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Sequence of the *relB* transcription unit from *Escherichia coli* and identification of the *relB* gene

Finn Wellner Bech, Steen Troels Jørgensen, Børge Diderichsen[1] and Olle H.Karlström

Institute of Microbiology, University of Copenhagen, Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark

[1]Present address: Novo Research Institute, DK-2880 Bagsværd, Denmark

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***Escherichia coli relB* mutants react to amino acid starvation by several abnormal responses, including accumulation of a translational inhibitor. We have isolated a *relB*-complementing plasmid from the Clarke and Carbon *E. coli* DNA library. From this plasmid we sequenced a 2140-bp segment which included the *relB* gene by the following two criteria: (i) it complements chromosomal *relB* mutations, (ii) the corresponding DNA segment cloned from chromosomal DNA of three *relB* mutants was defective in *relB* complementation. All three mutations fell within an open reading frame of 79 amino acids. A polypeptide of 9 kd compatible with this open reading frame was synthesized in maxicells and is in all probability the product of the *relB* gene. By nuclease S1 mapping we have determined the transcription start and stop of an 870 base transcript of the *relB* gene.**

Key words: *relB*/sequence/transcription mapping

Introduction

When *Escherichia coli* is starved for amino acids, RNA synthesis is greatly reduced (Gallant, 1979). Mutants lacking this so-called stringent regulation are called relaxed. The *relA* type of relaxed mutants are defective in a protein needed to synthesize 3′-pyrophosphoguanosine 5′-pyrophosphate in response to amino acid starvation. This important signal substance has many functions in the cell, one of which is to curtail stable RNA synthesis (Cashel, 1975; Gallant *et al.*, 1976). Relaxed mutants of the *relB* type are much less well understood.

RelB mutants (Lavallé, 1965; Lavallé *et al.*, 1976; Diderichsen *et al.*, 1977; Mosteller, 1978) which map at 34.4 min, are characterized by three phenotypes provoked by amino acid starvation. (i) RNA accumulates during amino acid starvation only after an initial period of ~10 min, during which RNA synthesis is under normal stringent control. This phenotype has been termed 'delayed relaxed' (Lavallé, 1965; Diderichsen and Desmarez, 1980). (ii) When starvation is terminated by addition of a required amino acid, growth is transiently inhibited (Lavallé *et al.*, 1976; Diderichsen and Desmarez, 1980). (iii) During starvation an inhibitor of translation accumulates. The inhibitor, presumably a protein, has been partially purified from a ribosomal wash fraction (Lavallé *et al.*, 1976; Diderichsen and Desmarez, 1980). The growth retardation following starvation appears to be closely correlated to the accumulation of active inhibitor (Diderichsen and Desmarez, 1980). A fourth phenotype, not as directly related to starvation, has been observed: *relB* strains are more sensitive than wild-type strains to several antibiotics that inhibit translation (Mosteller and Kwan, 1976; Diderichsen and Desmarez, 1980).

The present paper reports the nucleotide sequence of the *relB* region and identifies the *relB* gene with a stretch of DNA coding for a 79 amino acid polypeptide.

Results

Cloning of the relB gene

A mixture of the ColE1 plasmids of the Clarke and Carbon (Clarke and Carbon, 1976) colony bank was transferred to a *relB* strain by conjugation. The mixed exconjugants were subjected to a 5-fluorouracil selection for $RelB^+$ (see Materials and methods). Ten Rel^+ strains were tested and found to co-transfer ColE1 immunity and Rel^+ to a *relB* recipient in matings. Plasmids from six of these strains had the same size and the same pattern of restriction sites. One of the six, pBD602, was chosen

Table I. List of strains

Strain	Genotype	Source or reference
BD143	*argA his*Δ*(pro lac) thi nalA relB*102 *supE man*	Diderichsen *et al.* (1977)
BD639[a]	*thyA*112 *relB*35 *deoB/C tna*2	SK375 from Mosteller and Kwan (1976)
BD1020	*argA pheA thi trp relB*101 *rpsL supE tonA lac mal mtl xyl*	Diderichsen and Desmarez (1980)
BD1050	As BD1020, except *relB*102	Diderichsen and Desmarez (1980)
BD1401	*argA pheA his pyr*622 *thi supE* str^r *tonA lac mal mtl xyl*	This paper
BD1402	As BD1401, except *relB*101	This paper
BD2432	BD1401/F′*lac*I^{q1}-*lacZ*::Tn*5*	This paper
BD2433	BD1402/F′*lac*I^{q1}-*lacZ*::Tn*5*	This paper
MC1000		Casadaban and Cohen (1980)
7118	Δ(*pro-lac*)/F′ pro^+,*lac*I^q-*lacZ*ΔM15	Messing *et al.* (1977)
TC943	CSR603[b]/F′*lac*I^{q1}-*lacZ*::Tn*5*	Provided by T. Atlung

[a]*relB*35 were formerly designated *rel*-35 (Mosteller, 1978).
[b]Sancar *et al.* (1979).

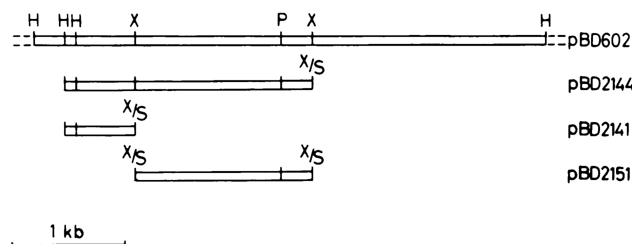

Fig. 1. Chromosomal DNA contained on pBD602 and subclones. The open bars specify the part of pBD602 DNA that lies between restriction sites identified as chromosomal by comparison with the ColE1 map (Armstrong and Helinski, 1977). The stippled lines denote that more chromosomal DNA may be found on pBD602. pBD2144, pBD2141 and pBD2151 were constructed by cloning the shown fragments into either the *Hin*dIII-*Sal*I sites or the *Sal*I site of pBR322. H: *Hin*dIII, X: *Xho*I, P: *Pst*I, X/S and S/X: *Xho*I-*Sal*I or *Sal*I-*Xho*I hybrid sites.

