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KorB protein of promiscuous plasmid RP4 recognizes inverted sequence repetitions in regions essential for conjugative plasmid transfer

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ABSTRACT

We have constructed a RP4 KorB overproducing strain and purified the protein to near homogeneity. KorB is a DNA binding protein recognizing defined palindromic 13-bp sequences (TTTAGCSGCTAAA). Inverted sequence repetitions of this type, designated O_B, are present on RP4 12 times. O_B-sequences are localized in replication and maintenance regions as well as in the regions Tra1 and Tra2 essential for conjugative transfer. All sites found in Tra regions by computer search act as targets for specific binding of KorB protein. KorB-DNA complexes were detected by DNA fragment retardation assay using polyacrylamide gels. The 13-bp symmetric arrangement of the consensus O_B-sequence constitutes the core for binding KorB protein since any truncation of this sequence prevents complex assembly or leads to a considerable destabilization of the KorB-DNA complexes. A hydroxyl radical footprint analysis demonstrated complex formation of KorB with the O_B-sequence directly and suggests the presence of an unusual DNA structure within the nucleoprotein complex.

INTRODUCTION

RP4 is a selftransmissible resistance plasmid (60 kb) that is capable of replication and stable maintenance in a wide variety of Gram-negative bacteria (for review see 1, 2). The plasmid is a member of *E. coli* incompatibility group P (IncPα) and it is indistinguishable from plasmids RP1, R68, R18 and RK2 (3, 4). IncP plasmids encode a regulatory network known as the *kil-kor* regulon (5, 6, 7). RK2/RP4 gene products KorA, KorB, KorC, KorE, and KorF have been described to act in different combinations as repressors of transcription in a series of operons on the RK2/RP4 genome. Since the genes that are regulated include essential maintenance functions (i.e. the determinant of the initiator protein of vegetative replication *trfA*), the *kil-kor* regulon is supposed to be involved in the IncP broad host range character (7, 8, 9, 10, 11). By genetic studies, genes *korA*, *incC*, *korB* and *korF* have been located in the *korA-korB* operon (for review see 1, 12, 13, 14). The *korB* nucleotide sequence has been determined by the Figurski and the Thomas laboratory (11, 14, 15, 16). The *korA-korB* operon seems to be regulated synergistically by proteins KorA and KorB (8). Genes *incC* and *korB* have been suggested to be involved in partitioning functions (18, 19). Inverted sequence repetitions found in a variety of control regions, including those of operons *trfA*, *kilC*, *kilE*, *kilA*, *korA-korB* and *kfrA*, have been proposed as sites of action for repressors KorA and KorB, designated operator O_A (KorA) and O_B (KorB) (9, 19). The O_B sequences were proposed to be 13 bp long, showing 2-fold rotational symmetry (19). In contrast to the O_A potential binding sequences, O_B have not only been found in the plasmid's regions involved in replication and maintenance but also in the Tra1 and Tra2 regions which are essential for conjugative plasmid transfer (20, 21). This finding suggests that KorB functions as a regulator protein in the expression of transfer genes.

Molecular cloning of *korB* led to an identifiable gene product. Following optimization of the *korB* translational initiation region the protein could be overproduced in large quantities. In this report we describe the characterization of KorB binding sites in the transfer regions of RP4 and on synthetic substrates by the fragment retention assay on polyacrylamide gels. Furthermore, the contacts of the protein with the sugar-phosphate backbone of the DNA were elucidated by hydroxyl radical footprinting.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Phages and Media

Escherichia coli K-12 strains SCS1 (derived from DH1, ref. 22) or S17-1 (23) were used as hosts for plasmids. Recombinant plasmids were: pMS208 (24); pJF166uΔ5 (25); pWP471 consists of pMS119EH Δ[*Xba*I-*Sph*I, kb 0.028–0.050] containing a 40-bp *Xba*I-*Nde*I fragment of pT7-7, that encloses the ribosomal binding site of T7 gene *10*, a synthetic 19-bp *Nde*I-*Hae*II adaptor and a 668-bp *Hae*II-*Nsph*I fragment of *Sph*I F fragment, kb 43.40–46.73 of the RP4 standard map (24); pJF162: pJF118EH Ω[*Sma*I; RP4, *Apa*I-*Dde*I, kb 55.70–51.43 of RP4], pML40 (21) and pDB776: pT7-7 Ω[*Nde*I; RP4, *Nde*I D (kb 16.83–20.00 of RP4)]; pMS470Δ8 consists of pMS119EH Δ[*Xba*I-*Sph*I, 0.028–0.050] containing a 40-bp *Xba*I-*Nde*I fragment of pT7-7,

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that encloses the ribosomal binding site of T7 gene *10*, a synthetic 10-bp *Nde*I-*Ava*I adaptor and an *Ava*I-*Sph*I fragment corresponding to kb 40.22−38.86 of R751 standard map (26, 27). Expression systems were: $P_{tac}/lacI^{Q}$ vectors pJF118EH (28); pMS119EH (pJF119EH Δ[*Nru*I-*Nde*I; 1.324 kb, ref. 28); pT7-7 (obtained from Stanley Tabor, Harvard Medical School) is a derivative of pT7-5 (29) containing the *ϕ10* promoter (one of six strong T7 promoters that is located upstream of the T7 gene *10*, ref. 30) and the translation initiation site for T7 gene *10* (kb 22.880−22.998 of T7 DNA, ref. 30) inserted between kb 2.065−4.360 of pBR322 (31). This plasmid also has the polylinker sequence of pUC12, inserted downstream of the initiation codon. Phage M13mp18 and recombinants derived were propagated in XL-1 Blue (32). Cells were grown in a shaking water bath at 37°C under the following conditions: YT-Medium (10 g of Tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) was supplemented with 3-(*N*-morpholino)propanesulfonic acid, sodium salt (25 mM, pH 8.0), glucose (0.1%, wt/vol) and thiamine-HCl (25 μg/ml). When appropriate, antibiotics ampicillin, sodium salt (100 μg/ml) or tetracycline-hydrochloride (10 μg/ml) were added.

