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Arginine Operator Binding by Heterologous and Chimeric ArgR Repressors from *Escherichia coli* and *Bacillus stearothermophilus*

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***Bacillus stearothermophilus* ArgR binds efficiently to the *Escherichia coli carAB* operator, whereas the *E. coli* repressor binds very poorly to the *argCo* operator of *B. stearothermophilus*. In order to elucidate this contradictory behavior between ArgRs, we constructed chimeric proteins by swapping N-terminal DNA-binding and C-terminal oligomerization domains or by exchanging the linker peptide. Chimeras carrying the *E. coli* DNA-binding domain and the *B. stearothermophilus* oligomerization domain showed sequence-nonspecific rather than sequence-specific interactions with *arg* operators. Chimeras carrying the *B. stearothermophilus* DNA-binding domain and *E. coli* oligomerization domain exhibited a high DNA-binding affinity for the *B. stearothermophilus argCo* and *E. coli carAB* operators and repressed the reporter-gene transcription from the *B. stearothermophilus* P*argCo* control region in vitro; arginine had no effect on, and indeed even decreased, their DNA-binding affinity. With the protein array method, we showed that the wild-type *B. stearothermophilus* ArgR and derivatives of it containing only the exchanged linker from *E. coli* ArgR or carrying the *B. stearothermophilus* DNA-binding domain along with the linker and the α4 regions were able to bind *argCo* containing the single Arg box. This binding was weaker than binding to the two-box operator but was no longer arginine dependent. Several lines of observations indicate that the α4 helix in the oligomerization domain and the linker peptide can contribute to the recognition of single or double Arg boxes and therefore to the operator DNA-binding specificity in similar but not identical ArgR repressors from two distant bacteria.**

Recent genome comparative analysis has revealed a global vision of *arg* genes transcription regulation in microorganisms (3, 23, 26, 30). However, experimental studies on distant organisms are required to elucidate the molecular mechanisms and to understand the evolutionary principles established for arginine regulatory systems in various microbes.

A substantial amount of functional and structural information regarding transcription regulation has accumulated for the *Escherichia coli* arginine repressor, ArgR (14, 22, 40). In this organism, the repressor, in cooperation with L-arginine, governs expression of the arginine biosynthesis genes (*arg*) and *carAB* genes, coding for carbamoylphosphate synthetase (EC 6.3.5.5), providing the carbamoylphosphate required for the synthesis of both arginine and pyrimidine residues. The ArgR protein consists of N-terminal DNA-binding and C-terminal oligomerization domains connected by a short protease-sensitive linker (15), and a three-dimensional structure has been separately resolved for each domain (41, 45). A winged helix-turn-helix (wHTH) motif of the DNA-binding domain (41) recognizes a 40-bp sequence which comprises two adjacent imperfect 18-bp palindromes, known as Arg boxes, that are separated by a 3-bp spacer in the majority of cognate operators or by a 2-bp spacer in the *argR* operator (6, 44, 46). ArgR monomers associate spontaneously to form trimers, and arginine, as a ligand, binds distinct amino acids located within the oligomerization domain and provides the transition of two identical apo-trimers to a holohexameric molecule (45).

Transcription of the arginine biosynthesis genes is also repressed in the moderate thermophilic bacterium *Bacillus stearothermophilus* (32, 47). The ArgR repressor binds a 42-bp operator comprising two Arg box-like sequences separated by a 2-bp spacer and overlapping the P*argC* promoter located upstream of the *argCJBD* operon (10, 35). The three-dimensional structure of a full-length aporepressor and arginine-bound C-terminal domain of *B. stearothermophilus* ArgR has recently been resolved (28). The DNA-binding and oligomerization domains of the *E. coli* and *B. stearothermophilus* repressors adapt similar folds despite the fact that these proteins show only 27% amino acid sequence similarity. Furthermore, arginine binding to distinct amino acids (four of the six residues involved are conserved in the oligomerization domain of both ArgR proteins; Fig. 1) leads to allosteric changes in both hexameric repressors (28, 41, 45), thereby increasing their *arg* operator DNA-binding affinity (5, 6, 8, 10, 21, 42, 43, 46). It has been shown that binding six arginine molecules, in addition to reinforcing interactions between monomers within a trimer

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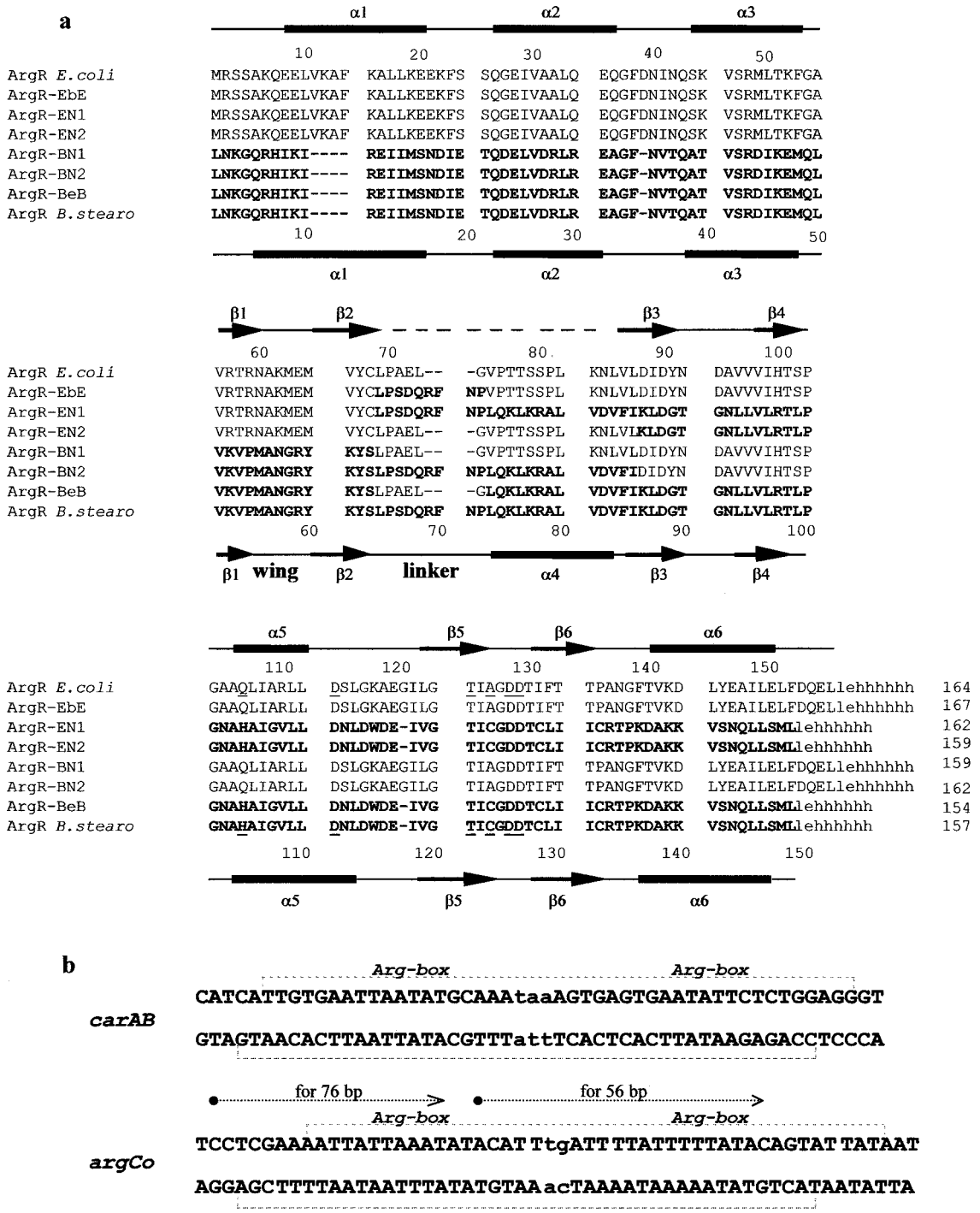