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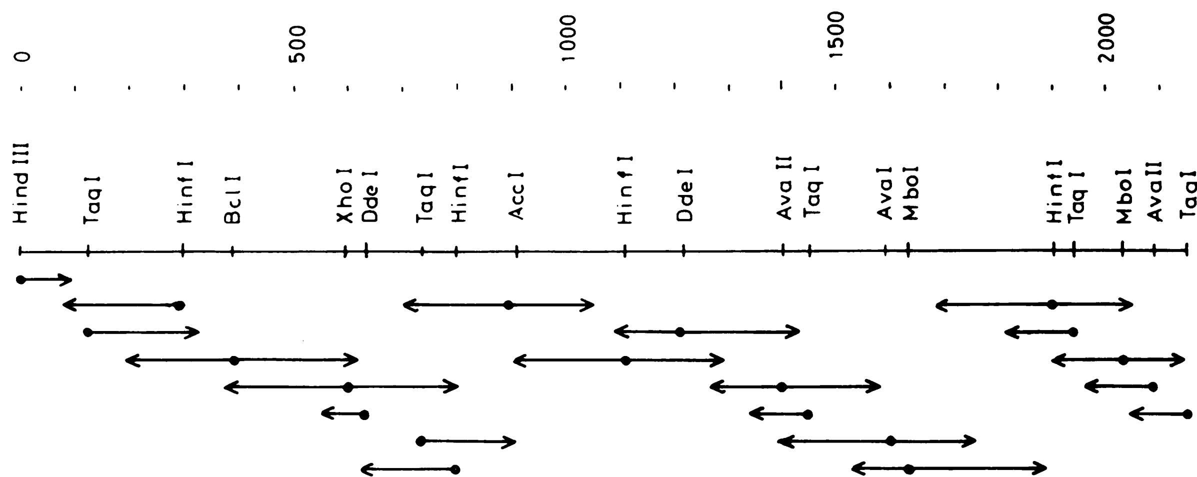

Fig. 2. Sequencing strategy for the chromosomal DNA from pBD2144. Only restriction sites used for labeling are indicated. Restriction fragments were ^{32}P-labeled at their 3′ end and the extent of sequence read from each site is indicated by arrows. The scale at the top shows number of base pairs, with position 1 arbitrarily assigned to the 4th base pair in the *Hind*III site.

for further study. pBD602 completely suppressed the RelB phenotypes growth retardation, RNA accumulation, and antibiotic sensitivity of strains harboring the *relB* mutations *relB*101, *relB*102, *relB*103 or *relB*35 (data not shown). Various derivatives of pBD602 were constructed by subcloning of fragments into pBR322 (Figure 1). Of these, pBD2144, which contains 2.2 kb of chromosomal DNA, suppressed the RelB phenotype. Since pBD2141 and pBD2151 did not, sequences on both sites of the *Xho*I site must be necessary for expression of the *relB* gene.

Nucleotide sequence determination

Using the Maxam and Gilbert protocol and 3′ end ^{32}P-labeling we determined the nucleotide sequence of the chromosomal DNA of pBD2144. The strategy is outlined in Figure 2 and the sequence is shown in Figure 3.

An open reading frame (designated ORF 1) from nucleotide 494 to nucleotide 730 is indicated in the sequence. It is preceded by a typical ribosome-binding site (Shine and Dalgarno, 1974), and 'Testcode' (Fickett, 1982) evaluated it as coding sequence with P=1.00 and a 'testcode indicator' of 1.19. Its position straddling the *Xho*I site suggested that it might be the *relB* gene. A terminator-like structure, consisting of a GC-rich region of dyad symmetry followed by a T-rich region is situated between 1306 and 1347.

Overlapping the stop codon of ORF 1 is a start codon of another open reading frame, ORF 2, and 70 bp downstream from the end of ORF 2 is the start codon of a third one, ORF 3. Both score as coding (test code indicators of 1.15 and 1.19, respectively). Just following the terminator-like sequence is a fourth open reading frame, ORF 4, which elicits the response 'no option' (test code indicator 0.80). All of these ORFs are indicated in Figure 3. In the present paper we shall however focus exclusively on ORF 1.

In vivo test of promoter activity

In a search for a possible promoter for ORF 1, *Taq*I fragments from pBD2144 were cloned in a promoter-detection plasmid, pTAC909. A 430-bp *Taq*I fragment from pBD2144 starting at position 167 and ending in the *Xho*I site at 596 was cloned into the *Cla*I site of pTAC909. In the resulting plasmid, DNA including the first part of ORF 1 immediately precedes the *tet* gene. It conferred tetracycline resistance (to 30–40 μg/ml) to transformants of MC1000. The corresponding plasmid where the *Taq*I fragment has the opposite orientation conferred no tetracycline resistance. These results indicate that a promoter with the same orientation as ORF 1 is contained in this *Taq*I fragment.

Nuclease S1 mapping of 5′ and 3′ ends of the transcript

To search for a transcript extending from the promoter detected *in vivo* to the terminator-like sequence around position 1322, we carried out a nuclease S1 analysis of the region.

(i) The 5′ end. RNA extracted from strains BD1401 (*rel*$^+$) and BD1402 (*relB*101), both from exponentially growing and from starved cultures, was hybridized to the 5′-labeled restriction fragment shown in Figure 4A. After exonuclease S1 digestion, the lengths of the resulting hybrids were determined from a 10% sequencing gel, using the Maxam-Gilbert A+G and G sequencing reactions as markers (Figure 5B). The 5′ end of the transcript is found at position 463 and 464 in the sequence. The two bands were seen also after prolonged S1 digestion at low temperature (5°C). Hybrids of exactly the same length were found with all of the RNA preparations. A 1.5-bp correction has been made, as chemical degradation eliminates the modified base and leaves a 3′-phosphate group whereas S1 nuclease leaves a 3′-OH group (Sollner-Webb and Reeder, 1979). Thus the 5′ end of the transcript precedes the start of ORF 1 by 30 bases. The sequence preceding the transcription start includes homology to the −35 and −10 sequences of known promoters. In fact, two partially overlapping versions of homology to the consensus sequence seem to be present.

(ii) The 3′ end. The 3′ end of the RNA was mapped using RNA derived from strains BD1401 (*rel*$^+$), BD1402 (*relB*101) and BD2456. The latter strain is BD2432 containing pBD2395 (Figure 4B).