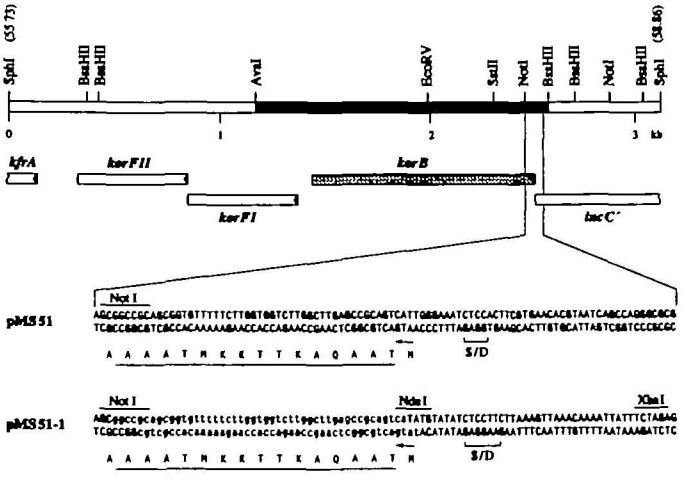


Fig. 1. Construction of a *korB* overexpressing plasmid. *Upper part:* The physical and genetic map of the RP4 *Sph*I fragment H is shown and restriction sites relevant for the cloning procedure have been included. Coordinates (kb) on the RP4 standard map are given at both ends of the fragment. The hatched and the bold portions correspond to fragments applied for cloning. The genetic loci are designated *incC'*, a gene expressing weak incompatibility towards IncP replicons (40), *korB*, the structural gene of KorB, *korFI* and *korFII*, genes encoding transcriptional repressors (11), *kfrA*, a gene locus regulated by *korF* gene products (41). Genes are drawn as bars, their direction of transcription is indicated by arrowheads. The *korB* gene is shaded. *Lower part:* Sequences flanking the *korB* initiation codon in the recombinant plasmids pMS51 and pMS51-1. Insertion of the filled-in 1407-bp *Ava*I-*Bss*HII fragment of pMS208 (hatched and bold portions of the physical map) including the original *korB* ribosomal binding site into the *Sma*I site of the $P_{tac}/lacI^{Q}$ expression vector pMS119EH, resulted in pMS51. pMS470Δ8, a derivative of pMS119EH, containing the T7 gene *10* ribosomal binding site, was used to construct pMS51-1. The 1333-bp *Not*I-*Hin*dIII fragment of pMS51 corresponding to the hatched portion of the physical map, was inserted into the *Nde*I-*Not*I digested pMS470Δ8 using two synthetic oligodeoxyribonucleotides as adaptor to restore the 5'-portion of *korB* gene. Ribosomal binding sites are indicated by S/D. The nucleotide sequences of synthetic oligodeoxyribonucleotides employed are marked lowercase. The *korB* initiation codon is shown by an arrow on pMS51 as well as on pMS51-1. The amino acid sequence of KorB was deduced from the nucleotide sequence (11, 14, 15). The underlined N-terminal part was confirmed by protein microsequencing.

DNA Techniques

Standard molecular cloning and labeling techniques were performed as described (33). DNA was sequenced by the dideoxynucleotide chain termination method (34) using [α-^{35}S]dATP (35) and 7-deaza-dGTP (36). Synthetic oligodeoxyribonucleotides were used as adaptors.

Fragment Retention Assay

DNA fragments were incubated with KorB protein for 30 min at 37°C in total volume of 20 μl of buffer containing 20 mM Tris-HCl (pH 7.6), 50 mM NaCl, and 10 μg/ml bovine serum albumin. For analysis of protein-DNA complexes, samples were electrophoresed in non-denaturating 3.5% polyacrylamide gels at 8 V/cm.

Protein Analysis

Cultures were centrifuged (4,000×g, 10 min) and one A_{600} unit of cells was resuspended in a volume of 30 μl 100 mM Tris-HCl (pH 6.8), 6% (wt/vol) sodium dodecyl sulfate (SDS), 12.5% (wt/vol) glycerol, and 10% (vol/vol) 2-mercaptoethanol. Suspensions were incubated 5 min at 60°C, 2 min at 100°C and centrifuged (81,000×g, 30 min). The supernatants were electrophoresed in SDS/15% (wt/vol) polyacrylamide gels (7.5 V/cm). By the aim of microsequencing its N-terminus, protein was electrophoretically transferred from a gel onto a PVDF-membrane (37). The protein containing portion of the membrane was excised and applied to sequencing in a model 477A of pulsed-liquid phase sequencer (Applied Biosystems) equipped with a model 120A PTH-amino acid analyzer (38).

Solid Phase Immuno Assay

Proteins were electrophoresed on SDS/15%(wt/vol) polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (12 V/cm, 90 min; ref. 39), followed

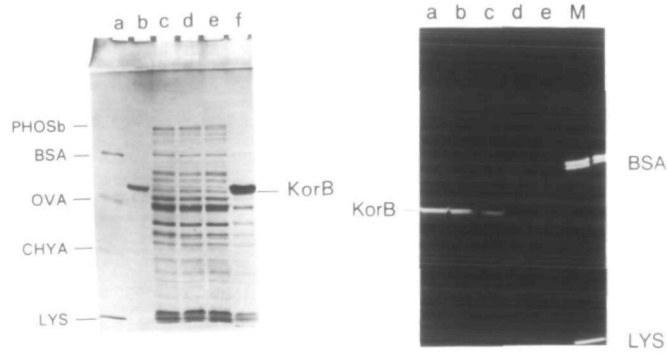


Fig. 2. Overproduction of RP4 KorB. *(Left)* Electrophoresis of cell extracts in a SDS/15% (wt/vol) polyacrylamide gel. Lanes: a, size markers phosphorylase b (PHOS b, 2 μg), 97.4 kDa; bovine serum albumin (BSA, 1 μg), 68 kDa; ovalbumin (OVA, 2 μg), 43.6 kDa; chymotrypsinogen A (CHY A, 1 μg), 25.7 kDa; lysozyme (LYS, 1 μg), 14.3 kDa; b, purified KorB protein (3 μg); crude cell extracts: c and d, SCS1(pMS51); c, non-induced; d, IPTG-induced; e and f, SCS1(pMS51-1); e, non-induced; f, IPTG-induced. *(Right)* Solid phase immunoblot assay of native and overproduced KorB. Extracts of SCS1 cells carrying various plasmids were electrophoresed in a SDS/15% (wt/vol) polyacrylamide gel. Anti-KorB serum raised in a rabbit was diluted 1:1000. Lane a, purified KorB (40 ng); b, pMS51-1, IPTG-induced (1 μl cell extract, 1:100); c, RP4 (5 μl cell extract); d, without plasmid; e, pMS119EH, IPTG-induced; M, molecular mass standards bovine serum albumin (BSA) and lysozyme (LYS) labeled with fluorescein isothiocyanate.