FIG. 1. Amino acid sequences of wild-type and domain- and linker-exchanged chimeric proteins constructed from *E. coli* and *B. stearothermophilus* ArgR repressors (a) and nucleotide sequences of *E. coli carAB* and *B. stearothermophilus argCo* operators (b). The amino acid sequence for *E. coli* is shown in lightface, and that for *B. stearothermophilus* is shown in boldface. Secondary-structure features of the wild-type ArgR repressors from *E. coli* (41, 45) and *B. stearothermophilus* (28) are shown above and below the corresponding sequences, respectively. The DNA-binding (amino acids 1 to 64) and oligomerization (amino acids 73 to 149) domains of *B. stearothermophilus* ArgR are connected by an 8-amino-acid-long linker peptide (28). The structure between the β2 and β3 sheets has not yet been determined for *E. coli* ArgR. The amino acid residues involved in arginine binding are underlined in the wild-type repressor sequences. The C-terminal His tag is shown in lowercase letters. Dashed brackets indicate operator sequences protected against DNase I cleavage upon wild-type repressor binding (35, 46). Spacer nucleotides between two Arg boxes are shown in lowercase letters. Dashed-line arrows indicate the sites for amplification of the *argCo* operator DNAs with double (76-bp) and single (56-bp) Arg boxes.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevent genotype or description[a]	Reference or source
E. coli		
Top 10	F′ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
BL21(DE3)	*hsdS gal* (λcIts857 *ind1 S*Am7 *nin-5 lac*UV5-T7 gene 1)	Novagen
B. stearothermophilus NCIB 8224	Wild type	31
Plasmids		
pHAV2	*bla*, pBTac2 carrying *B. stearothermophilus* P*argCo-argC* region	33
pET21d+	*bla*, T7 promoter expression vector	Novagen
pET-ArgR*Ec*	pET21d+ carrying wild-type *E. coli argR*	This work
pET-ArgR*Bst*	pET21d+ carrying wild-type *B. stearothermophilus argR*	This work
pET-ArgR-EN1	As pET-ArgR*Bst* but carrying *argR*-EN1	This work
pET-ArgR-EN2	As pET-ArgR*Bst* but carrying *argR*-EN2	This work
pET-ArgR-BN1	As pET-ArgR*Bst* but carrying *argR*-BN1	This work
pET-ArgR-BN2	As pET-ArgR*Bst* but carrying *argR*-BN2	This work
pET-ArgR-EbE	As pET-ArgR*Bst* but carrying *argR*-EbE	This work
pET-ArgR-BeB	As pET-ArgR*Bst* but carrying *argR*-BeB	This work
pCR-Blunt	*kan*, PCR cloning vector	Invitrogen
pCR-ArgR*Ec*	pCR-Blunt carrying wild-type *E. coli argR* transcribed from P*lac*	This work
pCR-ArgR*Bst*	pCR-Blunt carrying wild-type *B. stearothermophilus argR* transcribed from P*lac*	19
pCR-ArgR-EN1	As pCR-ArgR*Bst* but carrying *argR*-EN1	This work
pCR-ArgR-EN2	As pCR-ArgR*Bst* but carrying *argR*-EN2	This work
pCR-ArgR-BN1	As pCR-ArgR*Bst* but carrying *argR*-BN1	This work
pCR-ArgR-BN2	As pCR-ArgR*Bst* but carrying *argR*-BN2	This work
pCR-ArgR-EbE	As pCR-ArgR*Bst* but carrying *argR*-EbE	This work
pCR-ArgR-BeB	As pCR-ArgR*Bst* but carrying *argR*-BeB	This work

[a] Wild-type and chimeric proteins were fused in frame with a C-terminal His tag.

and between trimers within a hexamer, also provokes a 15° rotation of two of the trimers with respect to each other (28). Such an allosteric modification appears to expose four of the six wHTH modules properly with respect to four halves of both Arg box palindromes and therefore improve the operator-binding affinity (28).

The similarity of protein and operator organization suggests a common DNA recognition mechanism for the mesophilic *E. coli* and thermophilic *B. stearothermophilus* ArgR repressors. There is, however, a major difference between the DNA-binding properties of the two ArgR proteins. The *E. coli* repressor has a very low affinity for the *argCo* operator of *B. stearothermophilus* in vitro and is practically unable to repress transcription from the *B. stearothermophilus* P*argCo* promoter-operator region in *E. coli* host cells (32, 33). On the contrary, the *B. stearothermophilus* repressor binds *E. coli* operators with high efficiency (19, 32, 35). Furthermore, the overexpressed wild-type *B. stearothermophilus* ArgR repressor, unlike the *E. coli* protein (44), behaves as a superrepressor in *E. coli* host cells, which become auxotrophic for arginine due to repression of arginine and probably other biosynthesis genes, suggesting that it is unable to recognize a wider range of operator sequences (19).

In an effort to understand why two similar ArgR proteins possess divergent DNA-binding specificities, we constructed chimeric proteins comprising various regions of the *E. coli* and *B. stearothermophilus* repressors and analyzed their DNA-binding properties. We show that, depending on the presence of a substituted domain or a linker peptide, ArgR chimeric proteins differ in their ability to bind to cognate *arg* operators, in solubility, and in electrophoretic migration. Our data indicate that the *B. stearothermophilus* repressor is able to bind to the *argCo* operator comprising a single Arg box and that the α4 helix in the oligomerization domain is a major structural determinant contributing to the operator-binding specificity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. The strain *B. stearothermophilus* NCIB 8224 was grown with aeration at 60°C in a liquid Luria-Bertani (LB) medium (24). *E. coli* strains were grown with aeration at 28°C or 37°C on LB medium with appropriate antibiotics: ampicillin, 100 μg/ml, or kanamycin, 25 μg/ml.

To express His-tagged wild-type and chimeric ArgR proteins, the protease-deficient *E. coli* BL21(DE3) strain carrying a plasmid with a wild-type or chimeric *argR* gene was grown in LB with ampicillin at 28°C to an A_{600} of 0.8. Synthesis of T7 RNA polymerase in this strain was induced by addition of isopropylthiogalactopyranoside (IPTG) at a final concentration of 1 mM. The cells were harvested 3 h later by centrifugation and used for protein purification. Liquid M9 medium (24) was used to assess the arginine auxotrophy of *E. coli* strain Top10 harboring a pCR-Blunt vector derivative carrying a wild-type or chimeric *argR* gene by measuring the optical density of corresponding cultures after a 48-h incubation at 37°C.

Construction of recombinant DNAs and DNA labeling by PCR. The DNA-binding and oligomerization domains of ArgR are separated by an 8- and 5-amino-acid-long linker peptide in the *B. stearothermophilus* and *E. coli* repressors, respectively. Four domain-exchanged chimeric proteins were constructed in which the DNA-binding domain with the corresponding linker was taken from the same wild-type repressor or the linker peptide and oligomerization domain were taken from the same repressor (see Fig. 1).

The chimeric *argR* genes were constructed in two consecutive PCR steps by the overlap extension method (16). At the first PCR step, two separate DNA fragments corresponding to the N-terminal and C-terminal domains of the ArgR repressors were amplified on a chromosomal DNA template from the *B. stearothermophilus* NCIB 8224 and *E. coli* XA4 strains by the creation of overlapping sequences at expected junctions. The amplified DNA fragments were then combined in a subsequent fusion PCR, and the full-length *argR* hybrid DNAs were obtained with only two flanking primers, carrying in addition an *Nco*I or *Xho*I restriction site.

The oligonucleotide primer sequences used are described in Table 2. The amplified DNA fragments were treated with *Nco*I and *Xho*I and inserted into the pET21d(+) vector digested with the same enzymes in order to create a hybrid

TABLE 2. Oligonucleotide primers used for cloning or construction of wild-type and chimeric *argR* genes

Primer	Sequence	Protein(s) encoded by construction
Nco-Ec	5′CATGCCATGGGAAGCTCGGCTAAG	ArgR*Ec*, ArgR-EN1, ArgR-EN2
Xho-Ec	5′GTGCTCGAGGAGCTCCTGGTCGAACAG	ArgR*Ec*, ArgR-BN1, ArgR-BN2
NcoI-Bst	5′CATGCCATGGACAAAGGGCAAAGGCA	ArgR*Bst*, ArgR-BN1, ArgR-BN2
XhoI-Bst	5′GTGCTCGAGGAGCATGGACAGCAGCTGG	ArgR*Bst*, ArgR-EN1, ArgR-EN2
Oli 1	5′CTTCCGTCCGATCAGCG	ArgR-EN1
Oli 2	5′AAGCTTGACGGAACCG	ArgR-EN2
Oli 3	5′CTGCCAGCTGAACTG	ArgR-BN1
Oli 4	5′GATATCGACTACAACGA	ArgR-BN2
Cys68Leu	5′CGCTGATCGGACGGAAGGCAGTAAACCAT	ArgR-EN1
L87K86	5′CGGTTCCGTCAAGCTTCAGCACCAGATTCTTC	ArgR-EN2
S63L69	5′CCCAGTTCAGCTGGCAGGCTGTACTTATAG	ArgR-BN1
185D88	5′CATCGTTGTAGTCGATATCAATGAACACGTCAACGA	ArgR-BN2
EN1-BN1[a]	5′-CAGCGCTTCAACCCGGTACCAACCACCTCCAGTCC	ArgR-EbE
BN1-EN1[b]	5′-CCAGCTGAACTGGGTCTGCAAAAACTGAAGCGG	ArgR-BeB
BN1-down	5′-ACCCAGTTCAGCTGGCAG	ArgR-BeB
EN1-down	5′-CGGGTTGAAGCGCTGATC	ArgR-EbE
UP-pET	5′-CACCATACCCACGCCGAAAC	ArgR-EbE, ArgR-BeB
Down-pET	5′-ATCCGGATATAGTTCCTCC	ArgR-EbE, ArgR-BeB

[a] pArgR-BN1 DNA was used as a template for PCR.
[b] pArgR-EN1 DNA was used as a template for PCR.

argR gene fused in frame with six histidine codons (C-terminal His tag). The resulting ArgR chimeric proteins (see Fig. 1) contained (i) ArgR-EN1, a 68-amino-acid N-terminal portion fused to the next C-terminal 64 to 149 amino acids from the *E. coli* and *B. stearothermophilus* repressors, respectively; (ii) ArgR-EN2, an 87-amino-acid N-terminal portion fused to the next C-terminal 86 to 149 amino acids from the *E. coli* and *B. stearothermophilus* repressors, respectively; (iii) ArgR-BN1, a 63-amino-acid N-terminal portion fused to the next C-terminal 69 to 156 amino acids from the *B. stearothermophilus* and *E. coli* repressors, respectively; and (iv) ArgR-BN2, an 85-amino-acid N-terminal portion fused to the next C-terminal 88 to 156 amino acids from the *B. stearothermophilus* and *E. coli* repressors, respectively.

