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Mechanism of postsegregational killing by the *hok* gene product of the *parB* system of plasmid R1 and its homology with the *relF* gene product of the *E. coli relB* operon

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The *parB* region of plasmid R1 encodes two genes, *hok* and *sok*, which are required for the plasmid-stabilizing activity exerted by *parB*. The *hok* gene encodes a potent cell-killing factor, and it is regulated by the *sok* gene product such that cells losing a *parB*-carrying plasmid during cell division are rapidly killed. Coinciding with death of the host cell, a characteristic change in morphology is observed. Here we show that the killing factor encoded by the *hok* gene is a membrane-associated polypeptide of 52 amino acids. A gene located in the *Escherichia coli relB* operon, designated relF, is shown to be homologous to the *hok* gene. The *relF* gene codes for a polypeptide of 51 amino acids, which is 40% homologous to the *hok* gene product. Induced overexpression of the *hok* and *relF* gene products results in the same phenomena: loss of cell membrane potential, arrest of respiration, death of the host cell and change in cell morphology. The *parB* region and the *relB* genes were cloned into unstably inherited *oriC* minichromosomes. Whereas the *parB* region also conferred a high degree of genetic stability to an *oriC* minichromosome, the *relB* operon (with *relF*) did not; therefore the latter does not appear to 'stabilize' its replicon (the chromosome). The function of the *relF* gene is not known.

Key words: hok gene/*parB* of plasmid R1/plasmid stability/ *relB* operon/*relF* gene/gene homology/host cell killing/membrane potential

Introduction

Natural bacterial plasmids are genetically stable entities, indicating that precise mechanisms operate not only at the level of replication control, but also at the level of plasmid maintenance during cell division. The low copy number antibiotic resistance plasmid R1 is an example of a very stably maintained replicon, characterized by a loss rate in the range of 10^{-7}/cell/cell division (Nordström and Aagaard-Hansen, 1984). When the largest *Eco*RI fragment of this plasmid was deleted, the plasmid still replicated in a normal fashion,but the very stable maintenance during cell growth and division was lost, as shown by a loss rate in the range of 10^{-2}/cell/cell division (Nordström *et al.*, 1980). A more detailed genetic analysis showed that plasmid R1 encodes two stability loci, designated *parA* and *parB* respectively, both of which independently confer a high degree of genetic stability on mini-R1 plasmids (Gerdes *et al.*, 1985). The *parB* locus seemed particularly interesting, since many unrelated unstably inherited plasmids became efficiently stabilized by this locus in *cis* (Gerdes *et al.*, 1985, 1986; unpublished results).

Recently we found that the *parB* locus confers genetic stability to plasmids in *Escherichia coli* populations by a new mechanism termed 'postsegregational killing' (Gerdes *et al.*, 1986): cells which have lost a *parB*-carrying plasmid at cell division are rapidly killed. Thus the *parB* locus prevents the appearance of plasmid-free cells in a growing culture. Cells killed after losing the *parB* locus have a characteristic morphology (so-called 'ghost' cells). The killing factor was encoded by a small region within the *parB* locus, the *hok* gene (host killing). Induction of *hok* gene expression caused rapid killing of the host cells and yielded the same morphological changes which paralleled the killing concomitant with the loss of a *parB*-carrying plasmid. Expression of the *hok* gene is regulated by the nearby *sok* gene (suppressor of killing) such that the *hok* gene product is only expressed in cells which have lost the *parB*-carrying plasmid during the preceding cell division.

We demonstrate here that the killing controlled by the *parB* system is due to the synthesis of a 52 amino acid polypeptide encoded by the *hok* gene. Induced overproduction of the *hok* protein, which appears to be membrane bound, leads to a rapid collapse of the membrane potential and a concomitant arrest of respiration.