Total RNA from the three strains was hybridized to 3′-labeled restriction fragments (Figure 4A), the products were digested with exonuclease S1, and analysed on polyacrylamide gels. A 700-bp labeled hybrid was formed between RNA from BD1401 or BD1402 and the *Xho*I-*Nru*I fragment labeled in the *Xho*I site. With RNA from all three strains, a hybrid of ~90 bp was formed with the *Mst*II-*Nru*I fragment labeled in the *Mst*II site (sizes determined from a 5% polyacrylamide gel, non-denaturing conditions). Both of these results point at a 3′ end situated some 90 bp to the right of the *Mst*II site. To locate the 3′ end more precisely, the *Mst*II-labeled hybrids were run on a 10% sequen-

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                                                                                                      100
CTTAATTTCAGGCCCCATCGGATCACACATGGAGAGTTTTTATGAATAACCCCGTCTGTCTTGATGACTGGTTGATTGGCTTTAAAAGCTTGTTGACAGG
                                                                                                      200
GGTAAACGTTCGGCAATAATTTTCTGCCGCATGCGGGTGTTGCATAAAACGTGTTACGTTCCTTTATCGACAGGTCAGGTCACCGCTCACCCGCCGACGA
                                                                                                      300
GAAAGCAACACTGACATGCTAAAGCAAAAAATAGATGAATAAGTTGAGTTGTGCATATGTAGCCTGACCGTCACAAAGTATATGGTGTCTGTACCAGTAA
                                                                                                      400
GATGATGGCCGGACTCTTTAAAAACGAGCTGACCTGCACAATACAGGATGGACTTAGCAATGGCTGCTCCTGGCACAAAGCGGACAGTGATCACCGTTCT
                                                           **                                         500
TACGACTACTTTCTGACTTCCTTCGTGACTTGCCCTAAGCATGTTGTAGTGCGATACTTGTAATGACATTTGTAATTACAAGAGGTGTAAGACATGGGTA
                                                    -------->  -->  <--  <-------
                                                                                             fMetGlySer
                                                                                                      600
GCATTAACCTGCGTATTGACGATGAACTTAAAGCGCGTTCTTACGCCGCGCTTGAAAAAATGGGTGTAACTCCTTCTGAAGCGCTTCGTCTCATGCTCGA
 IleAsnLeuArgIleAspAspGluLeuLysAlaArgSerTyrAlaAlaLeuGluLysMetGlyValThrProSerGluAlaLeuArgLeuMetLeuGlu
                                                                                                      700
GTATATCGCTGACAATGAACGCTTGCCGTTCAAACAGACACTCCTGAGTGATGAAGATGCTGAACTTGTGGAGATAGTGAAAGAACGGCTTCGTAATCCT
TyrIleAlaAspAsnGluArgLeuProPheLysGlnThrLeuLeuSerAspGluAspAlaGluLeuValGluIleValLysGluArgLeuArgAsnPro
                                                                                                      800
AAGCCAGTACGTGTGACGCTGGATGAACTCTGATGGCGTATTTTCTGGATTTTGACGAGCGGGCACTAAAGGAATGGCGAAAGCTGGGCTCGACGGTACG
LysProValArgValThrLeuAspGluLeu fMetAlaTyrPheLeuAspPheAspGluArgAlaLeuLysGluTrpArgLysLeuGlySerThrValArg
                                                                                                      900
TGAACAGTTGAAAAAGAAGCTGGTTGAAGTACTTGAGTCACCCCGGATTGAAGCAAACAAGCTCCGTGGTATGCCTGATTGTTACAAGATTAAGCTCCGG
GluGlnLeuLysLysLysLeuValGluValLeuGluSerProArgIleGluAlaAsnLysLeuArgGlyMetProAspCysTyrLysIleLysLeuArg
                                                                                                     1000
TCTTCAGGCTATCGCCTTGTATACCAGGTTATAGACGAGAAAGTTGTCGTTTTCGTGATTTCTGTTGGGAAAAGAGAACGCTCGGAAGTATATAGCGAGG
SerSerGlyTyrArgLeuValTyrGlnValIleAspGluLysValValValPheValIleSerValGlyLysArgGluArgSerGluValTyrSerGluAla
                                                                                                     1100
CGGTCAAACGCATTCTCTGAACCAAAGCATGACATCTCTGTTTCGCACCGAAGGTGACACTTCTGCTTTGCGTTGACAGGAGAAGCAGGCTATGAAGCAG
 ValLysArgIleLeu
                                                                                             fMetLysGln
                                                                                                     1200
CAAAAGGCGATGTTAATCGCCCTGATCGTCATCTGTTTAACCGTCATAGTGACGGCACTGGTAACGAGGAAAGACCTCTGCGAGGTACGAATCCGAACCG
GlnLysAlaMetLeuIleAlaLeuIleValIleCysLeuThrValIleValThrAlaLeuValThrArgLysAspLeuCysGluValArgIleArgThrAsp
                                                                                                     1300
ACCAGACGGAGGTCGCTGTCTTCACAGCTTACGAACCTGAGGAGTAAGAGACCCGGCGGGGGAGAAATCCCTCGCCACCTCTGATGTGGCAGGCATCCTC
 GlnThrGluValAlaValPheThrAlaTyrGluProGluGlu
                                                                                                     1400
AACGCACCCGCACTTAACCCGCTTCGGCGGGTTTTTGTTTTTATTTTCAACGCGTTTGAAGTTCTGGACGGTGCCGGAATAGAATCAAAAATACTTAAGT
     ----->     ----->  <-----
                                                                                                     1500
AGCGCGCAGGGATAAGAGGGATGGTCCCTTAAAGGGGAGAGCTAATTATCCGGAAGGATTCTGATGATGAACATCGAAGAACTGCGTAAAATTTTTTGTG
                                                                 fMetMetAsnIleGluGluLeuArgLysIlePheCysGlu