Two other chimeric derivatives, ArgR-EbE and ArgR-BeB, were also constructed by mutually replacing the linker peptide in the wild-type *E. coli* and *B. stearothermophilus* His-tagged ArgR repressors (see Fig. 1). The pArgR-EN1 and pArgR-BN1 plasmids carrying chimeric *argR* genes and wild-type chromosomal *argR* genes were used as templates in these PCR amplifications with the corresponding oligonucleotides (Table 3).

For the mobility shift assay, two DNA fragments were synthesized: a 137-bp DNA fragment carrying the *argC* operator from *B. stearothermophilus* NCIB 8224 was amplified with nonlabeled 5′-GGCTGCCGGGACAAATCGG and digoxigenin-labeled 5′-CCCGTATGCCTCATGTAG oligonucleotide primers, and a 141-bp DNA fragment carrying the *carAB* operator (46) from *E. coli* XA4 was amplified with nonlabeled 5′-ACGTCATCATTGTGAATTAA and digoxigenin-labeled 5′-CAACCGCCGAACCTGTTG primers. The same operator DNA was labeled at a downstream terminus with biotin for surface plasmon resonance studies.

For testing protein-DNA interactions by the protein chip method, we used two probes, a 76-bp DNA carrying the entire two-Arg-box operator region and a 56-bp DNA carrying only the downstream Arg box of the *B. stearothermophilus argCo* operator region (see Fig. 1). Both DNA probes were amplified by PCR with 5′-CCTCGAAAATTATTAAAT or 5′-ACATTTGATTTTATTTTTATAC upstream primers with a 5′-CCCGTATGCCTCATGTAG downstream primer labeled at the first position with IRDye800. Concentrations of labeled DNAs were measured with a UV/VIS spectrometer (Perkin Elmer) and by comparison of fluorescent DNA bands in an agarose gel.

Overlap extension was carried out with *Pfu* DNA polymerase (Stratagene), whereas other PCR amplifications were performed with *Taq* DNA polymerase (Qiagen). The nucleotide sequences of domain- and linker-exchanged ArgR chimeras were verified by automatic sequencing.

Oligonucleotide primers were purchased from MWG Biotech.

Purification and molecular mass determination of His-tagged proteins. His-tagged wild-type and chimeric ArgR proteins were purified with minor modifications as follows. Bacterial cells were suspended in a buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 10 mM imidazole) and sonicated, and cell extracts were subjected to affinity chromatography on a nickel-nitrilotriacetic acid column (Qiagen). The column was equilibrated and washed with the buffer defined above, and ArgR proteins were eluted with the same buffer containing 250 mM imidazole. Protein samples of an *E. coli* wild-type ArgR, ArgR-BN1, and ArgR-

TABLE 3. Rate and equilibrium constants of wild-type ArgR repressors and domain-exchanged and linker-exchanged chimeras for *carAB* and *argCo* operators[a]

ArgR protein	Arginine added	k_{ass} (M^{-1} s^{-1})		k_{diss} (s^{-1})		K_d (M, monomer)	
		carAB	*argCo*	*carAB*	*argCo*	*carAB*	*argCo*
ArgR*Ec*	−	—	—	—	—	—	—
	+	6.6×10^5	1.5×10^3	2.4×10^{-3}	9.8×10^{-3}	3.6×10^{-9}	6.6×10^{-6}
ArgR*Bst*	−	1.3×10^6	8.0×10^5	1.4×10^{-2}	1.3×10^{-2}	1.1×10^{-8}	1.6×10^{-8}
	+	1.5×10^5	5.4×10^4	6.3×10^{-4}	2.4×10^{-4}	4.2×10^{-9}	4.4×10^{-9}
ArgR-BN1	−	1.0×10^5	9.4×10^4	3.0×10^{-4}	5.6×10^{-4}	3.0×10^{-9}	6.0×10^{-9}
	+	6.1×10^4	1.5×10^5	5.7×10^{-4}	9.4×10^{-4}	9.4×10^{-9}	6.4×10^{-9}
ArgR-BN2	−	1.5×10^5	1.2×10^5	1.5×10^{-4}	1.4×10^{-4}	1.0×10^{-9}	1.2×10^{-9}
	+	3.1×10^4	3.6×10^4	1.3×10^{-4}	1.3×10^{-4}	4.4×10^{-9}	3.6×10^{-9}
ArgR-EbE	−	—	—	—	—	—	—
	+	4.9×10^5	6.7×10^3	6.0×10^{-4}	6.0×10^{-3}	1.2×10^{-9}	8.8×10^{-7}
ArgR-BeB	−	9.3×10^4	1.4×10^5	1.9×10^{-4}	7.1×10^{-4}	2.0×10^{-9}	5.2×10^{-9}
	+	6.2×10^6	3.3×10^5	8.3×10^{-3}	7.2×10^{-4}	1.3×10^{-9}	2.2×10^{-9}

[a] —, not detectable.

BN2 were dialyzed against a low-salt-concentration buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol, whereas those of a *B. stearothermophilus* wild-type ArgR, ArgR-EN1, ArgR-EN2, ArgR-BeB, and ArgR-EbE were dialyzed against a high-salt-concentration buffer of the same composition except that 1 M NaCl was used.

Molecular masses of His-tagged ArgR proteins were determined by size exclusion chromatography on a Sephacryl S200 column (Amersham Pharmacia). Columns were calibrated with molecular mass markers (Amersham Pharmacia), consisting of bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsin A (25 kDa), and RNase A (13.7 kDa). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed as described by Ausubel et al. (2). Protein concentration was measured by the described method (4) with bovine serum albumin as the standard.

Computer-assisted characterization of proteins was conducted with the MacDNAsis V3.6 program (Hitachi Software).

Mobility shift assay. Binding reactions were performed in 10 mM Tris-HCl (pH 7.5)–250 mM KCl–5 mM $MgCl_2$–2.5 mM $CaCl_2$–2.5% glycerol–0.5 mM dithiothreitol–10 mM L-arginine for the wild-type *B. stearothermophilus* ArgR, ArgR-EN1, and ArgR-EN2 chimeric proteins and in 10 mM Tris-HCl (pH 7.5)–100 mM KCl–10 mM $MgCl_2$–2.5% glycerol–0.5 mM dithiothreitol–10 mM L-arginine for the wild-type *E. coli* ArgR, ArgR-BN1, and ArgR-BN2 chimeric proteins. Alternatively, the binding reaction was carried out in 20 mM Tris-HCl (pH 7.9)–50 mM NaCl–50 mM KCl–0.1 mM dithiothreitol–0.005% surfactant P20 as described previously (27). Binding buffer contained ~0.03 pmol of digoxigenin-labeled operator DNA and a 100-fold excess of unlabeled sonicated herring sperm DNA. The incubation was performed at 37 or 55°C for 30 min.

Samples were loaded on a 2% agarose gel prepared in TAE buffer (40 mM Tris-base, 10 mM sodium acetate, 1 mM EDTA [pH 8.0]) with 10 mM L-arginine and migrated by electrophoresis in the same buffer at room temperature at 12 $V \cdot cm^{-1}$ for 1 h. The DNA-protein complexes were transferred onto nylon membranes by the capillary method (36), and the immunological detection of bound DNA was carried out with CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3,3,1,1^{3,7}]decan}-4-yl)phenyl phosphate)-mediated luminescence (Boehringer Mannheim, Mannheim, Germany). Quantification of free and retarded DNA in gels was done by scanning densitometry of chemiluminograms (Molecular Analyst; Bio-Rad). A weaker chemiluminescent signal for protein-DNA complexes relative to free DNA could be due to differences in the efficiency of transfer and/or fixation to the membrane. An apparent dissociation constant (K_d monomer equivalent) of protein-DNA complexes formed was estimated by eye as the point at which 50% of the probed DNA remained free (average data from graphic representations of three experiments). Other details were described previously (19).

Surface plasmon resonance. The surface plasmon resonance method was used to study the real-time interaction of wild-type and chimeric ArgR proteins with operator DNAs. The uniquely end-biotinylated DNA fragments corresponding to the *B. stearothermophilus argCo* and *E. coli carAB* operators were purified from an excess of primers and deoxynucleoside triphosphates by passage across a spin cartridge containing a silica-based membrane (Life Technologies, Gibco-BRL), and their immobilization was carried out on streptavidin-captured biosensor chips (Biacore AB). Biotinylated operator DNA at concentrations of 5 μg/ml in the binding buffer described above at pH 7.9 was injected over the sensor chip at a flow rate of 5 μl/min at 25°C. Immobilization was controlled manually to about 150 resonance units (RU). Binding assays were carried out in the same buffer with and without 10 mM L-arginine by injection of a wild-type or chimeric ArgR protein at 10 to 100 nM (monomer equivalent) for 2 to 8 min (association phase) at a flow rate of 20 μl/min at 25°C, followed by the injection of a proteinless buffer for 15 min (dissociation phase).