Surprisingly we found that the *relF* gene (previously designated *orf3*) of the *E. coli relB* operon (Bech *et al.*, 1985) is related to the *hok* gene both structurally and functionally. The *relF* gene

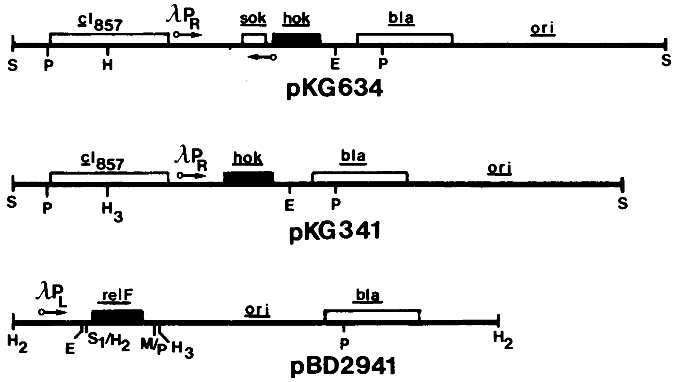

Fig. 1. Physical and genetic maps of the plasmids used in the induction of the *hok* and *relF* genes. Plasmid pKG634 is a pBR322 derivative which contains the *parB* region extending from +194 to +580 (P.B.Rasmussen, K.Gerdes and S.Molin, in preparation) linked to the λ pR promoter (indicated by →). The *hok* and *sok* genes are indicated with filled and open bars respectively. The presumed *sok* gene promoter is indicated by ←. The *bla* and cI857 genes are indicated with open bars. Plasmid pKG341 is a similar plasmid containing the *parB* region from +268 to +580 linked to the λ pR promoter. This plasmid derivative is devoid of the *sok* gene. Plasmid pBD2941 is a pBR322 derivative containing the *relF* gene (filled bar) linked to the λ pL promoter. The *relB* derived region of this plasmid extends from +1070 (*Hinc*II site) to +1348 (*Mlu*I site) [*relB* coordinates as in Bech *et al.* (1985); see also Figure 6b]. The *bla* gene (open bar) and origin of replication are also indicated. The figure is not drawn to scale. Restriction sites are shown as S (*Sal*I), P (*Pst*I), H_3 (*Hin*dIII), E (*Eco*RI), S_1 (*Sma*I), H_2 (*Hinc*II), M (*Mlu*I).

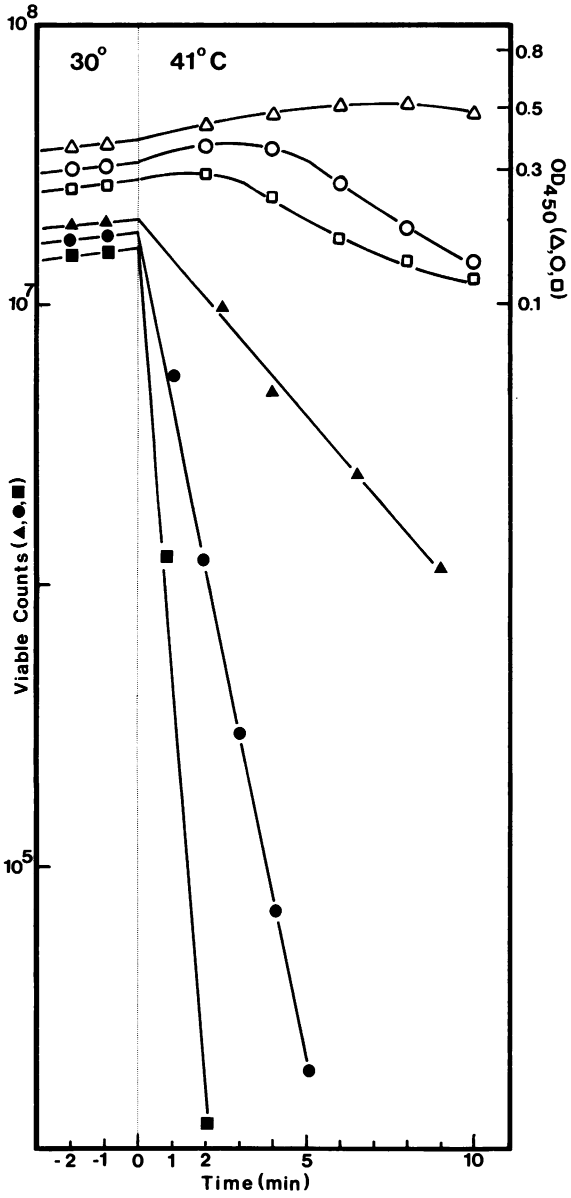

Fig. 2. Killing kinetics after induction of the *hok* and *relF* genes. The strains were grown exponentially at 30°C in A+B medium (Clark and Maaløe, 1967) supplemented with casamino acids. At time zero the temperature was shifted to 41°C. Open symbols show OD450, closed symbols are viable counts. △, ▲: JC411 (KG634 *sok*$^+$ *hok*$^+$), ○, ●: JC411 (pKG341 *hok*$^+$), and □, ■: JC411 [pBD2941 (*relF*$^+$)/pNF2690 (contains cI857)].