                                                                                                     1600
AAGATGGCCTCTATGCTGTGTGCGTTGAAAATGGAAATCTTGTTAGTCATTACCGCATTATGTGTTTGCGAAAGAATGGGGCTGCGTTAATTAATTTTGT
 AspGlyLeuTyrAlaValCysValGluAsnGlyAsnLeuValSerHisTyrArgIleMetCysLeuArgLysAsnGlyAlaAlaLeuIleAsnPheVal
                                                                                                     1700
GGATGCTCGGGTCACGGACGGATTTATCTTGCGCGAAGGTGAGTTTGTCACTTCATTACAGGCATTGAAAGAGATCGGAATAAAAGCTGGCTTTTCTGCT
 AspAlaArgValThrAspGlyPheIleLeuArgGluGlyGluPheValThrSerLeuGlnAlaLeuLysGluIleGlyIleLysAlaGlyPheSerAla
                                                                                                     1800
TTTTCAGGAGAATAAACTCATCTACAATCTTGCGCGGGGCTGAACTCCCGCTGAGTAACACCGTGCCACCGGAGAAAACCGATGGCACGCAACGCAAAAT
PheSerGlyGlu
                                                                                                     1900
ATTACAATTCTGATAATTCGCCCGTTCTTGCCTGCACGCACGGGCGGTATTCTCACGCATTCAAGTCTGAATGGTTCCAGCACCCTCCATGCACTGCAGA
                                                                                                     2000
ACAGCCGAATGGCTGATTCATTCTTACCGCAGGCGCGGGTTCGAGGTTAAGAAAGCTCTCAGTCTCGACTATCGGCACTGGATAATCTCTGTCAGGCTGC
                                                                                                     2100
CTTATTCCGAACGCCCACCACGTGCGTCCCGCACTTCCAGCAACGGATCTGGAGGTAACGTGCGGGTATTACTTAGACCTGTTCTGGTGCCTGAGCTTGG
                                       2142
GCCGGTGGACCCTTAAGCCGGGCCGTGAATCCATACAGATAT
```

Fig. 3. Nucleotide sequence of the chromosomal DNA from pBD2144. The sequence is numbered as in Figure 2. The amino acid sequence corresponding to the four open reading frames, starting at position 494, 733, 1092 and 1464, respectively, is shown. Also indicated are regions of dyad symmetry (marked by arrows beneath the sequence) and the site of transcription initiation (marked by **).

cing gel, with a Maxam-Gilbert sequencing reaction A+G on the *Mst*II-labeled DNA-fragment as a marker (Figure 5A).

The plasmid-derived transcripts (Figure 5A, lane 1) end at position 1331 – 1335 whereas transcripts from BD1401 and from BD1402 end at position 1334 – 1338 (Figure 5A, lane 3 and data not shown). The reason for this slight difference is unknown. The positions of the hybrid bands have been corrected by 1 bp as the sequencing reaction eliminates the modified base (Sollner-Webb and Reeder, 1979).

The detected 3′ end is situated at or in a run of Ts following

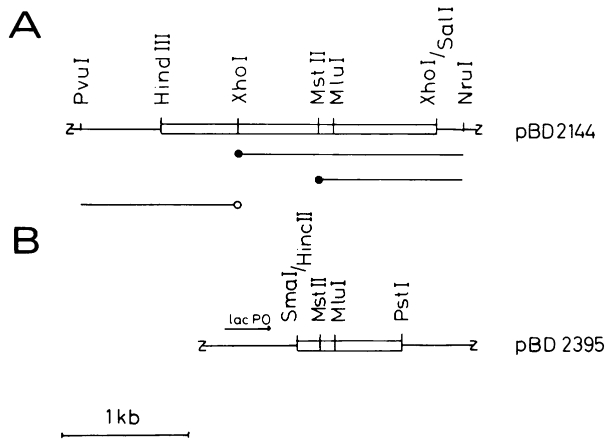

Fig. 4. (**A**) DNA probes for nuclease S1 mapping. To search for mRNA 3′ ends, *Xho*I-*Nru*I or *Mst*II-*Nru*I fragments from pBD2144 were 3′-labeled at the *Xho*I or *Mst*II site using DNA polymerase I (Klenow fragment) and [α-^{32}P]dTTP. For mapping of 5′ ends, the *Xho*I-*Pvu*I fragment was treated with phosphatase and 5′-labeled at the *Xho*I site using [γ-^{32}P]ATP and T4 polynucleotide kinase. (**B**) Structure of pBD2395. The thin line denotes vector DNA, pBR322 in the case of pBD2144 and pUC8 in the case of pBD2395. ○: 5′-labeling, ●: 3′-labeling.

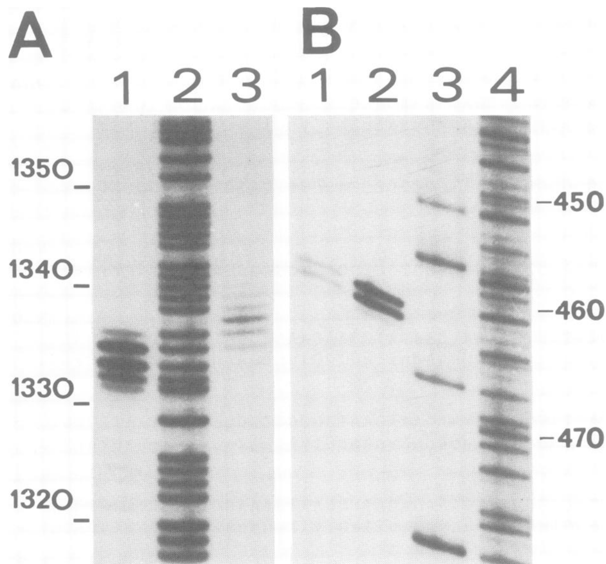

Fig. 5. Nuclease S1 map of transcripts from the *relB* region. The numbers correspond to positions in the DNA sequence (Figure 3). (**A**) Mapping of 3′ end by hybridization to DNA fragments 3′-labeled at the *Mst*II site (Figure 4). (**1**) Hybrid formed with RNA from the plasmid carrying strain BD2456. (**2**) A+G sequencing reaction on the labeled DNA fragment. (**3**) Hybrid formed with RNA from strain BD1402 (*relB*101). (**B**) Mapping of 5′ end by hybridization to DNA fragments 5′-labeled at the *Xho*I site (Figure 4). (**1**) Hybrid formed with RNA from BD1401 (*relB*$^+$). (**2**) Hybrid formed with RNA from BD1402 (*relB*101). (**3**) and (**4**) G and A+G sequencing reactions on the labeled DNA fragment.

position 1331. Immediately before the run of Ts there is a GC-rich region with two obvious possibilities of stem-loop formation within the mRNA. The sequence ACCCGC occurs both from position 1306 to 1311 and from 1317 to 1322; the complementary sequence GCGGGT occurs from position 1327 to position 1332. Since transcription termination often occurs in a run of Ts following a stem-loop (Kolter and Yanofsky, 1982), we conclude that the 3′ end corresponds to a transcription termination site.

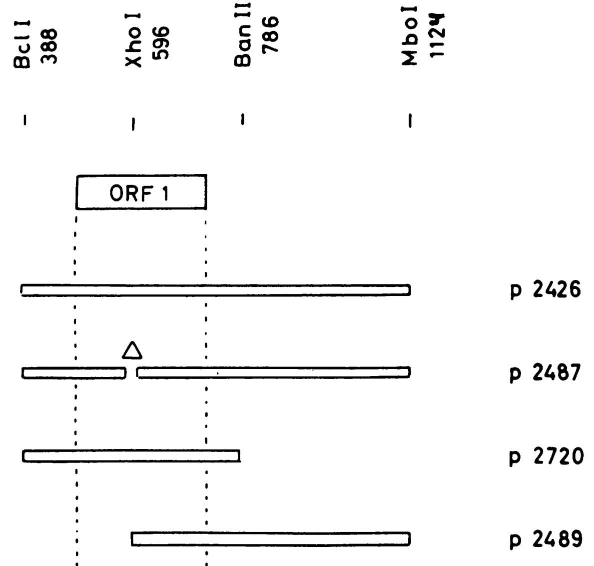

Fig. 6. Chromosomal DNA contained on pUC8 derived plasmids used for complementation analysis and maxicell experiments. The numbers at the top refer to positions in the sequence. The position of ORF 1 is indicated. pBD2487 was constructed from pBD2426 by digestion with *Xho*I, followed by nuclease S1 treatment and religation. pBD2487 thus contains a small deletion that destroys the *Xho*I site.

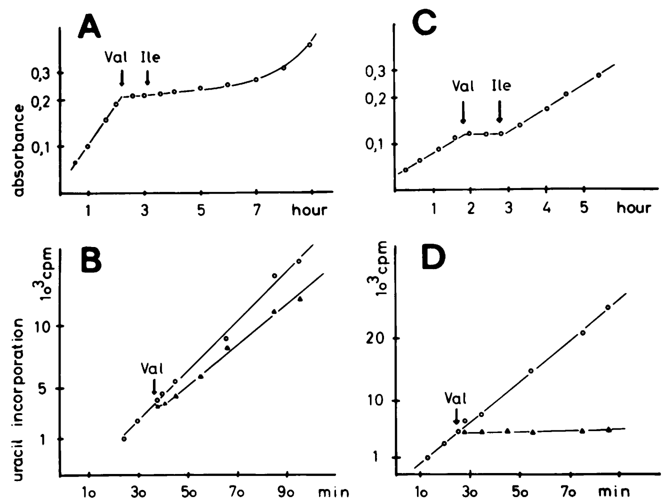