Surface plasmon resonance measurements were conducted in parallel channels with a Biacore 2000 or Biacore 3000 (Biacore AB). The sensor chip was regenerated by washing with 1 M NaCl at a flow rate of 10 μl/min. The channel without immobilized DNA was used as a reference signal for each cycle. Data for binding of ArgR repressors and chimeric proteins were evaluated from sensorgrams with the 1:1 binding model (BIAevaluation Software Handbook, 1999), and K_d values were estimated for the ArgR monomer equivalents. The surface plasmon resonance signal is directly proportional to the mass changes at the sensor chip surface and is expressed in resonance units: 1,000 RU correspond to a surface concentration of approximately 1 ng mm^{-2} for a globular protein.

Coupled transcription-translation in vitro. The *argC* gene (coding for *N*-acetylglutamate-5-semialdehyde dehydrogenase, EC 1.2.1.38) from *B. stearothermophilus* NCIB 8224 (31) is transcribed from a strong P*argC* promoter which largely overlaps the *argCo* operator (32, 33). In preliminary experiments, ArgC protein synthesis from this promoter was found to be rather high in a coupled transcription-translation system of *E. coli* with a corresponding kit purchased from Promega. However, further experiments were carried out in S30 extracts prepared as described by Chen and Zubay (7) with minor modifications.

Plasmid pHAV2 carrying the P*argC-argCo* DNA region from *B. stearothermophilus* (32) was used as a circular DNA template. Protein synthesis was performed at 37°C for 90 min with 10 μCi of L-[^{35}S]methionine (specific activity, 1,000 Ci/mmol, 37 TBq/mmol; Amersham-Pharmacia Biotech). The synthesized products were separated by SDS-PAGE, and the gels were electroblotted on polyvinylidene difluoride membranes (Bio-Rad) or fixed on 3MM paper. Autoradiography was performed after treatment of gels with an Amplifyer solution (Amersham Pharmacia Biotech). Analysis of the synthesized ArgC protein was carried out by densitometric analysis of autoradiograms (BioMax MR film; Kodak) with Molecular Analyst software (Bio-Rad).

Preparation of protein array and fluorescence detection of protein-DNA interactions. Purified His-tagged proteins were dissolved in phosphate-buffered saline with 130 mM NaCl and 10% glycerol and serially fourfold diluted in the same buffer, and 50 to 500 pl was printed with a GMS 417 microarrayer (Affymetrix) onto a BA83 nitrocellulose membrane (Schleicher & Schuell) attached manually to a glass slide. Membranes were incubated in a DNA-binding buffer at pH 7.9 containing 25 μg of sonicated salmon DNA per ml at room temperature for 30 min. Then, an IRDye-labeled DNA probe (10 ng/ml) was added to the solution and incubated for 12 h with slow rotation at room temperature overnight. If necessary, 10 mM L-arginine was included in all buffers. The membranes were washed three times with phosphate-buffered saline, and fluorescent signals from bound DNA-protein complexes were detected at 800 nm with an Odyssey Imager (LI-COR, Inc.). Fluorescent signals were analyzed with GenePix Pro4.0 (Axon Instruments) software.

RESULTS

Rationale for construction of domain-exchanged ArgR chimeras. We were first interested in examining whether the exchanged domains of the *E. coli* and *B. stearothermophilus* ArgR repressors might have a similar effect on the function of the chimeric proteins. The availability of three-dimensional structural data on domain boundaries of the *B. stearothermophilus* arginine repressor (28) allowed us to design an accurate reciprocal domain exchange between the *E. coli* and *B. stearothermophilus* proteins by swapping the oligomerization domain along with a corresponding linker peptide from one ArgR to the last amino acid in the DNA-binding domain of the other ArgR. The amino acid sequences of the two resulting ArgR-EN1 and ArgR-BN1 chimeras, carrying the N-terminal DNA-binding domains from *E. coli* and *B. stearothermophilus*, respectively, are shown in Fig. 1a.

The linker peptide in *E. coli* ArgR appears to be 3 amino acid residues shorter than that in the *B. stearothermophilus* repressor. Therefore, in order to conserve the integrity of an α-helix sequence with respect to the upstream-located DNA-binding domain sequence, we constructed two more chimeras, ArgR-EN2 and ArgR-BN2, in which the N-terminal regions were taken from the *E. coli* and *B. stearothermophilus* repressors, respectively, but extended to the Leu87 and Ile85 residues, respectively, located in a β3 sheet of the C-terminal oligomerization domain (see Fig. 1a).

Recently, two other ArgR chimeras were constructed by mutual swapping of the DNA-binding and oligomerization domains from the *E. coli* and *B. subtilis* arginine repressors (17) with junction sites close to those of the ArgR-EN2 and ArgR-BN2 chimeras described in this study (see Fig. 1). The *B. subtilis* protein also repressed *E. coli arg* operators, but not vice versa (34). However, since essential differences exist between *B. subtilis* and *B. stearothermophilus* regulatory systems and since different DNA targets have been used (10, 17, 19, 25, 32,

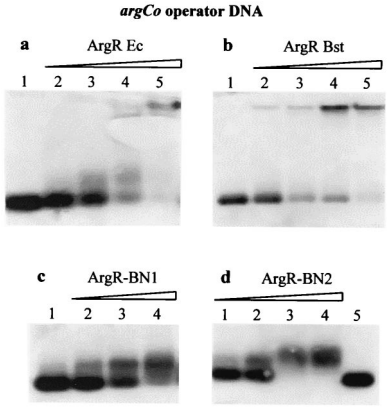


FIG. 2. Mobility shift assay of wild-type ArgR from *E. coli* (a) and *B. stearothermophilus* (b) and chimeric proteins ArgR-BN1 (c) and ArgR-BN2 (d) bound to *B. stearothermophilus argCo* operator DNA in the presence of arginine. *argCo* operator DNA was incubated with increasing amounts of His-tagged proteins at 37°C for 30 min and electrophoresed in a 2% agarose gel in the presence of arginine. (a) Lanes 2 to 5 contained 170 nM, 340 nM, 680 nM, and 1,360 nM *E. coli* ArgR, respectively; (b) lanes 2 to 5 contained 86 nM, 102 nM, 118 nM, and 130 nM *B. stearothermophilus* ArgR, respectively; (c) lanes 2 to 4 contained 15 nM, 25 nM, and 40 nM ArgR-BN1, respectively; (d) lanes 1 to 4 contained 12 nM, 24 nM, 48 nM, and 96 nM ArgR-BN2, respectively. Lane 1 in a, b, and c and lane 5 in d contained only operator DNA.

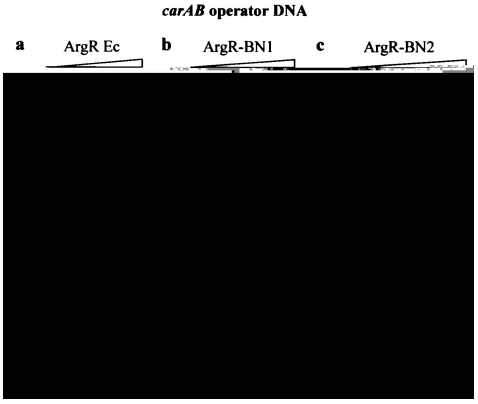


FIG. 3. Mobility shift assay of wild-type ArgRs of *E. coli* (a) and *B. stearothermophilus* (d) and domain-exchanged chimeric proteins ArgR-BN1 (b), ArgR-BN2 (c), and ArgR-EN2 (e) bound to *E. coli carAB* operator DNA in the presence of arginine. *carAB* operator DNA was incubated with increasing amounts of His-tagged proteins at 37°C for 30 min and electrophoresed in a 2% agarose gel in the presence of arginine. (a) Lanes 2 to 4 contained 12.4 nM, 24.8 nM, and 37.2 nM of *E. coli* ArgR protein, respectively; (b) lanes 2 to 5 contained 2.1 nM, 5.3 nM, 7.4 nM, and 9.5 nM ArgR-BN1, respectively; (c) lanes 2 to 5 contained 6 nM, 12 nM, 18 nM, 24 nM, and 48 nM ArgR-BN2, respectively; (d) lanes 2 to 5 contained 26 nM, 52 nM, 104 nM, and 208 nM *B. stearothermophilus* ArgR, respectively; (e) lanes 2 to 4 contained 600 nM, 1,200 nM, and 1,900 nM ArgR-EN2, respectively. Lane 1 in a, b, c, d, and e contained only operator DNA.

39; this study), it is impossible to extrapolate data obtained from these two studies.

Domain-exchanged chimera binding to operator DNAs. Previously it was shown that the wild-type *E. coli* ArgR is soluble in a low-salt-concentration buffer (21), whereas the wild-type *B. stearothermophilus* ArgR is soluble in a high-salt-concentration buffer (10). We observed that both ArgR-BN1 and, to a lesser extent, ArgR-BN2 were optimally soluble in low-salt buffer, as opposed to ArgR-EN1 and ArgR-EN2, which were more soluble in high-salt buffer. Consequently, DNA-binding reactions were performed for each protein in the optimum buffer (see Materials and Methods).