by reaction with anti-KorB serum (1:1000, 2 h) and then with dichlorotriazinylamino-fluorescein-conjugated goat anti-rabbit IgGs (Jackson ImmunoResearch; 1:100, 1 h).

RESULTS

Molecular Cloning of the RP4 *korB* Gene

The *korB* gene of RP4 is part of the *korA-korB* operon mapping between kb-coordinates 57.09 and 58.16 on the RP4 standard map (14, 15, 16, 24, 26). Plasmid pMS208 carrying the RP4 *Sph*I H fragment, served to isolate a 1407-bp *Ava*I-*Bss*HII fragment containing the complete *korB* gene (Fig. 1). This fragment was inserted blunt ended into the *Sma*I site of the multicloning region of the P_{tac} expression vector pMS119EH resulting in pMS51. Clones carrying recombinant plasmids with the *korB* containing fragment in the appropriate orientation were inspected for protein overproduction after IPTG induction on denaturating polyacrylamide gels (Fig. 2 *left*, lane c, d). The gel pattern of extracts of IPTG-induced cells shows a weak additional band corresponding to a protein of about 53 kDa. A molecular mass of 52 kDa has been estimated for KorB protein previously (15).

Construction of a KorB Overproducing Strain

Initial attempts to purify the overproduced protein resulted in sufficient material for microsequencing its N-terminus (Fig. 1). Except for the missing methionine in the first position, the following 15 residues were found to be identical to the N-terminus deduced from the nucleotide sequence (14, 15, 16). To improve overexpression of the *korB* gene achieved with SCS1(pMS51), the *Not*I-*Hin*dIII fragment (1296 bp) of pMS51 was isolated and fused to the efficient translation initiation signal of phage T7 gene *10* via synthetic oligodeoxyribonucleotide adaptors resulting in plasmid pMS51-1 (Fig. 1). The restoration of the structural gene was confirmed by restriction analyses with *Nde*I and *Not*I as well as by nucleotide sequencing (not shown). Upon IPTG-induction of bacteria containing plasmid pMS51-1, KorB overproduction was increased 10-fold as compared to strains harboring pMS51, facilitating the purification procedure of KorB (Fig. 2 *left*, lanes e, f; Fig. 3). A polyclonal KorB-specific antiserum was prepared using the purified protein as antigen. Both in SCS1(pMS51-1) and SCS1(RP4) cell extracts the crossreacting product, which is not detectable in cells harboring only the vector plasmid, corresponds to the size of the purified KorB protein (Fig. 2 *right*, lanes a-c). This suggests that KorB protein overproduced from SCS1(pMS51-1) is identical to the native RP4 gene product. From immunoblotting data, the presence of approximately 1000 KorB molecules per RP4 harboring cell was deduced.

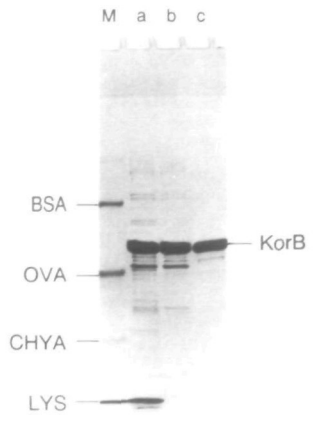


Fig. 3. Purification of KorB protein. Samples containing KorB protein were electrophoresed in a SDS/15% (wt/vol) polyacrylamide gel. A photograph of the gel stained with Coomassie Brilliant blue R-250 is shown. Lanes a–c contain fractions I–III. a, I (25 μg); b, II (20 μg); c, III (10 μg). Lane d: For molecular mass standards see Fig. 2.(*left*).

Purification of KorB Protein

KorB protein was purified in a two-step procedure involving initial column chromatography of Brij-lysozyme extracts of IPTG induced SCS1(pMS51-1) cells on DEAE-Sephacel. Additional affinity chromatography on heparin-Sepharose CL-6B as an established purification step for DNA binding proteins rendered the KorB protein 98% pure achieving an exceptional high recovery of 46% (Table 1 and Fig. 3).

Physical Properties of the KorB Protein

The N-terminal sequences were determined to be NH_2-TAAQALTTLLNTAAA- (Fig. 1). This matches the amino acid sequence predicted from the nucleotide sequence following the ATG codon (15, 16, 31). The methionine residue specified by the initiation codon was not present. The apparent molecular mass measured on denaturating polyacrylamide gels was found to be 53 kDa, which is much higher than that calculated from sequence data (39,011 Da). Such a discrepancy was observed for several acidic proteins including the KorB protein (15). The calculated isoelectric point of KorB is 4.5 and the net charge of the protein at pH 7.0 is −21.