Fig. 7. Complementation of the *relB*101 mutation. **A** and **C** are a test for the starvation-lag phenotype whereas **B** and **D** are RNA accumulation measured as [^{14}C]uracil incorporation (Materials and methods). Strains were starved by valine addition (to 500 μg/ml) and starvation was terminated by isoleucine addition (to 1000 μg/ml). The strains were BD2433 (*relB*101) containing either pUC8 (**A** and **B**) or pBD2720 (see Figure 6) (**C** and **D**).

Subcloning of the relB$^-$ complementing activity

A 734-bp fragment from the *Bcl*I site at 388 to the *Mbo*I site at 1124 was cloned in both orientations into the *Bam*HI site of pBR322. Both resulting plasmids eliminated the delayed-relaxed as well as the retarded phenotype of a *relB* background strain (data not shown), in accordance with the presence of the promoter deduced from the S1 mapping. Replacement of the *relB*$^+$ plasmids with an incompatible plasmid pTAC692, which does not carry *relB*-related DNA, restored the RelB phenotype.

To test whether ORF 1 was responsible for the RelB complementation we cloned the fragments shown in Figure 6 into

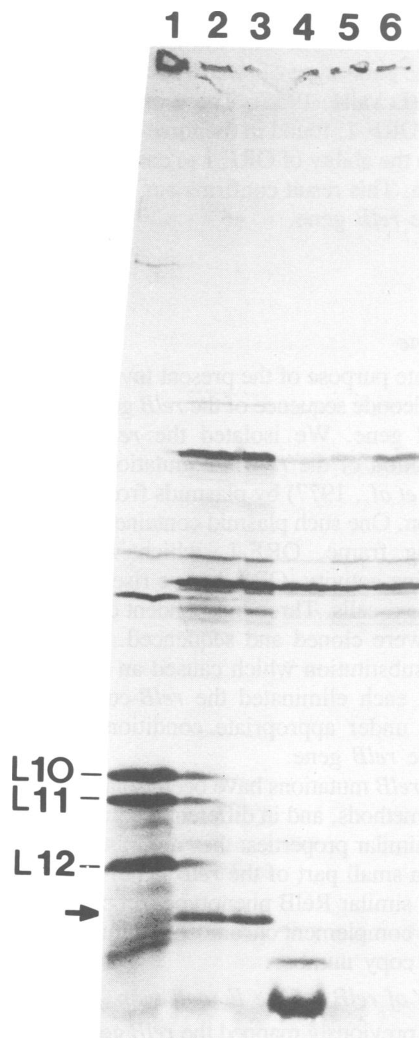

Fig. 8. Labeled polypeptides synthesized in maxicell experiments with the following plasmids: **(1)** a plasmid coding for ribosomal proteins L10, L11 and L12 [mol. wts. 18.1, 15.5 and 13.3 kd, respectively (Post *et al.*, 1976)]; **(2)** pBD2426; **(3)** pBD2720; **(4)** pBD2487; **(5)** pBD2489; **(6)** pUC8. See Figure 6 for plasmid description. The arrow marks the position of the polypeptide product from ORF 1.

pUC8 such that the direction of transcription was the same as that from the *lac* promoter of pUC8. In strain BD2433, which is *relB*101 and F′*lacI*^{q1}, the plasmids were tested for the delayed-relaxed and retarded phenotypes in the presence and absence of the *lac* inducer IPTG (isopropyl-β-D-thiogalactoside). The result shown in Figure 7, and the results obtained in similar experiments with the other plasmids (data not shown), show that plasmids pBD2426 and pBD2720, which have an intact ORF 1, eliminated the RelB phenotypes whereas pBD2487 and pBD2489 did not. Addition of IPTG had no effect. Thus ORF 1 preceded by the promoter appears responsible for the complementation.

A protein product from ORF 1

The plasmids described in Figure 6 were introduced into maxicell strain TC943. Plasmid-encoded ^{35}S-labeled polypeptides were analysed by SDS-polyacrylamide gel electrophoresis (Figure 8). Both plasmids with an intact ORF 1 produced a labeled polypeptide migrating with an apparent mol. wt. of 9 kd in good agreement with that expected for the product from ORF 1, a 79 amino acid polypeptide. The plasmid pBD2489, missing the promoter and the 5′-terminal part of ORF 1, did not produce any detectable band from the *relB* region. The plasmid pBD2487, which contains ORF 1 interrupted by a short deletion, had lost the 9-kd band but produced instead two smaller bands.

The product of ORF 2 is probably more weakly expressed than that of ORF 1 and is therefore not seen in Figure 8. Protein products from all four open reading frames, have, however, been detected in other maxicell experiments (unpublished).

Table II. Plasmids containing ORF 1 DNA from *relB* strains

Plasmid	Vector	Cloned fragment	*relB* allele
pBD2603	pKK2247	*Hin*dIII-*Xho*I	*relB*35
pBD2606	pKK2247	*Hin*dIII-*Xho*I	*relB*101
pBD2682	pKK2247	*Hin*dIII-*Xho*I	*relB*102
pBD2605	pKK2247	*Xho*I-*Xho*I	*relB*35
pBD2680	pKK2247	*Xho*I-*Xho*I	*relB*101
pBD2681	pKK2247	*Xho*I-*Xho*I	*relB*102
pBD2430	pUC8	*Bcl*I-*Pst*I	*relB*$^+$
pBD2684	pUC8	*Bcl*I-*Pst*I	*relB*35
pBD2686	pUC8	*Bcl*I-*Pst*I	*relB*101
pBD2688	pUC8	*Bcl*I-*Pst*I	*relB*102
pBD2742	pRE432	*Bcl*I-*Pst*I	*relB*$^+$
pBD2736	pRE432	*Bcl*I-*Pst*I	*relB*35
pBD2738	pRE432	*Bcl*I-*Pst*I	*relB*101
pBD2740	pRE432	*Bcl*I-*Pst*I	*relB*102

Cloning and sequencing of three relB mutations

To settle the question whether ORF 1 is in fact the *relB* gene, this region was cloned from three different *relB* strains and sequenced. The strains chosen were BD1020 (*relB*101), BD1050 (*relB*102) and BD639 (*relB*35).

(i) Cloning. To avoid any difficulties that might arise from having a mutated *relB* gene expressed from a plasmid, the presumed *relB* gene was cut in two parts, and each part was cloned separately, using a two-step digestion/isolation scheme. As seen from Figure 1, ORF 1 is contained on a 4.3-kb *Hin*dIII fragment. Digestion of this fragment with *Xho*I produces a 510-bp *Hin*dIII-*Xho*I fragment containing the first part of ORF 1 and a 1550-bp *Xho*I-*Xho*I fragment containing the second part of ORF 1.

Chromosomal DNA was digested with *Hin*dIII, the products were separated on an agarose gel, and DNA fragments of ~4.3 kb were isolated. These fragments were then digested with *Xho*I, and DNA fragments of ~500 bp and ~1500 bp were isolated. *Xho*I-*Xho*I fragments were ligated into *Xho*I-digested plasmid vector pKK2247 whereas *Xho*I-*Hin*dIII fragments were ligated into pKK2247 digested with *Xho*I and partially with *Hin*dIII. The recombinant clones were analysed with restriction endonucleases. Plasmids with DNA from either half of the ORF 1 region were obtained in this way from each mutant strain (Table II).