The DNA-binding properties of the four ArgR chimeras constructed and two wild-type repressors were first studied by the mobility shift assay. In the presence of arginine, the wild-type *B. stearothermophilus* ArgR repressor bound the *B. stearothermophilus argCo* (Fig. 2b), and the *E. coli carAB* (Fig. 3d) operators at 37°C with similar efficiencies (an apparent dissociation constant [K_d] was ~100 nM, close to the value observed for the untagged protein). The wild-type *E. coli* repressor bound the *carAB* operator with an apparent K_d of close to 120 nM (Fig. 3a), whereas it bound the *B. stearothermophilus argCo* operator with an apparent K_d in excess of 1 μM (Fig. 2a). Thus, in the presence of arginine, the DNA affinity of the wild-type *E. coli* repressor was more than 50 times lower for the heterologous *argCo* operator than for its homologous *carAB* operator under the conditions tested.

No binding was detected for the ArgR-EN1 chimeric protein to either of the two operators. Binding was also not detected between ArgR-EN2 and the *argCo* operator, and binding to the *carAB* operator was very weak (Fig. 3e). Two other chimeras, ArgR-BN1 and ArgR-BN2, both carrying the N-terminal domain from *B. stearothermophilus* ArgR, bound efficiently to the *argCo* (Fig. 2c and 2d) and *carAB* operators (Fig. 3b and 3c). Apparent K_d values were calculated to be 29 nM and 5 nM, respectively, for ArgR-BN1 and 34 nM and 11 nM, respectively, for ArgR-BN2 for these operators. However, when binding reactions were carried out at 55°C, no retarded bands could be detected for ArgR-BN1 or ArgR-BN2, indicating that these chimeras did not gain in thermostability compared to the wild-type *B. stearothermophilus* ArgR (10).

Karaivanova et al. previously described that the wild-type *B. stearothermophilus* ArgR repressor and the *argCo* operator DNA-bound complexes barely enter a nondenaturing agarose gel (19) (Fig. 2b). A similar behavior was observed for wild-type *B. stearothermophilus* ArgR-*carAB* complexes but not for *E. coli* ArgR-*carAB* complexes. ArgR-BN1 and ArgR-BN2 bound to *carAB* or *argCo* complexes also migrated as compact retarded bands (see Fig. 2 and 3). Therefore, different migration of chimeric protein-DNA complexes appeared to be related to the oligomerization domain of the corresponding parent repressors rather than to target DNA particularities. Indeed, the deduced isoelectric points of His-tagged proteins were found to be 5.6, 5.2, and 5.5 for wild-type *E. coli* ArgR, ArgR-BN1, and ArgR-BN2, respectively, whereas they were 8.0, 8.2, and 7.3 for wild-type *B. stearothermophilus* ArgR, ArgR-EN1, and ArgR-EN2, respectively. Consequently, their electrophoretic migration in a 6% nondenaturing polyacrylamide gel was in accordance with their deduced isoelectric point values, i.e., proteins possessing the basic oligomerization domain of *B. stearothermophilus* ArgR migrated slowly (data not shown).

The mobility shift assay detects DNA-protein interactions at equilibrium, although this analysis may be restricted by desalting and cage effects that modify off and on rates in an unpre-

dictable fashion. The use of surface plasmon resonance allows access to rate constants for the on and off processes under more constant buffer conditions and also allows a comparison between apparent K_d values obtained either by the ratio of apparent kinetic constants or by the half-saturation concentrations at steady state (29). Therefore, we used the latter method to compare apparent kinetic constants characteristic of wild-type repressor and chimeric protein interactions to operator DNAs. Binding reactions were carried out in a buffer at pH 7.9.

Both the *E. coli* and *B. stearothermophilus* wild-type repressors exhibited arginine dependence for *carAB* operator binding, but the shapes of the corresponding sensorgrams differed significantly. As can be seen in Fig. 4, the association phase for *E. coli* ArgR stabilized rapidly after a 2-min injection, whereas the *B. stearothermophilus* ArgR association peak was rather high for both the *carAB* and *argCo* operators after short injections but decreased gradually during longer injections. A similar picture was observed for *B. stearothermophilus* ArgR interactions in the absence of arginine. Therefore, assuming a possible stabilizing effect of the prolonged injection on the formation of the *B. stearothermophilus* ArgR protein-DNA complexes, we performed further binding reactions for 8 min (association phase), followed by the injection of a proteinless buffer for 15 min (dissociation phase).

As expected, the wild-type *E. coli* ArgR displayed strong binding to the *carAB* operator in the presence of arginine, whereas no binding could be detected in the absence of arginine, and the protein appeared to be unable to bind the *argCo* operator irrespective of the presence of arginine (all surface plasmon resonance sensorgrams are available from the authors upon request). The wild-type *B. stearothermophilus* ArgR repressor bound the *carAB* and *argCo* operators in the absence of arginine, but arginine improved the binding to both operators. Quantitative analysis of sensorgrams showed that the wild-type *B. stearothermophilus* ArgR repressor bound the *carAB* and *argCo* operators in the absence of arginine, but arginine improved its affinity nearly fourfold (Table 3). On the other hand, wild-type *E. coli* ArgR repressor binding to the *carAB* operator was found to be strongly dependent on arginine; even in the presence of arginine, the *E. coli* protein bound to the heterologous *argCo* operator with a 1,000-fold lower affinity than to the homologous operator, a greater relative difference than was detected by the mobility shift assay.

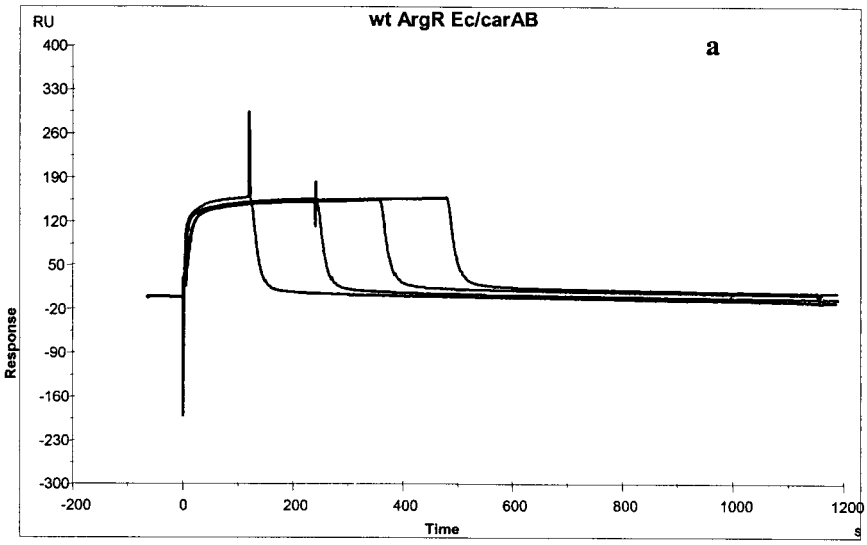
Resonance signals were detected for all domain-exchanged chimeric proteins when passed over sensor chips with coupled *carAB* or *argCo* operator DNAs. In the absence of arginine, the ArgR-BN1 and ArgR-BN2 chimeras bound the *carAB* and *argCo* operators with a higher affinity than wild-type *B. stearothermophilus* ArgR did under the same conditions. When chimeric proteins were passed over the immobilized *carAB* or *argCo* operator DNA in the presence of arginine, ArgR-BN1 and especially ArgR-BN2 binding affinities were lower for both operators compared to those in the absence of arginine (see Table 3). Sensorgrams with injected ArgR-EN1 and ArgR-EN2 chimeras indicated a possible interaction with both operators in the presence or absence of arginine. However, fits with a simple single-binding model used to calculate apparent rate constants were poor (for example, a standard χ^2 value was in excess of 1,000 in two independent experiments). Therefore, we concluded that sensorgrams of these chimeras reflect a more complex binding mode that could not be accessed for meaningful and comparable rate constants.

Thus, the surface plasmon resonance method showed that wild-type and domain-exchanged chimeras have different kinetics of interactions with operator DNAs and that arginine differentially affects these interactions.

To clarify the oligomeric nature of the chimeras, we determined their molecular masses by gel filtration chromatography. In the absence of arginine, two peaks were eluted for ArgR-BN1 and ArgR-BN2. A major peak was close to 41 ± 5 kDa, and another peak was approximately 200 kDa. On the basis of these experiments, no clear distinction could be made between dimeric and trimeric molecules for the major peak, whereas the second peak probably reflects aggregated proteins under these conditions. In the presence of arginine, a peak of 100 ± 8 kDa was additionally detected for purified ArgR-BN1 and ArgR-BN2 protein samples, which is consistent with the appearance of hexameric molecules. No clear peaks could be detected for ArgR-EN1 and ArgR-EN2, suggesting their tendency to aggregate and indicating that the resolution of the gel filtration technique is insufficient to determine the apparent molecular masses of these chimeric proteins.

