different live style, including extremophiles, may lead to a better understanding of the structure, function and evolution relationship of this family of enigmatic proteins.

Acknowledgments—*Psychrobacter* TAD1 strain was kindly provided by Dr. C. Gerday. We are thankful to V. V. Parfenova for providing us with the Baikal bacterial strain and to G. Feller for helpful advice. We are indebted to M. Monot, S. Fermandjian, and M. Nicaise for CD experiments and analyses. We are grateful to J. Potier for helpful advice concerning the protein structure analyses. We thank K. Sun and F. Hommais for technical assistance.

REFERENCES

1. Margesin, R., and Schinner, F. (1994) *J. Biotechnol.* **33,** 1–14
2. Thieringer, H. A., Jones, P. G., and Inouye, M. (1998) *Bioessays* **20,** 49–57
3. Gounot, A. M. (1991) *J. Appl. Bacteriol.* **71,** 386–397
4. Gerday, C., Aittaleb, M., Bentahir, M., Chessa, J. P., Claverie, P., Collins, T., D'Amico, S., Dumont, J., Garsoux, G., Georlette, D., Hoyoux, A., Lonhienne, T., Meuwis, M. A., and Feller, G. (2000) *Trends Biotechnol.* **18,** 103–107
5. Feller, G., and Gerday, C. (1997) *Cell. Mol. Life Sci.* **53,** 830–841
6. Berger, F., Morellet, N., Menu, F., and Potier, P. (1996) *J. Bacteriol.* **178,** 2999–3007
7. Michel, V., Lehoux, I., Depret, G., Anglade, P., Labadie, J., and Hebraud, M. (1997) *J. Bacteriol.* **179,** 7331–7342
8. Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T., Prentice, M. B., Sebaihia, M., James, K. D., Churcher, C., Mungall, K. L., Baker, S., Basham, D., Bentley, S. D., Brooks, K., Cerdeno-Tarraga, A. M., Chillingworth, T., Cronin, A., Davies, R. M., Davis, P., Dougan, G., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Karlyshev, A. V., Leather, S., Moule, S., Oyston, P. C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., and Barrell, B. G. (2001) *Nature* **413,** 523–527
9. May, B. J., Zhang, Q., Li, L. L., Paustian, M. L., Whittam, T. S., and Kapur, V. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 3460–3465
10. Simpson, A. J., Reinach, F. C., Arruda, P., Abreu, F. A., Acencio, M., Alvarenga, R., Alves, L. M., Araya, J. E., Baia, G. S., Baptista, C. S., Barros, M. H., Bonaccorsi, E. D., Bordin, S., Bove, J. M., Briones, M. R., Bueno, M. R., Camargo, A. A., Camargo, L. E., Carraro, D. M., Carrer, H., Colauto, N. B., Colombo, C., Costa, F. F., Costa, M. C., Costa-Neto, C. M., Coutinho, L. L., Cristofani, M., Dias-Neto, E., Docena, C., El-Dorry, H., Facincani, A. P., Ferreira, A. J., Ferreira, V. C., Ferro, J. A., Fraga, J. S., Franca, S. C., Franco, M. C., Frohme, M., Furlan, L. R., Garnier, M., Goldman, G. H., Goldman, M. H., Gomes, S. L., Gruber, A., Ho, P. L., Hoheisel, J. D., Junqueira, M. L., Kemper, E. L., Kitajima, J. P., Krieger, J. E., Kuramae, E. E., Laigret, F., Lambais, M. R., Leite, L. C., Lemos, E. G., Lemos, M. V., Lopes, S. A., Lopes, C. R., Machado, J. A., Machado, M. A., Madeira, A. M., Madeira, H. M., Marino, C. L., Marques, M. V., Martins, E. A., Martins, E. M., Matsukuma, A. Y., Menck, C. F., Miracca, E. C., Miyaki, C. Y., Monteriro-Vitorello, C. B., Moon, D. H., Nagai, M. A., Nascimento, A. L., Netto, L. E., Nhani, A., Jr., Nobrega, F. G., Nunes, L. R., Oliveira, M. A., de Oliveira, M. C., de Oliveira, R. C., Palmieri, D. A., Paris, A., Peixoto, B. R., Pereira, G. A., Pereira, H. A., Jr., Pesquero, J. B., Quaggio, R. B., Roberto, P. G., Rodrigues, V., de, M. R. A. J., de Rosa, V. E., Jr., de Sa, R. G., Santelli, R. V., Sawasaki, H. E., da Silva, A. C., da Silva, A. M., da Silva, F. R., da Silva, W. A., Jr., da Silveira, J. F., et al. (2000) *Nature* **406,** 151–157
11. Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J. C., Cattolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thebault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J., and Boucher, C. A. (2002) *Nature* **415,** 497–502
12. Tendeng, C., Badaut, C., Krin, E., Gounon, P., Ngo, S., Danchin, A., Rimsky, S., and Bertin, P. (2000) *J. Bacteriol.* **182,** 2026–2032
13. Goyard, S., and Bertin, P. (1997) *Mol. Microbiol.* **24,** 815–823
14. Dorman, C. J., Hinton, J. C., and Free, A. (1999) *Trends Microbiol.* **7,** 124–128
15. Bertin, P., Hommais, F., Krin, E., Soutourina, O., Tendeng, C., Derzelle, S., and Danchin, A. (2001) *Biochimie* (*Paris*) **83,** 235–241
16. Renzoni, D., Esposito, D., Pfuhl, M., Hinton, J. C. D., Higgins, C. F., Driscoll, P. C., and Ladbury, J. E. (2001) *J. Mol. Biol.* **306,** 1127–1137
17. Shindo, H., Iwaki, T., Ieda, R., Kurumizaka, H., Ueguchi, C., Mizuno, T., Morikawa, S., Nakamura, H., and Kuboniwa, H. (1995) *FEBS Lett.* **360,** 125–131

[2] C. Tendeng, E. Krin, O. A. Soutourina, A. Marin, A. Danchin, and P. N. Bertin, unpublished results.