(ii) Sequence. Sequencing of the cloned DNA fragments revealed that each mutant contained a missense mutation in ORF 1, just downstream of the *Xho*I site. The fragments were 3′-labeled at this site and the sequences derived corresponded to all or nearly all of the DNA contained in the minimal DNA fragment that we had previously found sufficient for RelB complementation, i.e., the *Bcl*I to *Ban*II fragment. In *relB*101 the G at position 608 was replaced by an A, such that an alanine residue is converted to a threonine. In *relB*35 the C at 626 is converted to an A and in *relB*102 the neighbouring C at 627 is converted to a T. These mutations affect the same proline residue converting it to a

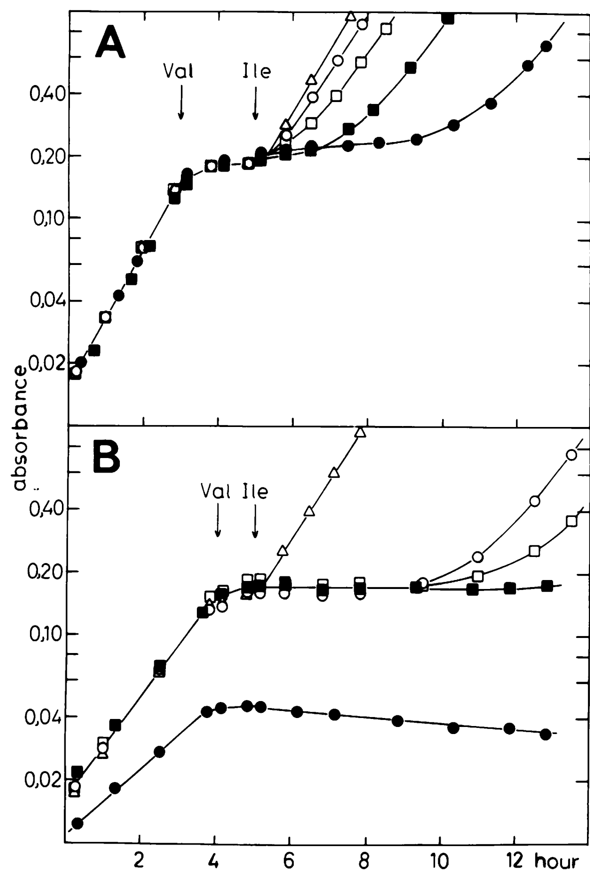

Fig. 9. Effect of plasmids containing *relB* mutations on growth retardation of a *relB* strain. Strains were grown in minimal media and subjected to isoleucine starvation as in Figure 7. **(A)** Growth at 37°C, 2 h starvation. **(B)** Growth at 42°C, 1 h starvation. Strains in both experiments were BD1402 (*relB*101) containing the following plasmids, all mini-F derivatives: △–△ pBD2742 (*relB*$^+$); ○–○ pBD2738 (*relB*101); □–□ pBD2740 (*relB*102); ■–■ pBD2736 (*relB*35); ●–● pRE432.

threonine and a leucine, respectively. Thus each mutant has one mutation in ORF 1 and two mutants with certainty have no other mutations within this minimal complementing fragment (the *relB*102 mutant was however not sequenced from position 424 to *Bcl*I). Considering also that a small deletion in the *Xho*I site within ORF 1 destroys the RelB complementing ability we conclude that ORF 1, coding for a 79 amino acid polypeptide, is indeed the *relB* gene.

(iii) Phenotypes. To show directly that the three mutations found were responsible for the RelB phenotype, we constructed plasmids that carried the *relB* region with the *relB* mutations from each of the mutant strains (see Materials and methods). The resulting plasmids were introduced in strain BD2433 (*relB*101), in order to test for complementation of the retarded phenotype. Surprisingly, all plasmids carrying a mutant *relB* gene complemented the chromosomal mutation as well as did the *relB*$^+$ plasmid.

Being unable to detect any effect of the *relB* mutations when the mutated gene was on a high copy number plasmid, we transferred wild-type and mutant *relB* genes to a low copy mini-F plasmid. These plasmids were tested in strain BD1402 (*relB*101), with the result shown in Figure 9. The strains were grown either at 37°C, with 2 h of starvation, or at 42°C, with 1 h of starvation. At 37°C, the three mutant plasmids were partially able to complement the chromosomal *relB*101 mutation, each mutant complementing to a different degree. At 42°C this complementation was less efficient. Interestingly, it has previously been shown that the phenotype of *relB*101 is more pronounced at high temperature (Lavallé, 1965). These experiments show that the mutations in ORF 1, found in the three *relB* strains investigated, interfere with the ability of ORF 1 to complement a chromosomal *relB* mutation. This result confirms our previous conclusion that ORF 1 is the *relB* gene.

Discussion

The relB gene

The immediate purpose of the present investigation was to determine the nucleotide sequence of the *relB* gene region and to identify the *relB* gene. We isolated the *relB* gene selecting for complementation of the *relB*102 mutation (which is recessive, Diderichsen *et al.*, 1977) by plasmids from the Clarke and Carbon collection. One such plasmid contained a 79 amino acid long open reading frame, ORF 1, which was required for the complementing activity. ORF 1 gave rise to a 9-kd polypeptide product in maxi-cells. Three independent chromosomal *relB* mutant alleles were cloned and sequenced. Each mutation was a single base substitution which caused an amino acid change in ORF 1, and each eliminated the *relB*-complementing activity when tested under appropriate conditions. We conclude that ORF 1 is the *relB* gene.

The three *relB* mutations have been isolated in different strains by different methods, and in different laboratories. Yet, they have remarkably similar properties: they are missense mutations which map within a small part of the *relB* gene. On the chromosome, they cause a similar RelB phenotype, on plasmids they are partially able to complement chromosomal mutations depending on the plasmid copy number.

Localization of relB on the E. coli map