ArgR-BN1 and ArgR-BN2 chimeras provide gene repression. To assess whether the wild-type ArgR and domain-exchanged chimeric proteins were able to mediate gene repression, we evaluated *argC* reporter gene expression from the P*argCo* promoter-operator region in a coupled transcription-translation system with S30 extracts of *E. coli* (arginine was present in the reaction mixture). An abundant band of the ArgC protein could still be detected after addition of 10 pmol of wild-type *E. coli* ArgR (Fig. 5a), confirming a weak repression effect of this protein on the heterologous promoter. No repression of ArgC synthesis was detected after addition of 10 pmol of the ArgR-EN1 or ArgR-EN2 chimeric protein to the reaction mixture. However, according to expectations, ArgC protein synthesis strongly decreased when smaller quantities of wild-type *B. stearothermophilus* ArgR were added to the reaction mixture (decreasing nearly fourfold with 2.5 pmol of protein). Moreover, the two other chimeras, ArgR-BN1 and ArgR-BN2, exhibited stronger repression of ArgC synthesis than wild-type *B. stearothermophilus* ArgR. The most pronounced repression was detected for ArgR-BN1; 0.5 pmol of this chimeric protein almost completely abolished reporter *argC* gene expression in vitro.

FIG. 4. Sensorgrams of binding of wild-type *E. coli* and *B. stearothermophilus* ArgR repressors to operator DNAs. The curves represent binding of 50 nM wild-type (wt) ArgR to the 141-bp *carAB* operator DNA of *E. coli* (a and b) and the 137-bp *argCo* operator DNA of *B. stearothermophilus* (c) 2 min, 4 min, 6 min, and 8 min after injection of the protein. The signals were monitored for *E. coli* ArgR in the presence of arginine (a) and for *B. stearothermophilus* in the presence of arginine (thick lines) and in the absence of arginine (thin lines) in b and c. No binding of *E. coli* ArgR to *carAB* was detected in the absence of arginine or to *argCo* irrespective of the presence of arginine.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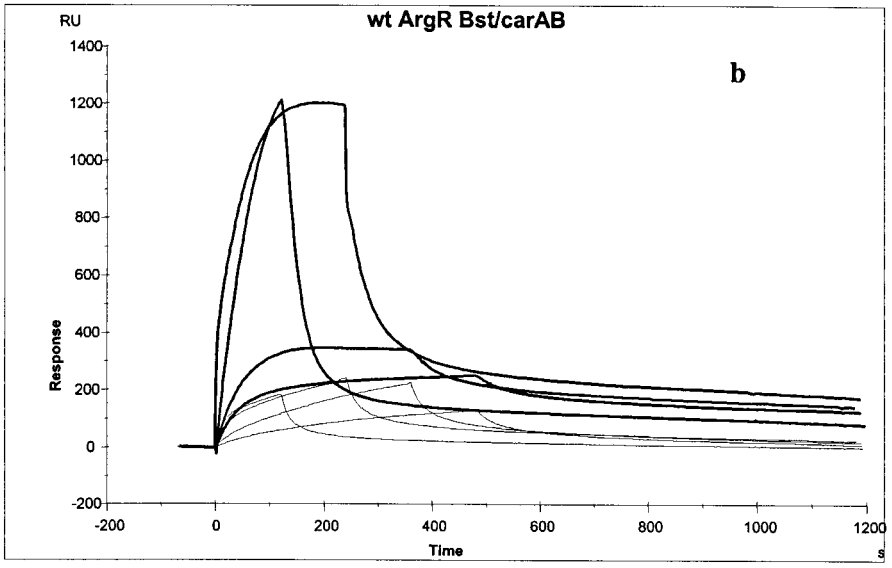
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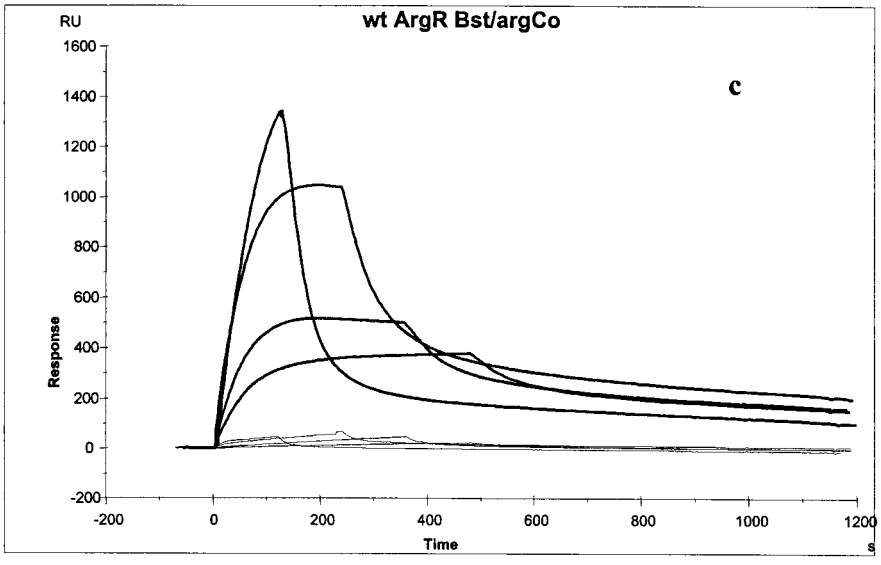
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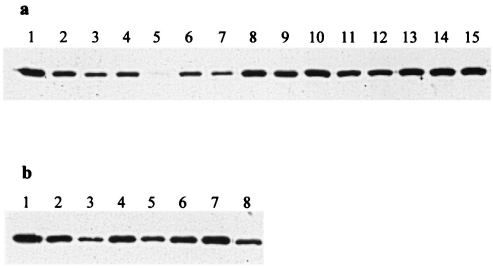

FIG. 5. Autoradiogram of *B. stearothermophilus* ArgC protein synthesis in a coupled transcription-translation system with *E. coli*. pHAV2 plasmid DNA (10 μg/ml) was used as the template for protein synthesis from a P*argC* promoter, and 1 μl of wild-type ArgR or chimeric proteins at different concentrations (indicated for the hexameric molecule) was added to the mixture containing a premixed solution, unlabeled amino acids, and L-[^{35}S] methionine, and the reaction was initiated by adding S30 extracts (a 30-μl total volume). (a) Lanes 1 and 10, without addition of exogenous ArgR protein to the reaction mixture. Other samples show ArgC synthesis after addition to the reaction mixture as follows: lanes 2 and 3, 1 pmol and 2.5 pmol, respectively, of wild-type *B. stearothermophilus* ArgR; lanes 4 and 5, 0.1 pmol and 0.5 pmol, respectively, of ArgR-BN1; lanes 6 and 7, 1 pmol and 2.5 pmol, respectively, of ArgR-BN2; lanes 8 and 9, 2.5 pmol and 10 pmol, respectively, of ArgR-EN1; lanes 11 and 12, 5 pmol and 10 pmol, respectively, of wild-type *E. coli* ArgR; lanes 13, 14, and 15, 2.5 pmol, 5 pmol, and 10 pmol, respectively, of ArgR-EN2. (b) Lane 1, without ArgR; lanes 2 and 3, 1 pmol and 5 pmol, respectively, of wild-type *B. stearothermophilus* ArgR; lanes 4 and 5, 1 pmol and 5 pmol, respectively, of ArgR-BeB; lane 6, 20 pmol of wild-type *E. coli* ArgR; lanes 7 and 8, 5 pmol and 20 pmol, respectively, of ArgR-EbE.

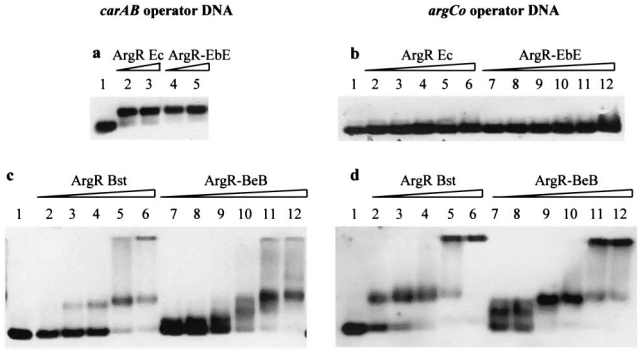

FIG. 6. Mobility shift assay of wild-type *E. coli* and *B. stearothermophilus* ArgR repressors and linker-exchanged ArgR-BeB and ArgR-EbE chimeras with respect to the *E. coli carAB* (a and c) and *B. stearothermophilus argCo* (b and d) operator DNAs. Operator DNAs were incubated with increasing amounts of His-tagged proteins at 37°C for 30 min in 20 mM Tris-HCl (pH 7.9)–50 mM NaCl–50 mM KCl–0.1 mM dithiothreitol–0.005% surfactant P20 in the presence of arginine and electrophoresed in a 2% agarose gel with arginine. (a) Lane 1 contained *carAB* DNA only; lanes 2 and 3 contained 3.2 nM and 6.4 nM *E. coli* ArgR, respectively; lanes 4 and 5 contained 3.2 nM and 6.4 nM ArgR-EbE, respectively. (b) Lane 1 contained *argCo* DNA only; lanes 2 to 6 contained 416 nM, 832 nM, 1,664 nM, 3,328 nM, and 6,656 nM *E. coli* ArgR, respectively; lanes 7 to 12 contained 416 nM, 832 nM, 1,664 nM, 3,328 nM, 6,656 nM, and 13,312 nM ArgR-EbE, respectively. (c and d) Lanes 1 contained *carAB* and *argCo* DNAs only, respectively; lanes 2 to 6 contained 6.5 nM, 13 nM, 26 nM, 52 nM, and 104 nM *B. stearothermophilus* ArgR, respectively; lanes 7 to 12 contained 6.5 nM, 13 nM, 26 nM, 52 nM, 104 nM, and 208 nM ArgR-BeB, respectively.