Table 1. Purification of KorB Protein

Fraction	Step	Protein mg	Recovery %	Purity[1] %
I	Crude extract[2]	1407	100	57
II	DEAE-Sephacel	884	96	88
III	Heparin-Sepharose	380	46	98

[1]From laser densitometrical measurements on gels stained with Coomassie Brilliant blue R-250

[2]From 16 g of cells (wet weight)

Cultures (1.2 liters) of SCS1(pMS51-1) were grown at 37°C in YT medium in a shaking water bath. At A_{600}=0.5, IPTG was added to a concentration of 1 mM. Shaking was continued for 4 h. After centrifugation (4,000×g, 10 min), cells (1 g, wet weight) were resuspended in 5 ml of 30 mM spermidine tris(hydrochloride), 200 mM NaCl, and 2 mM EDTA (pH 7.5), and frozen in liquid N_2. Frozen cells (16 g in 60 ml) were thawed and 90 ml of 10% (wt/vol) sucrose in 100 mM Tris-HCl (pH 7.6), 3 ml of lysozyme (70 mg/ml), 6.75 ml of 10% (wt/vol) Brij-58, and 140 ml 5% sucrose (wt/vol) in 100 mM NaCl, 50 mM Tris-HCl (pH 7.6) were added. After 1 h at 0°C the highly viscous lysate was centrifuged at 70,000×g for 90 min. *DEAE-Sephacel chromatography*. The supernatant (280 ml, fraction I) was applied to a DEAE-Sephacel column (3.8×9.5 cm) equilibrated with 105 ml of buffer A (20 mM Tris-HCl [pH 7.6], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% [wt/vol] glycerol). The column was washed with 250 ml of buffer A. Proteins were eluted with a 750 ml gradient from 50 to 600 mM NaCl in buffer A at a flow rate of 100 ml/h. KorB protein eluted around 200 mM NaCl (fraction II, 210 ml). *Heparin-Sepharose chromatography*. Fraction II was diluted to 850 ml by addition of buffer A (NaCl omitted) and applied to heparin-Sepharose Cl-6B column (1.6×12.5 cm) equilibrated with buffer A. Proteins were eluted with a 1000 ml gradient from 50 to 600 mM NaCl in buffer A at a flow rate of 100 ml/h. KorB eluted at about 350 mM NaCl. The peak fractions (360 ml) were pooled, concentrated by dialysis at first against 20% (wt/vol) polyethylene glycol 20,000 and 10% (wt/vol) glycerol in buffer A, then dialyzed against buffer A containing 50% (wt/vol) glycerol, and stored at −20°C without loss of specific DNA binding ability for at least one year (fraction III, 5.6 ml).

To address the question whether native KorB protein exists in an oligomeric form in solution, cross-linking studies were performed using glutaraldehyde (Fig. 4). A low protein concentration (1.7 μM) ensured that cross-linking of KorB monomers contained within a potential complex was favoured. At increasing concentrations of glutaraldehyde the amount of the monomer diminished and a band corresponding to the dimer's apparent molecular mass was detected. Even at a 300-fold molar excess of reagent, when monomers were almost quantitatively converted to dimers, no bands equivalent to higher molecular masses were visible. Therefore, KorB protein forms predominantly a dimer in solution.

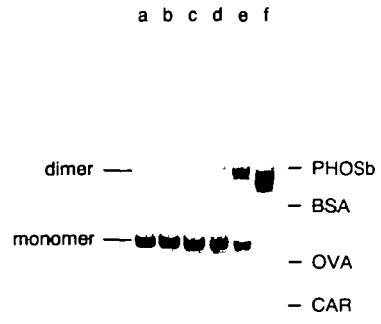
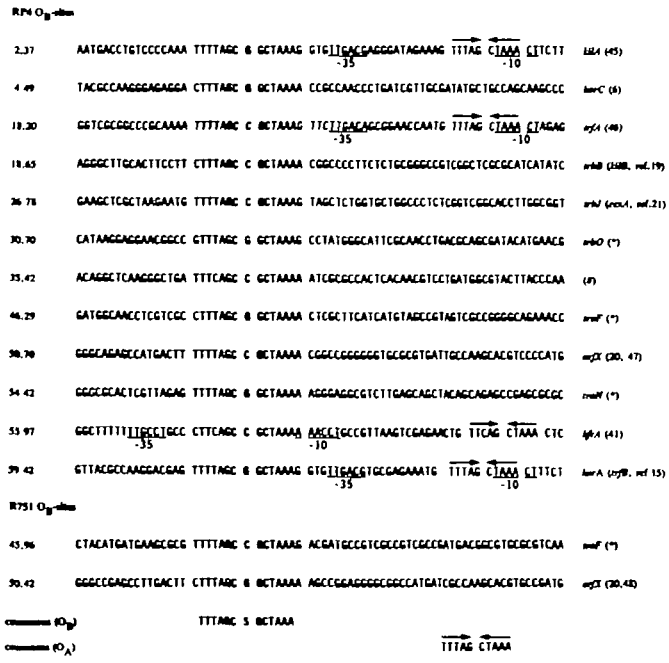


Fig. 4. Cross-linking of KorB monomers. KorB protein (1.7 μM) was treated with increasing concentrations of glutaraldehyde in a buffer consisting of 20 mM NaH_2PO_4 (pH 7.0), 100 mM NaCl, 1 mM DTT and 5% glycerol. After 1 h incubation at 37°C, the reaction was stopped by adding urea to a concentration of 5 M. The products were reduced, heat-denatured and electrophoresed on a 5% (wt/vol) polyacrylamide gel containing 0.1 M NaH_2PO_4 pH 7.2, 0.1% (wt/vol) SDS, and 6 M urea. Lane a, glutaraldehyde omitted; b, 10 μM; c, 50 μM; d, 100 μM; e, 200 μM; f, 500 μM glutaraldehyde. Migration positions of molecular mass standards are marked and given as in Fig. 2. (CAR, carbonic anhydrase, 28 kDa).

KorB Protein Binds to RP4 DNA Containing O_B-Sites

Proposed KorB binding sites are called O_B according to Thomas and Smith (42). Sequences and locations of O_B-sites on the RP4/RK2 genome are shown in Fig. 5. Computer analysis of nucleotide sequences of RP4 regions Tra1 and Tra2 have revealed six O_B-type palindromic sequences (Fig. 5, ref. 43). In contrast to O_B-sites previously found, none of the O_B-sites in transfer regions occur close to a promoter (Fig. 5). O_B-sites map in the Tra2 region upstream of the *trbB* (*kilB*) gene and within the *trbJ* (*eexA*) gene as well as between *trbN* and *trbO*. In the Tra1 region, O_B-sites exist within *traF* as well as between *traJ* and *traI* and upstream of the *traN* gene. Additionally, two O_B-sites have been found in the Tra1 region of the distantly related IncPβ plasmid R751 matching those of RP4 at corresponding positions (2). RP4 and R751 show considerable sequence divergence yet a large degree of functional conservation. The O_B-sequences reveal a 13-bp consensus that shows an interchangeability between C and G on its center of symmetry. In some cases, the consensus is flanked by an A or T resulting in a 15-bp inverted repeat structure (Fig. 5). The only two mismatches occur in one arm of the O_B-sequences located upstream of *kfrA* and between Par/Mrs and IS*8*, where a C instead of a T at corresponding positions was found.