Diderichsen previously mapped the *relB* gene to 34.4 min on the *E. coli* genetic map, near the terminus of DNA replication (Bachmann, 1983; Diderichsen *et al.*, 1977). A restriction enzyme map of the *E. coli* terminus region has been published (Bouché, 1982). At a position corresponding to 400 kb on this map, a series of fragments sizes reproduce those found on pBD602, disregarding a 100-bp fragment which we found in the beginning of the sequenced region (compare the *Hind*III and the *Pst*I sites on Figure 1 with those on Figure 6 of Bouché). The position at 400 kb on the terminus map, i.e., 34.6 min on the genetic map, is in excellent agreement with the previous mapping of *relB* to 34.4 min. Since no other region of the terminus map contains a similar run of restriction enzyme sites, we conclude that this is the position of the *relB* region. The direction of transcription of *relB* is counter-clockwise relative to the *E. coli* map.

A cryptic lambdoid prophage is located very close to *relB* (Bouché *et al.*, 1982; Espion *et al.*, 1983). The known *lambda* homology (Bouché *et al.*, 1982), begins within a fragment whose *relB*-proximal end lies some 2 kb downstream of the sequenced region. The detected lambdoid remnants include a *Q* gene analog and the extreme left and right ends of the lambdoid vegetative map. The direction of transcription of this *lambda* segment is the same as that of *relB*.

We have probed for similarities to *lambda* in our sequence by computer searches on two levels. On the one hand we have compared all of our DNA sequence with the lambda DNA sequence (Sanger *et al.*, 1982); on the other we have compared amino acid sequences of all right-ward genes and open reading frames of

lamdba's right arm with the amino acid sequences of the *relB* gene and the three other open reading frames mentioned here. We found no evidence that the *relB* gene region is of lambdoid origin.

As yet the role of the *relB* gene in the cell is unknown. It has previously been shown that the translational inhibitor which *relB* mutants accumulate upon amino acid starvation has the same heat inactivation kinetics when prepared from three different *relB* mutants. Hence, different *relB* mutants possibly produce identical inhibitors (Diderichsen and Desmarez, 1980). We show here that the *relB* products of different mutants have different complementation properties. We conclude that the *relB* gene product is not the inhibitor. We suspect that it has a negative regulatory function somehow connected to the synthesis and/or activation of the inhibitor and that this function is defective in *relB* mutants.

Finally, the sequence offers two pieces of information concerning the other two open reading frames of the *relB* transcript: the N-terminal end of ORF 3 is very similar to known signal sequences, and ORF 2 corresponds to an extraordinarily basic protein. We are investigating the hypothesis that ORF 2 is the gene of the translational inhibitor.

Materials and methods

Strains and plasmids

The strains used, derivatives of *E. coli* K-12, are listed in Table I.

The following plasmids were used as cloning vectors.

pUC8 (Vieira and Messing, 1982). pUC8 derived plasmids were constructed in strain 7118.

pBR322 (Bolivar *et al.*, 1977).

pKK2247, a derivative of pACYC184 (Chang and Cohen, 1978) that carries the *kan* gene from Tn903 inserted in the *Eco*RI site of pACYC184, thus conferring kanamycin resistance and destroying chloramphenicol resistance. The *kan* gene contains a *Hind*III site and a *Xho*I site (Oka *et al.*, 1981). This plasmid was obtained from J.Vieira *via* S.Brown.

pRE432, a 13.5-kb mini-F derivative with sites for *Sal*I, *Bam*HI, *Hind*III and *Eco*RI, obtained from R.Eichenlaub *via* S.Pedersen. Cloning of fragments between *Hind*III and *Eco*RI destroys tetracyclin and chloramphenicol resistance but leaves ampicillin resistance (up to 75 μg/ml).

pTAC909 (Atlung *et al.*, 1984), a promoter-detection plasmid constructed by deletion of the *Hind*III site of pBR322, thereby eliminating *tet*-promoter activity.

pTAC692, a pBR322 derivative conferring only tetracyclin resistance (provided by T.Atlung), was used in one experiment to replace another pBR322 derivative.

For constructed plasmids see Table II, Figures 1, 4 and 6.

Media and growth experiments

Growth experiments were performed in minimal glucose medium (Clark and Maaløe, 1967), at 37°C except where otherwise indicated. Amino acids and uracil were added to final concentrations of 50 μg/ml and 25 μg/ml, respectively, when required. Growth inhibition and RNA accumulation was measured as described (Diderichsen and Desmarez, 1980). Growth was followed using an Eppendorf spectrophotometer 1101 M at a wavelength of 436 nm.

Rich medium used for growing cells for transformation and plasmid preparation was NY (von Meyenburg *et al.*, 1982).

For resistant strains antibiotics were added in the following concentrations: ampicillin 60 or 100 μg/ml, kanamycin 20 μg/ml, tetracycline 20 μg/ml.

Enzymes and radioactivity