We also found that the ArgR-BN1 and ArgR-BN2 chimeras inhibited the growth of *E. coli* Top10 host cells in minimal medium irrespective of the presence of arginine. Neither ArgR-EN1 nor ArgR-EN2 displayed such a property. Consequently, the ArgR-BN1 and ArgR-BN2 chimeras, as described previously for wild-type *B. stearothermophilus* ArgR (19), behave as superrepressors in the heterologous *E. coli* host.

Therefore, in summary, the ArgR-BN1 and ArgR-BN2 chimeras but not ArgR-EN1 or ArgR-EN2 act as strong repressors both in vitro and in vivo.

Analysis of linker-exchanged ArgR chimeras. Taking into consideration the positions of junction sites between domains in the four chimeras studied, it was reasonable to assume that, along with the α4 structure, the interdomain-linker peptide might affect the DNA-binding properties of ArgRs. Therefore, we designed two new derivatives, ArgR-EbE and Arg-BeB, in which only the linker sequence was replaced in wild-type ArgR repressors (see Fig. 1a).

The purified ArgR-BeB protein was found to be soluble in high-salt buffer and hardly entered nondenaturing polyacrylamide gels, properties similar to those of the wild-type *B. stearothermophilus* ArgR. ArgR-EbE was somewhat more soluble in high-salt buffer, but its migration was closer to that of the wild-type *E. coli* repressor (data not shown). The mobility shift assay was performed in the pH 7.9 buffer used for the surface plasmon resonance studies. Under these conditions, wild-type *B. stearothermophilus* protein-DNA complexes entered a nondenaturing agarose gel more easily, forming compact retarded bands, and moreover, the affinity of the repressor was almost twice that seen in the previous buffer. In the presence of arginine, ArgR-EbE bound only the *carAB* operator with an affinity similar to the wild-type *E. coli* repressor in the presence of arginine (Fig. 6a and 6b), whereas ArgR-BeB bound both *carAB* and *argCo* operator DNAs with an affinity higher than that of the wild-type *B. stearothermophilus* ArgR repressor (Fig. 6c and 6d). The ArgR-BeB binding was accompanied by the appearance of an additional band(s) that might reflect trimeric or dimeric protein-DNA-bound complexes.

As further shown by surface plasmon resonance, the ArgR-EbE protein bound perfectly to *carAB* in the presence of arginine but was practically unable to bind the *carAB* or *argCo* operator in the absence of arginine (see Table 3). The ArgR-BeB protein was able to bind both *carAB* and *argCo* operator DNAs with affinities higher than that of the wild-type *B. stearothermophilus* ArgR repressor, and the binding was less affected by arginine.

The linker-exchanged chimeric proteins were tested for their ability to repress *B. stearothermophilus argC* gene transcription from the P*argCo* promoter-operator region in vitro (see Fig. 5b). The same quantity of added ArgR-EbE protein appeared to decrease ArgC synthesis more than the wild-type protein from *E. coli*, whereas the ArgR-BeB protein appeared to be slightly weaker compared to the wild-type *B. stearothermophilus* protein.

The linker-exchanged chimeras ArgR-EbE and ArgR-BeB were thus found to be reminiscent of the wild-type *E. coli* and *B. stearothermophilus* repressors, respectively. Nevertheless, minor differences detected in their properties indicated that the linker peptide could modulate the DNA-binding properties of the chimeric proteins.

Wild-type *B. stearothermophilus* ArgR protein can recognize a single Arg box. Since the stimulating effect of arginine on DNA binding was less noticeable for the wild-type *B. stearothermophilus* ArgR, absent for ArgR-BN1 and, moreover, decreased for the ArgR-BN2 chimeric protein, we assumed that target DNA recognition by these proteins is less dependent on the double Arg box organization of operators. To address this question, we applied the protein array method, which permits simultaneous analysis of numerous proteins (11).

Eight wild-type ArgR and chimeric proteins were serially fourfold diluted and spotted onto a nitrocellulose membrane. Two IRDye800-labeled DNA probes, a 76-bp fragment carrying the entire *B. stearothermophilus argCo* operator and a shorter 56-bp fragment lacking the upstream Arg box of the operator (see Fig. 1b), were incubated with duplicate membranes in the presence and absence of arginine. Membranes were washed thoroughly, and protein-DNA binding was monitored by detection of a fluorescent signal.

As expected, no response was detected from the wild-type *E. coli* ArgR and chimeric ArgR-EbE, ArgR-EN1, and ArgR-EN2 proteins with either of the two probes (Fig. 7). A clear signal was detected from the wild-type *B. stearothermophilus* ArgR and chimeric ArgR-BeB, ArgR-BN1, and ArgR-BN2 proteins with respect to a 76-bp DNA probe. The linker-exchanged ArgR-BeB chimera exhibited better affinity for the *argCo* operator than the wild-type protein under the conditions used. A signal was still detectable for 0.93 pg of spotted ArgR-BeB or wild-type ArgR, indicating the extreme sensitivity of the detection method (see Fig. 7a). Arginine increased the binding affinity of the wild-type *B. stearothermophilus* repressor (except for a 238-pg protein spot) and probably the ArgR-BN1 chimera for the operator DNA. However, this amino acid decreased the DNA-binding efficiency of the ArgR-BN2 chimera and probably the ArgR-BeB chimera under the conditions used (see Fig. 7b).

A weaker signal was detected from the wild-type *B. stearothermophilus* ArgR and the ArgR-BeB and ArgR-BN2 chimeras bound to a 56-bp DNA probe, and no signal was detectable from ArgR-BN1 spots (see Fig. 7c and 7d). It was clearly visible that the presence of arginine had no more stimulating effect on the binding of wild-type *B. stearothermophilus* ArgR, suggesting that allosteric modifications of the wild-type repressor are not necessary to recognize the single-Arg-box operator DNA.

Analysis of fluorescent spots with the GenePix Pro4.0 program confirmed the visual observations (see Fig. 7e). Though it is impossible quantify binding constants by the protein chip method, the data obtained from titrated protein samples are, in general, in agreement with the results of the mobility shift assay and surface plasmon resonance data. The difference in the physicochemical characteristics of the chimeras, especially in solubility, can explain variations in binding affinities detected by various methods, as optimal conditions differed for each protein. Consequently, comparative study of such proteins is a laborious task. Therefore, the ability to detect DNA-binding responses from numerous immobilized proteins with high sensitivity and in parallel experiments with various probes might give a great advantage to the protein array method compared to other approaches.

DISCUSSION

Arginine metabolism in two bacterial species, the gram-negative mesophilic *E. coli* and the gram-positive thermophilic *B. stearothermophilus*, is governed by a similar transcription-regulatory system. However, the data presented in this study show that the wild-type *E. coli* arginine-bound or unbound repressor is practically unable to recognize the heterologous *argCo* operator, whereas the *B. stearothermophilus* repressor binds both the *argCo* and the *carAB* operators irrespective of the presence of arginine (see also the introduction).

By applying different methodological approaches, we have shown that DNA-binding and gene repression functions have been affected in domain-exchanged and linker-exchanged ArgR chimeras. We have found that, due to structural perturbations, the ArgR-EN1 and ArgR-EN2 chimeras, both conserving the DNA-binding domain of *E. coli* ArgR, were unable to achieve sequence-specific recognition, even with respect to *carAB* in the presence of arginine. Regardless of expectations, ArgR-BN2 and probably ArgR-BN1, both harboring the oligomerization domain from the *E. coli* repressor, bound more weakly to the *carAB* and *argCo* operators in the presence of arginine than in its absence, indicating that their hexameric molecules have lower affinity for a target than other oligomeric forms, and in this respect both chimeras resembled the wild-type ArgR protein of the hyperthermophilic bacterium *Thermotoga neapolitana* (9). Furthermore, both chimeras display stronger repression of the *argC* reporter gene than the wild-type *B. stearothermophilus* repressor did, indicating that they may differ in other protein-protein interactions involved in transcriptional regulation from the *B. stearothermophilus argC* promoter-operator region (M. Snapyan, M. Lecocq, L. Guével, M.-C. Arnaud, A. Ghochikyan, and V. Sakanyan, unpublished data). Finally, the replacement of the linker peptide in the wild-type *E. coli* and *B. stearothermophilus* repressors slightly changed the DNA-binding parameters of the corresponding chimeras.

In agreement with observed modulations in DNA-binding properties, the six chimeras constructed also possessed various solubility, electrophoretic migration, and thermostability properties depending on the region substituted. Given the junction sites between substituted domains and the observed differences in solubility and velocity within the pairs ArgR-BN1 and ArgR-BN2, ArgR-EN1 and ArgR-EN2, and ArgR-BeB and ArgR-Ebe, we attribute the particularities of the chimeric proteins to their oligomerization domain, in particular to the region covering the α4 helix and, to a lesser extent, the linker peptide. It has already been shown that mutations in the linker peptide of the *E. coli* CytR and LacI repressors can affect the operator-binding affinity (12, 18).