codes for a polypeptide of 51 amino acids which has a degree of homology with the *hok* gene product. Induction of *relF* gene expression leads to the same corollary of responses as when the *hok* gene is overexpressed: collapse of the membrane potential, arrest of respiration, change in morphology and cell death.

Cell death caused by the *hok* gene product appears to be due to the elimination of a vital function of the cell membrane, so does cell death caused by the *relF* gene product, however, the physiological role of the latter is as yet unknown.

Results

Host cell killing caused by induced expression of the hok and relF genes

Plasmids pKG341 and pKG634 are pBR322 derivatives carrying the *hok* gene inserted downstream of the λ-pR promoter (Figure 1). These plasmids also carry the cI857 allele of the λ repressor gene, thereby allowing temperature induction of the λ pR promoter. At low temperature (30°C) the *hok* genes of pKG341 and pKG634 are not transcribed, whereas a shift to high temperature (41°C) greatly increases the transcription of the genes due to inactivation of the λ repressor. Upon a temperature shift from 30 to 41°C of strain JC411, containing either pKG341 or pKG634, the numbers of viable cells in the culture decrease rapidly (Figure 2). Thus induction of *hok* gene expression leads to rapid cell death in agreement with results shown previously (Gerdes *et al.*, 1986). The killing with pKG634, which carries the *sok* gene in addition to the *hok* gene, is slower than with pKG341, which is Sok$^-$ (Figure 1).

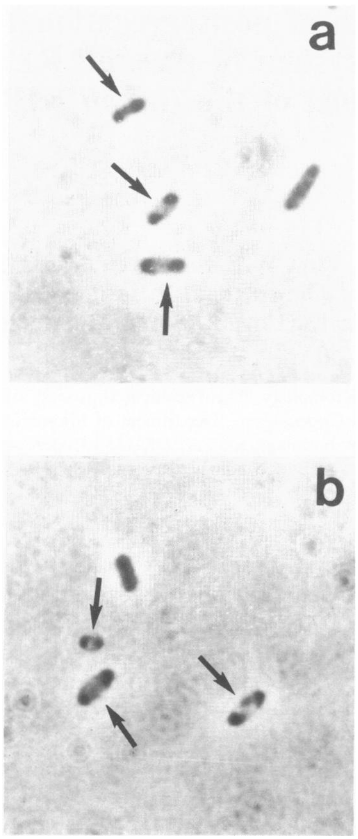

Fig. 3. Photograph obtained by phase contrast microscopy of cells from the *hok* and *relF* induction experiments (Figure 2). Arrows point at cells with a clearly changed morphology. Cells with a normal morphology are also seen. **(a)** Shows cells obtained from strain JC411 (pKG341) 10 min after induction of the *hok* gene of the plasmid; **(b)** shows cells obtained from strain JC411 containing pBD2941/pNF2690 also 10 min after induction of the *relF* gene of pBD2941. Magnification × 1600.

A similar response was found for strain JC411, harbouring plasmid pBD2941 (Figure 1), with the *relF* gene under λ pL control (Figure 2), and pNF2690 (with the heat-sensitive cI857 λ repressor gene). The cells killed by overproduction of the *relF* gene product exhibited the same morphological changes as the cells killed by overexpression of the *hok* gene product (Figure 3a and b). The central parts of the cells appear completely translucent, whereas the poles apparently contain condensed material. Also, the dead cells appear somewhat larger than normal cells. By Coulter counter experiments we showed that the cell size is 70–90% larger than normal (data not shown). Thus induction of the *hok* and *relF* genes leads to similar biological phenomena: a rapid killing of the host cells and a change to an unusual cell