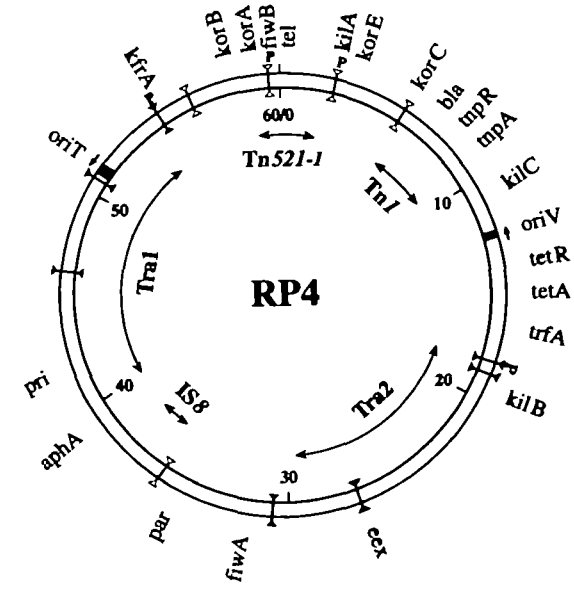


Fig. 5. Nucleotide sequences and map locations of RP4 KorB binding sites. *(Left) List of IncP O_B-sites.* RP4 coordinates (kb) of the O_B-sequence regions are at the left, the O_B-sequences enclosing or following gene loci are designated on the right hand side of the list (**manuscript in preparation/# located between* Mrs/Par *and* IS*8*, *H. Schwab, unpublished).* The O_B-sequences including A or T nucleotides on their flanks are rendered prominent by boldface letters, the palindromes additionally by spacing. Potential KorA binding sites (O_A) are also indicated by spacing. The inverted repeats are marked by arrows. The consensus sequences are shown at the bottom of the list. Promoters are indicated by underlining the −35 and −10 regions. *(Right) Map of RP4 O_B-sites.* Locations of O_B-sites are shown by dumbbells; sites studied in this work are designated by filled triangles. Promoters close to O_B-sites are indicated by a P. Genes are designated as described previously (42, 44).

To detect specific KorB-O_B-DNA complexes, the fragment retention assay was applied (Fig. 6; refs. 49, 50). DNA fragments carrying O_B-sequences were obtained by digestion of recombinant plasmids containing parts of Tra1 or Tra2 regions with suitable enzymes to generate a well-resolved restriction pattern. DNA was incubated with KorB protein at molar protein-DNA ratios up to 24:1 and both free DNA and the KorB-treated samples were analyzed in non-denaturating gels. The pattern of pDB776n, a plasmid containing the DNA region between the *trfA* operon and *trbB* (*kilB*) gene reveals, that in presence of KorB two fragments show significant retardation (Fig. 6, lanes I, I'). Both fragments carry an O_B-sequence. In the other patterns, all fragments shifted include the 13-bp sequence. Plasmids applied are pML40, containing DNA of *trbJ/trbK* (*eexA/eexB*) genes (Fig. 6, lanes II, II'), pJF166uΔ5 comprising genes *traJ* and the 5'-terminal part of *traI* (Fig. 6, lanes III, III') and pJF162 embodying a region between genes *traO* and *traN* (Fig. 6, lanes IV, IV'), and the *traF* and *trbN/trbO* containing plasmids (not shown). The retardation pattern of pJF162 shows, that the shifted fragment E splits into two separable species that could be induced

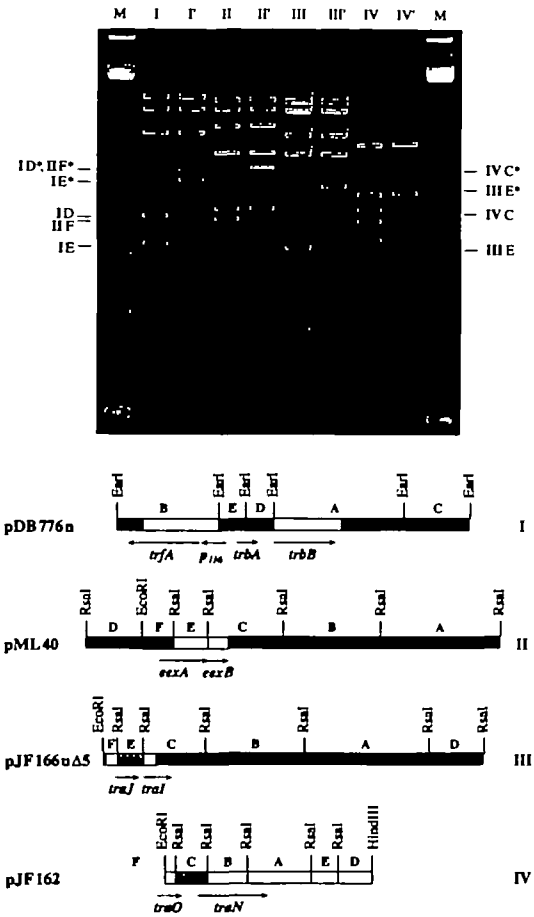


Fig. 6. Gel electrophoresis of KorB protein-O_B-DNA complexes. Recombinant plasmids that carry DNA subfragments of RP4 transfer regions containing the proposed O_B-sequences were digested with restriction endonucleases as indicated and incubated with KorB. Maps of the resulting patterns visualized on the gel are shown at the bottom of the figure. Fragments are designated in alphabetical order according to their length. Applied plasmids are: pDB776n (I), cleaved by *Ear*I; pML40 (II) and pJF166uΔ5 (III), *Eco*RI and *Rsa*I digested; pJF162 (IV), *Eco*RI-*Hin*dIII fragment, *Rsa*I digested. The fragments containing O_B-sequences are hatched. The vector portions are indicated by bold black segments. Genes are shown by arrows. Fragments corresponding on map and photograph of the gel are designated I-IV for both their plasmid origin and their individual fragment name character. Bands corresponding to KorB-DNA complexes are marked by asterisks. Lanes: I/I', pDB776n, II/II', pML40; III/III', pJF166uΔ5; IV/IV', pJF162; I, II, III, IV protein omitted, I', II', III', IV' DNA fragments in the presence of KorB protein; M, 123-bp ladder.