Restriction endonucleases, T4 DNA ligase, DNA polymerase I (large fragment) and S1 nuclease were from New England Biolabs, Inc. Bacterial alkaline phosphatase was from Sigma and polynucleotide kinase was from Cambridge Biotechnology Laboratories. The enzymes were used according to the manufacturers instructions.

[α-^{32}P]dNTP and [γ-^{32}P]dATP, 3165 Ci/mmol, were from New England Nuclear. [2-^{14}C]uracil, 58 mCi/mmol, was from Amersham International Ltd., UK. [L-^{35}S]methionine, 1095 Ci/mmol, was from New England Nuclear.

DNA sequencing

The DNA sequencing reactions were performed according to Maxam and Gilbert (1980) and fragments separated on 0.6 mm thick 6% or 10% polyacrylamide gels containing 8 M urea.

S1 mapping

RNA extraction, RNA/DNA hybridization and nuclease S1 treatment was essentially as described (Aiba *et al.*, 1981; Brown *et al.*, 1982).

For strain BD2456, IPTG to a final concentration of 5×10^{-4} M was added 5 min prior to RNA extraction. For strains BD1401 and BD1402, RNA was extracted both from exponentially growing cultures and from starved cultures. The latter samples were taken 20 min after valine-induced isoleucine starvation.

Maxicell experiments

Plasmids were introduced in strain TC943 and polypeptide products labeled with [^{35}S]methionine as described (Sancar *et al.*, 1979). The polypeptides were separated on SDS-polyacrylamide gels containing 18% acrylamide and 0.1% bis-acrylamide according to Laemmli (1970).

Selection of relB$^+$ plasmids

The selection was based on the deleterious effect of 5-fluorouracil on relaxed strains (Diderichsen *et al.*, 1977). The Clarke and Carbon colony bank (Clarke and Carbon, 1976), as a mixture of ~2000 plasmid carriers, was mated with BD143 (*relB*102), and the exconjugants were grown in minimal medium for 3 h. The culture was then starved for arginine, histidine and proline, and 10 min later 10 μg/ml 5-fluorouracil was added. After 15 h of starvation, 10^7 cells were plated on plates containing colicin E1 (prepared after Spudich *et al.*, 1970). Of the 300 resulting colonies, 5% showed Rel$^+$ phenotype as determined by filter assay (Diderichsen and Desmarez, 1980).

Preparation of plasmid DNA

Plasmid DNA was purified from 10 ml bacterial cultures as described by Birnboim and Doly (1979), with slight modifications.

Preparation of chromosomal DNA

Cells were lysed with a combination of lysozyme and SDS, essentially as described by Schleif and Wensinck (1981), followed by phenol extraction (repeated several times), $CHCl_3$ extraction and removal of SDS by KCl-precipitation (0.2 M KCl). Nucleic acids were precipitated with ethanol, rinsed, dried and resuspended in 10 mM Tris pH 8, 1 mM EDTA.

Isolation of DNA fragments

DNA fragments were isolated during agarose gel electrophoresis by collecting onto pieces of DEAE-membrane (Schleicher and Schüll NA 45). The DNA was recovered by immersing the DEAE-membrane in 10 mM Tris pH 8, 1 mM EDTA, 1.5 M NaCl at 65°C for 15 min (repeated once). After precipitations with ethanol, this DNA was used for ligation or for a second restriction endonuclease digestion.

Construction of relB mutant plasmids

Plasmids carrying the *relB* mutations were constructed from pBD2430, a pUC8 derivative with the *relB*$^+$ region from *Bcl*I (position 388) to *Pst*I (position 1894), inserted in the *Bam*HI and *Pst*I sites in the pUC8 polylinker. Plasmid pBD2430 constructed in this manner thus contains the entire *relB* transcription unit.

The fragment from *Xho*I (596) to *Ban*II (786), containing the second part of the *relB* gene, was removed from pBD2430, and in its place the corresponding *Xho*I-*Bam*II fragment from each of the mutant clones (see Table II) was inserted. In each case, the *Xho*I-*Ban*II fragment contains the mutation and includes only sequenced DNA. Since it would be impossible to verify, by use of restriction endonucleases, that the *Xho*I-*Ban*II fragment from pBD2430 had actually been replaced by the corresponding mutation-containing fragment, the construction was done in two steps. First, the 190-bp *Xho*I-*Ban*II fragment from pBD2430 was replaced with a 450-bp *Xho*I-*Ban*II fragment of unrelated DNA. This 450-bp fragment was then replaced with the mutation-containing fragments.

The entire *relB* region from each of these plasmids and from pBD2430 were then excised with *Eco*RI and *Hind*III, and transferred to a low copy number vector, pRE432. The plasmids resulting from these constructions are listed in Table II.

Computer analysis

For detection of restriction sites and open reading frames Queen and Korn's program (Queen and Korn, 1980) was used on a Univac OS-1100 computer.

Nucleotide sequence homology and complementarity was analysed on the same computer primarily using a program by Morten Johnsen (Johnsen, unpublished).

Protein homology was sought using a HP-125 and a program by Olle Karlström (Karlström, unpublished).

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