18. Williams, R. M., and Rimsky, S. (1997) *FEMS Microbiol. Lett.* **156,** 175–185
19. Bertin, P., Benhabiles, N., Krin, E., Laurent-Winter, C., Tendeng, C., Turlin, E., Thomas, A., Danchin, A., and Brasseur, R. (1999) *Mol. Microbiol.* **31,** 319–330
20. Schroder, O., and Wagner, R. (2002) *Biol. Chem.* **383,** 945–960
21. Hommais, F., Krin, E., Laurent-Winter, C., Soutourina, O., Malpertuy, A., Le Caer, J. P., Danchin, A., and Bertin, P. (2001) *Mol. Microbiol.* **40,** 20–36
22. De Biase, D., Tramonti, A., Bossa, F., and Visca, P. (1999) *Mol. Microbiol.* **32,** 1198–1211
23. Soutourina, O., Kolb, A., Krin, E., Laurent-Winter, C., Rimsky, S., Danchin, A., and Bertin, P. (1999) *J. Bacteriol.* **181,** 7500–7508
24. Soutourina, O. A., Krin, E., Laurent-Winter, C., Hommais, F., Danchin, A., and Bertin, P. N. (2002) *Microbiology* **148,** 1543–1551
25. La Teana, A., Brandi, A., Falconi, M., Spurio, R., Pon, C. L., and Gualerzi, C. O. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 10907–10911
26. Dersch, P., Kneip, S., and Bremer, E. (1994) *Mol. Gen. Genet.* **245,** 255–259
27. Sun, K., Camardella, L., Di Prisco, G., and Herve, G. (1998) *FEMS Microbiol. Lett.* **164,** 375–382
28. Bruni, C. B., Colantuoni, V., Sbordone, L., Cortese, R., and Blasi, F. (1977) *J. Bacteriol.* **130,** 4–10
29. Laurent-Winter, C., Ngo, S., Danchin, A., and Bertin, P. (1997) *Eur. J. Biochem.* **244,** 767–773
30. Krin, E., Hommais, F., Soutourina, O., Ngo, S., Danchin, A., and Bertin, P. (2001) *FEMS Microbiol. Lett.* **199,** 229–233
31. Miller, J. H. (1972) *Experiments in Molecular Genetics*, p. 456, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
32. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
33. Frishman, D., and Argos, P. (1996) *Protein Eng.* **9,** 133–142
34. Marin, A., Pothier, J., Zimmermann, K., and Gibrat, J. F. (2002) in *Protein Stucture Prediction; Bioinformatic Approach* (Tsigelny, I., ed) pp. 227–262, International University Line, La Jolla, CA
35. Soutourina, O. A., Semenova, E. A., Parfenova, V. V., Danchin, A., and Bertin, P. (2001) *Appl. Environ. Microbiol.* **67,** 3852–3859
36. Di Fraia, R., Wilquet, V., Ciardiello, M. A., Carratore, V., Antignani, A., Camardella, L., Glansdorff, N., and di Prisco, G. (2000) *Eur. J. Biochem.* **267,** 121–131
37. Cavanagh, J., Austin, J. J., and Sanderson, K. (1996) *Int. J. Syst. Bacteriol.* **46,** 841–848
38. Lejeune, P., Bertin, P., Walon, C., Willemot, K., Colson, C., and Danchin, A. (1989) *Mol. Gen. Genet.* **218,** 361–363
39. Gianese, G., Argos, P., and Pascarella, S. (2001) *Protein Eng.* **14,** 141–148
40. Smyth, C. P., Lundback, T., Renzoni, D., Siligardi, G., Beavil, R., Layton, M., Sidebotham, J. M., Hinton, J. C., Driscoll, P. C., Higgins, C. F., and Ladbury, J. E. (2000) *Mol. Microbiol.* **36,** 962–972
41. Pelton, J. T., and McLean, L. R. (2000) *Anal. Biochem.* **277,** 167–176
42. Tippner, D., and Wagner, R. (1995) *J. Biol. Chem.* **270,** 22243–22247
43. Feller, G., Narinx, E., Arpigny, J. L., Aittaleb, M., Baise, E., Genicot, S., and Gerday, C. (1996) *FEMS Microbiol. Rev.* **18,** 189–202
44. Gerday, C., Aittaleb, M., Arpigny, J. L., Baise, E., Chessa, J. P., Garsoux, G., Petrescu, I., and Feller, G. (1997) *Biochim. Biophys. Acta* **1342,** 119–131
45. Taguchi, S., Ozaki, A., Nonaka, T., Mitsui, Y., and Momose, H. (1999) *J. Biochem. (Tokyo)* **126,** 689–693
46. Xu, Y., Zhang, Y., Liang, Z., Van de Casteele, M., Legrain, C., and Glansdorff, N. (1998) *Microbiology* **144,** 1435–1441
47. Spurio, R., Falconi, M., Brandi, A., Pon, C. L., and Gualerzi, C. O. (1997) *EMBO J.* **16,** 1795–1805
48. Shindo, H., Ohnuki, A., Ginba, H., Katoh, E., Ueguchi, C., Mizuno, T., and Yamazaki, T. (1999) *FEBS Lett.* **455,** 63–69
49. Jacquet, M., Cukier-Kahn, R., Pla, J., and Gros, F. (1971) *Biochem. Biophys. Res. Commun.* **45,** 1597–1607