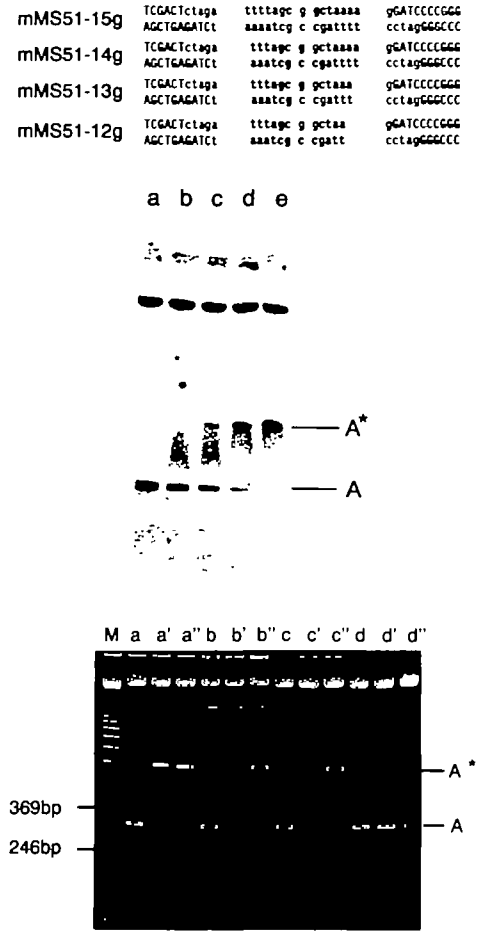


Fig. 7. Complex formation between O_B-sequences and KorB protein. *(Upper) Construction of recombinant M13 phages.* M13 mp18 was cleaved by *Xba*I and *Bam*HI and an oligodeoxyribonucleotide adaptor, that contains a 15-, 14-, 13-, or 12-bp O_B symmetric arrangement carrying a G in its center of symmetry, was inserted resulting in recombinant phages mMS51-15g, -14g, -13g and -12g, respectively. Sequences corresponding to the M13mp18 multicloning region are indicated by capital letters, inserted oligodeoxyribonucleotide adaptors are designated lowercase. The remaining part of the O_B-sequence is indicated boldface. Recombinant phages are designated on the left hand side. *(Middle) Gel electrophoresis revealing the affinity of KorB protein to O_B-DNA.* RF-DNAs of selected phages were digested with *Ava*II and *Pvu*I supplying a fragment that carried the O_B-sequence, 5'-labeled with ^{32}P and incubated with increasing amounts of KorB protein and electrophoresed. Bands corresponding to the free DNA and to the DNA-protein complex are designated A and A*, respectively. Lanes: a-e, mMS51-13g (from the total amount of 1,9 μg DNA applied 0,3 μg were labeled with 5 μCi/μg) a, KorB protein omitted; b, 60 ng c, 90 ng d, 120 ng; e, 180 ng. *(Lower) Gel electrophoresis of DNA containing different symmetric arrangements of O_B treated with KorB protein.* The recombinant mMS51 phages described are digested with *Pvu*II supplying fragments of about 330 bp that contains the O_B-arrangements. Lanes: a, b, c, d, KorB protein omitted; a', b', c', d', digestion of recombinant M13 phages (1,9 μg) incubated with 110 ng of KorB protein; a", b", c", d", digestion of recombinant M13 phages (1,9 μg) incubated with 350 ng of KorB protein; a, a', a", mMS51-15g; b, b', b", mMS51-14g; c, c', c", mMS51-13g; d, d', d", mMS51-12g; M, DNA fragment size marker. For designation of bands see above.

by an additional O_B-like structure (TTTTCGCnnnnn-CGATTT) on the fragment (Fig 6, lanes IV, IV'). Moreover, the vector fragment D of pJF162 shows affinity for KorB protein. Although this fragment does not contain an O_B-sequence, inspection of its DNA sequence revealed a run of nucleotides (GCG-AAAAnnnnnnnnn-TTTTGCA) that might function as an inefficient recognition signal.

A 13-bp Consensus Sequence Constitutes the KorB Binding Site

To determine the minimal size of the palindrome that fulfills the requirements for KorB binding, a series of complementary oligodeoxyribonucleotides was synthesized resulting in 15-, 14-, 13-, 12-bp or smaller symmetric arrangements of the O_B-palindrome with a G or a C as center of symmetry, respectively. The adaptors were inserted into the multicloning region of M13mp18, so that the adjacent DNA for each of the derivatives was identical (Fig. 7). Recombinant phage DNA was sequenced to confirm the sequence of the synthetic oligodeoxynucleotides (not shown). After suitable cleavage of the resulting recombinant RF DNAs the fragment retardation assay was applied (Fig. 7). The electrophoretic analysis revealed that mobility of the fragments carrying 15-, 14, or 13-bp O_B-sequences was reduced. Fragments containing a 12-bp or smaller central part of O_B do not form the specific O_B-KorB-complexes under the conditions applied (Fig. 7). These results were obtained regardless whether the center of symmetry was occupied by G or C. Therefore, the 13-bp consensus sequence constitutes the minimal region for recognition by KorB protein.