The three-dimensional structure of the region covering the linker peptide and α4 helix is not yet available for *E. coli* ArgR. The linker peptide sequences in the *E. coli* and *B. stearothermophilus* repressors are completely different, and therefore it is difficult to obtain a model structure even for ArgR-EbE in order to make confident interpretations of apparent functional modifications in linker-substituted chimeras. However, a model simulation of *B. stearothermophilus* ArgR clearly predicts that the α4 helix substitution within the oligomerization

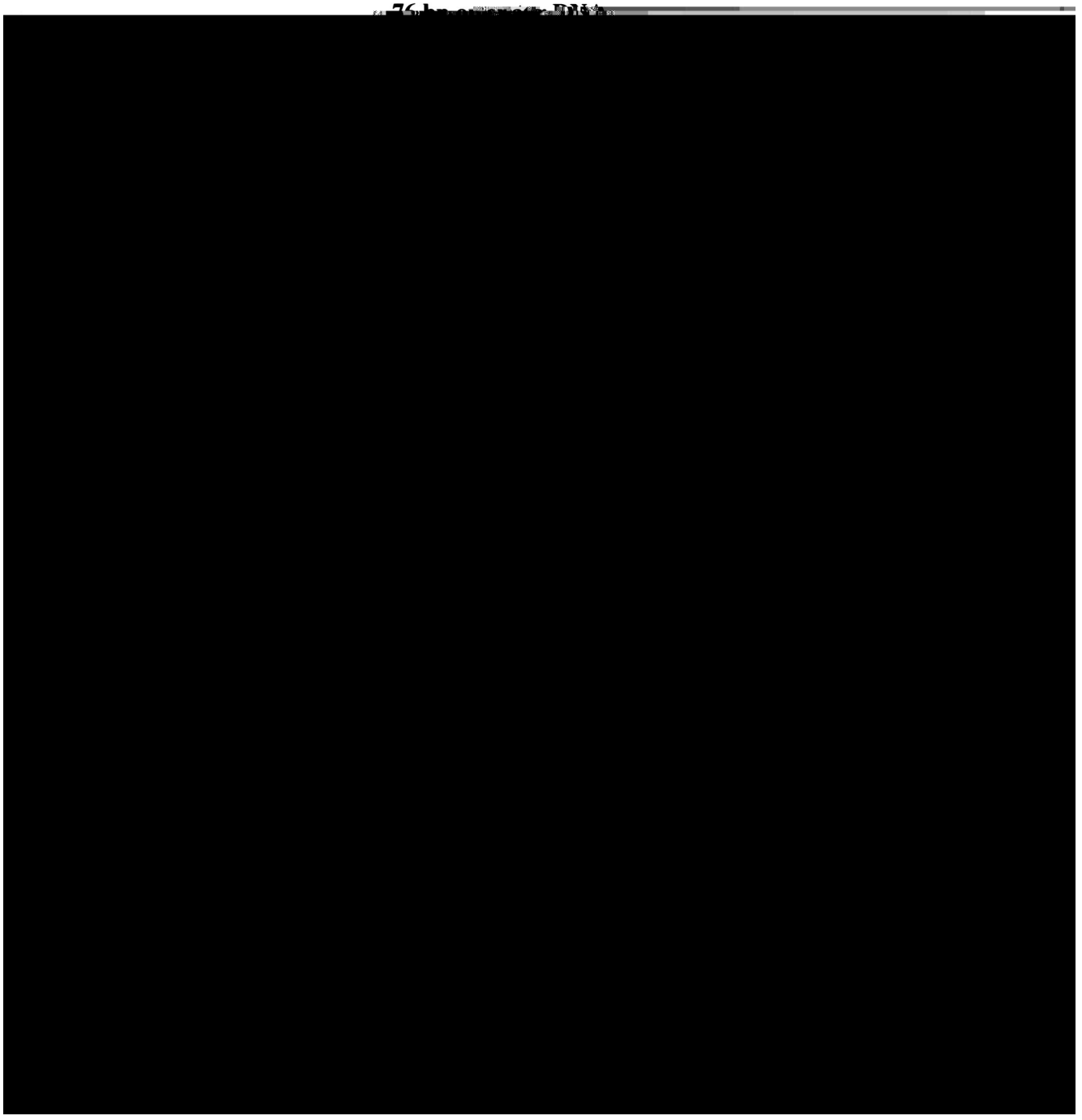

FIG. 7. Fluorescence detection of DNA-protein interactions with protein arrays (a to d) and relative intensity of fluorescence signals (e). His-tagged purified proteins were serially fourfold diluted and spotted on duplicate nitrocellulose membranes. The nondiluted sample corresponds to 238 pg of spotted protein. Binding reactions were carried out with equal quantities of IRDye800-labeled probes, a 76-bp DNA (a and b) and a 56-bp DNA (c and d), for 12 h at 18°C. L-Arginine (10 mM) was included in the protein dilution and DNA-binding buffers (b and d). Membranes were washed and scanned with an Odyssey Imager (LI-COR).

domain can affect interdomain interactions in chimeric proteins (V. Duyne, personal communication).

In the wild-type *B. stearothermophilus* hexameric protein, each DNA-binding domain of one trimer is in contact with the oligomerization domain of the neighboring trimer, i.e., amino acids of the α1 helix interact with residues of the α4 helix (28). The α4 helix structure in *E. coli* ArgR appears to be about one turn shorter than its *B. stearothermophilus* homologue, or this structure is of the same length but kinked due to a Pro76 residue that might make a loop shorter by 3 to 4 residues. Therefore, the α4 helix substitution should change the spacing between the DNA-binding and oligomerization domains in

trimers of domain-exchanged chimeras and thereby affect wHTH module positioning with respect to the Arg boxes in the operator sequence. Moreover, arginine-mediated allosteric modification in the α4 helix-substituted ArgR-BN2 and probably ArgR-BN1 chimeras can lead to worsening of the cooperative compliance between the four DNA-binding motifs in the hexamers and the four patches in the two Arg boxes of the operator and thereby to decreasing the DNA-binding affinity. Though these structural predictions need to be confirmed, it follows from our data that the oligomerization domain of *E. coli* and *B. stearothermophilus* ArgRs have a different impact on the physicochemical and DNA-binding properties of the corresponding repressors.

DNA-protein interactions are highly dynamic processes that depend on both partners, and local folding transition of a transcription-regulatory protein can be coupled to site-specific DNA binding (37). DNA-binding affinity varies for different *arg*-specific operators and is considerably reduced for a single Arg box of *E. coli* operators (6, 8, 42, 43). A single Arg box has been identified for several arginine-related and nonrelated genes (13, 20, 25, 26, 30, 38), though binding of ArgR to these targets has not yet been proven. We confirmed that arginine-stimulated allosteric transition is essential in the molecular recognition of operators carrying two adjacent Arg boxes by the wild-type *B. stearothermophilus* repressor. In addition, we show that, contrary to *E. coli* ArgR, the wild-type *B. stearothermophilus* repressor is able to bind a single Arg box of the *B. stearothermophilus argCo* operator with a rather high efficiency, and arginine-mediated allosteric modification appears not to be necessary for this recognition, since arginine has no activating effect on the binding of such a target.

In the *B. stearothermophilus* repressor, the oligomerization domain appears to contribute, via interdomain contacts, to the adaptation of the DNA-binding domain to various geometries in target DNAs. In particular, protein contacts between residues of the oligomerization domain and the DNA-binding domain in hexamers or trimers of the thermophilic ArgR protein may modulate the position of wHTH with respect to a single or a double Arg-box. Consequently, the binding of wild-type *B. stearothermophilus* ArgR repressor to a single Arg-box can reflect its ability to bind to a wider range of operators, whereas binding of the wild-type *E. coli* repressor to the two-Arg-box-associated operator reflects its restricted ability to recognize DNA targets.

It is worth mentioning that the ArgR repressor of *T. neapolitana* displays similar affinity for single- and double-Arg-box operators (M.-C. Arnaud and V. Sakanyan, unpublished data), and its own *argRo* operator can be efficiently recognized by the *B. stearothermophilus* but not by the *E. coli* repressor (35). Therefore, the capacity of bacterial ArgR repressors to distinguish and bind a single Arg box appears to be a basic condition for interactions with a wide range of *arg*-specific and other operator sequences.

Maas has postulated that the arginine repressor might play the role of a global regulatory protein in bacterial cells (22), which is supported by the presence of many single-Arg-box-like sequences in the *E. coli* genome (30). Therefore, we explain the superrepressor behavior of *B. stearothermophilus* ArgR and some chimeras in *E. coli* as the result of repression of single-box-carrying operators for metabolic genes encoding proteins involved in synthesis of a growth factor(s) other than arginine growth factors and that this repression can arrest host cell reproduction in synthetic medium supplied with this amino acid.

Since thermophilic bacteria branched from the 16S rRNA tree earlier than mesophilic enterobacteria (1), we put forward the hypothesis that the ArgR-mediated regulatory mechanism evolved from a less-specific single-box to a highly gene-specific double-box operator organization in microbial genomes and that the oligomerization domain α4 structure-determined interactions with the DNA-binding domain might reflect a molecular adaptation mechanism of various ArgR regulators to the corresponding targets.

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