To quantify the affinity of KorB to O_B-DNA, specific complexes were formed at a variety of molar protein/DNA ratios using a mixture of unlabeled and ^{32}P-labeled 509-bp fragment that contains the 13-bp O_B-consensus (Fig. 7). Following electrophoresis, KorB-complexed and free DNA was evaluated quantitatively applying the storage phosphor technology (51). The apparent equilibrium constant K_{app} of KorB and O_B was estimated as described by Halfman and Nishida (52), using Scatchard plot analysis (53). K_{app} is approximately 70 nM at pH 7.8 and 37°C for a KorB dimer. The analysis also reveals that four KorB molecules might bind to one O_B-site. KorB protein does not show any significant deviation on its binding behavior to a 15-bp O_B-sequence (not shown).

KorB Protein Embeds O_B and Alters the Structure of the Target DNA

To evaluate the contacts of KorB protein with the sugar-phosphate backbone of its target, a hydroxyl radical footprinting analysis was performed. KorB-O_B complexes assembled on a DNA fragment containing the 15-bp O_B-sequence were subjected to limited hydroxyl radical degradation. The resulting cleavage pattern was analyzed for protected nucleotide positions by densitometrical tracing of the autoradiographs (Fig. 8A and 8B). The KorB footprint on both strands encompasses approximately 22 nucleotides including the 15-bp O_B-target and part of the surrounding restriction sites *Bam*HI and *Xba*I (Fig. 9). In accordance to the fragment retention data this result suggests that KorB recognizes the minimal 13-bp O_B-sequence, binds to it, and in addition, contacts unspecifically deoxyribose residues of nucleotides flanking O_B. In the upper strand, there is a continuous stretch of protection, whereas in the lower strand two times a band of enhanced intensity interrupts the protected patch. This indicates, that the corresponding deoxyribose residues become hyperreactive to hydroxyl radicals upon binding of KorB (Fig. 8 B and C, lanes d and e). The hypersensitive nucleotide residues are interspaced by 7 bp. The sites are arranged asymmetrically with respect to the symmetry center of the O_B inverted repeat sequence (Fig. 9). This finding suggests that as compared to free DNA, the DNA within the KorB-O_B complex is altered in its secondary structure. The unusual large domain of protection in both strands covering approximately two helical turns of B-DNA suggests i) that more than one KorB monomer is involved in complex formation and ii) that KorB protein embeds O_B by binding round about the DNA double helix.

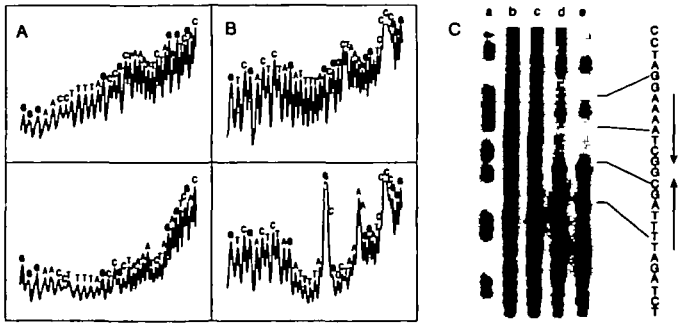


Fig. 8. Hydroxyl radical protection pattern of O_B. The *Eco*RI-*Bst*NI fragment of recombinant phage mMS51-15g (see Fig. 7) was labeled at either 5'-end applying T4 polynucleotide kinase. Complex formation was done as given in *Materials and Methods*. Limited cleavage of the complexes by hydroxyl radicals was performed as described by Tullius and Dombroski (54) with slight modifications (20). In contrast to the latter reference, the H_2O_2 concentration applied for cleavage was reduced tenfold. Hydroxyl radical generated cleavage products were separated on 8% denaturing sequencing gels and the cleavage pattern was visualized by autoradiography. Panels A and B: Laserdensitometrical scans of KorB footprints generated by hydroxyl radicals. A: upper strand; B: lower strand (Fig. 9). *Upper:* Cleavage of DNA in the absence of KorB. *Lower:* Cleavage of KorB-O_B complexes. The letters above each peak represent the base whose deoxyribose residue was degraded by reaction with the radical. The sequences are given from left to right in 5' to 3' direction. Panel C: Autoradiograph of the KorB footprint obtained from the lower strand (Fig. 9). Lanes are: a, Maxam-Gilbert purine specific sequencing reaction; b, KorB omitted; c, 7.5 ng, d, 75 ng; e, 750 ng KorB protein.

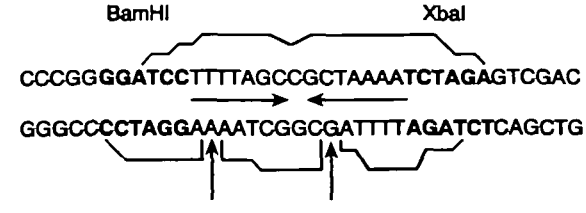


Fig. 9. KorB protected nucleotides at O_B. The nucleotide sequence of the O_B-containing adaptor carried by mMS51-15g between the *Bam*HI and *Xba*I site (bold face letters) and adjacent vector sequences are shown. The inverted repeat is represented by arrows between the sequence lines. Note that the outermost A/T basepairs do not belong to the O_B consensus sequence. The brackets outline nucleotides whose deoxyriboses are protected from radical attack (± 1 nucleotide). The distance between the brackets and the corresponding sequence row schematically indicates the relative strength of protection at the respective sugar residue. Vertical arrows designate nucleotides whose deoxyriboses became hypersensitive against radical cleavage upon KorB has bound.

DISCUSSION

We have shown that the purified KorB protein recognizes and binds to a 13-mer palindromic sequence in RP4. This is consistent with data of genetic experiments demonstrating that KorB is one of the key regulator proteins of replication and maintenance functions of RP4. KorB acts by negatively controlling transcription of several cistrons including the *korA-korB*, *trfA* and *kilA* operons (1, 15, 16). Each of the associated promoters contains an O_B-sequence. The presence of *korB* however, causes only weak transcriptional repression. When *korA* was provided in addition to *korB*, the effect of *korB* increases strongly indicating that KorB and KorA proteins act as a corepressor system that synergistically enhances repression (16). The proposed binding sites for KorA (O_A) and O_B are approximately 20 bp apart. It remains to be shown whether both proteins interact physically with each other on the DNA. Formation of a KorA-KorB-DNA complex more efficiently could prevent (i) binding of RNA polymerase or (ii) initiation of transcription, or (iii) it could dissociate the transcribing enzyme-DNA complex more readily.

An additional clue to biological functions of KorB might consist in the observation that recombinant plasmids carrying the functional Tra2 region can only be stably maintained in cells harboring the *korA-korB* operon in *trans*. Since KorB for itself was observed to reduce transcription in the direction of Tra2 gene loci and we have found three O_B-sites in the Tra2 region, a central role of KorB in the regulation of Tra2 genes is suggested (19, 44, 55). Recently, it was proposed that *korB* also plays a role in the partitioning system of the plasmid, because the organization of the operon encoding *korA, incC* and *korB* resembles those coding for partitioning functions in P1 and F (56).

Within the known 60-kb sequence of the plasmid RP4, we found twelve O_B-sites consisting of a highly conserved 13-bp inverted repeat showing dyad symmetry. The center is always a G/C or C/G base pair. Ten out of twelve O_B-sequences perfectly fit the consensus, whereas the remaining two show a T→C mismatch at identical positions (Fig. 5). We did not find any more symmetric arrangements that correspond to O_B allowing additional mismatches. Eight out of twelve O_B-sites apparently are not associated with promoter regions of the *E. coli* σ^{70}-type. Therefore, in addition to the repression of transcription, KorB might have further functions. It could not be ruled out that KorB requires a co-factor to act as a transcriptional terminator or attenuator. It is also possible that KorB binds to mRNA playing a role in controlling translation. Interestingly, on both strands the O_B-consensus encompasses the translation stop signals TAG and TAA in different reading frames. Thus, mRNA can be translated across an O_B-site in only one reading frame.

The remarkable low electrophoretic mobility of KorB-O_B complexes formed on relatively long (>300 bp) DNA fragments (Figs. 6 and 7) might not be caused by simply binding of a KorB monomer. Compared to free DNA, the mass of the nucleoprotein structure formed would increase only to a small extent. Furthermore, the high negative net charge of KorB should counteract the effect of increasing mass. Interestingly, the estimation of the apparent dissociation constant by a Scatchard plot (53) reveals that likely four KorB molecules are contributing to complex formation. Since KorB protein exists in solution as a dimer, it is conceivable that two dimers bind to one O_B-sequence.

Typical hydroxyl radical footprints of relative small DNA binding proteins like repressors contain small protected patches comprising approximately four nucleotides separated by a small segment still cleavable by the radical. In contrast, KorB protects a continuous stretch of about 22 nucleotides on both strands. Another untypical feature of the KorB-O_B complex is the existence of two sharply defined deoxyriboses hypersensitive to radical-initiated degradation, which are located in the same strand, asymmetrically to the rotation center of the inverted repeat. This may indicate an asymmetrical structure of the complex forcing the respective sugar moieties into an unfavorable energy-rich conformation that may cause hyperreactivity. For the CAP-DNA complex, it was shown that the 90° bend of the complexed DNA almost entirely results from two kinks (57). Despite the finding that none of the deoxyribose residues within the CAP-DNA complex show enhanced reactivity with hydroxyl radicals (58) it is conceivable that the hypersensitive deoxyriboses within O_B are due to an unusual DNA conformation that is different from kinks induced by KorB binding. However, comparison to high resolution footprints of other DNA-binding proteins reveals that KorB seems to have binding properties not yet described for other polypeptides interacting with DNA.

Helix-turn-helix (HTH) motifs found in many specific DNA-binding proteins, in several cases were demonstrated to be the domains responsible for specific protein-DNA interaction (59, 60). Such motifs were also suggested for KorB as possible DNA-binding domains (15, 16). Analysis of the deduced amino acid sequence of KorB by an improved method for detecting HTH DNA-binding motifs did not reveal a structure with a significant standard deviation score (SD score, ref. 61). However, we could detect a candidate of indifferent SD score (2.2, amino acids pos. 172−193) already mentioned (16). Further predictions on secondary structure obtained by the method of Chou and Fasman (62) do not reveal a HTH-domain in the corresponding region. KorB lacks cysteine and histidine residues; therefore a 'zinc finger' (63) as an O_B-recognizing segment can be excluded. Also neither a 'bacterial zipper' (64) nor a 'leucine zipper' (65) associated with an adjacent basic region was found. Thus, more different complex structural features than those mentioned might be responsible for KorB protein to recognize and stably bind to the target.

Three out of the twelve O_B-sites found on RP4 were shown to play a role in downregulating transcription in the presence of KorB protein. Thus, the KorB/O_B-system is another example of regulatory arrangements with conserved small DNA segments scattered on a genome, which are of functional relevance in combination with an appropriate protein bound: (i) In phage P1, at operator (Op) sites *c*1 protein represses transcription of a variety of cistrons (66); (ii) in *E. coli*, at CAP-sites CAP protein activates transcription of different operons encoding enzymes required in sugar metabolism (67); (iii) in *E. coli*, DnaA protein bound to *DnaA* boxes distributed on the chromosome terminates transcription (68) (iv) the LexA protein which binds to many SOS boxes spread over the *E. coli* chromosome downregulates functions which are expressed efficiently only during the SOS response (69). A feature of all these systems is the conservation of the recognition sequences for the respective proteins. However, the degree of conservation differs: Whereas *c*1 binding regions and SOS boxes do not deviate strongly from the consensus sequence, CAP- and *DnaA* boxes are degenerated. In contrast, the O_B-sites are highly conserved. This implies, that the

proposed regulatory function of KorB should not be modulated by different binding affinities of the protein to the target, provided that adjacent nucleotide sequences do not influence binding of KorB. Slight differences in the immediate neighborhood of the O_B-consensus have no effect on complexation, because KorB binds to fragments containing the 13-bp consensus as well as to the extended derivative of 15-bp (Fig. 7) with indistinguishable binding constants. However, effects of the overall DNA context on complex formation could not be excluded.

The methods for overproduction and purification of KorB protein described in this report facilitate the isolation of KorB in large quantities required for crystallization of the KorB-O_B complex. Solving the crystal structure of the KorB-O_B complex would allow to decide whether KorB interacts with the DNA via a HTH-region or another unknown DNA binding motif.

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