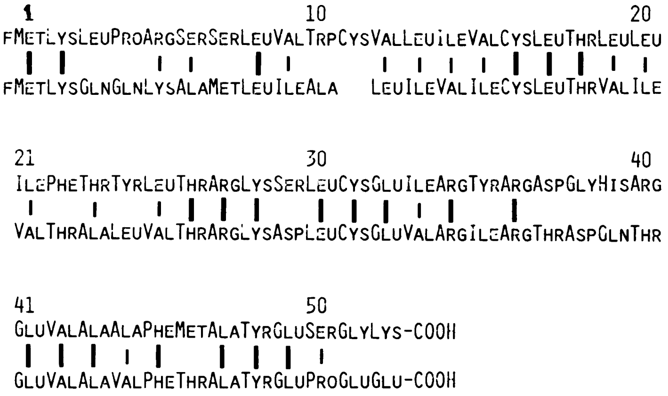

Fig. 4. Alignment of the amino acid sequences of the *hok* and *relF* proteins. Identical amino acids are indicated with bold vertical lines, conservative changes [as defined by Stragier *et al.* (1984)] are indicated with thin lines.

morphology, which is also reflected in a decrease in the buoyant density of the cells (Gerdes *et al.*, 1986).

Comparison of the hok gene of plasmid R1 and the chromosomal relF gene and their products

The presumptive products of the *hok* (P.B.Rasmussen, K.Gerdes and S.Molin, in preparation) and *relF* (Bech *et al.*, 1985) genes of 52 and 51 amino acid residues respectively, have strikingly similar structures as shown in Figure 4. The proteins share 40% direct homology besides a number of conservative replacements. Three segments exhibiting strong homology are apparent, namely 12–21, 26–36 and 41–49 (numbering is for the amino acids of the *Hok* protein).

Several characteristics of the two proteins are conserved (Figure 4). Firstly, both proteins are basic, most of the charged amino acids being in conserved positions: 9 of the 11 charged amino acids of the *Hok* protein are conserved in the *relF* encoded protein. Secondly, both proteins have hydrophobic sequences in the same regions (amino acids 8–15 and 19–25). Figure 5 shows a hydrophilicity plot of the two proteins. The patterns are very similar: the N termini of the proteins are hydrophobic, whereas the C termini are hydrophilic; also the N-terminal ends of the two proteins bear resemblance to signal peptides (Hall and Silhavy, 1981), although no typical cleavage site for signal peptidase is present. Thirdly, the cysteine residues at positions 16 and 31 are conserved.

In the coding region extending from +304 to +460, there is 52% nucleotide sequence homology (Figure 6). Also in the region surrounding the Shine & Dalgarno sequence (Shine and Dalgarno, 1974) there is a remarkable sequence conservation (13 out of 14 bp are identical).

The regions downstream of both coding sequences show resemblance to 'extragenic palindromic units' (EPUs; Gilson *et al.*, 1984). Nucleotides in accordance with the consensus EPU sequence are marked with asterisks in Figure 6 in both sequences.

Cellular localization of the Hok and RelF proteins

The *hok* and *relF* gene products were radioactively labelled after heat induction, using the maxicell technique (Materials and methods). After labelling, disintegrated cells were fractionated into crude membranes and cytoplasm using the method of Russel and Model (1982). The fractions were run on an SDS–polyacrylamide gel and autoradiographed (Figure 7). Both the *hok* and *relF* gene products migrate with apparent mol. wts of 11–12 kd. No bands appear in this part of the gel if the cells are labelled prior to heat induction (not shown). Large quantities of the *relF* and *hok* gene products are found in the membrane fraction, as is also found for the smallest subunit of the ATP synthase (the c-subunit product of the *atpE* gene; Figure 7, lane 8 and 9), the synthesis of which was induced in an analogous way in strain CSR603 carrying plasmid pCMC1073 (von Meyenburg *et al.*, 1985). The *rom* gene product of the vector plasmid appeared almost exclusively in the cytoplasmic fraction (Figure 7). Thus these results indicate that the *relF* and *hok* gene products are localized in a cellular membrane, presumably the cytoplasmic membrane.

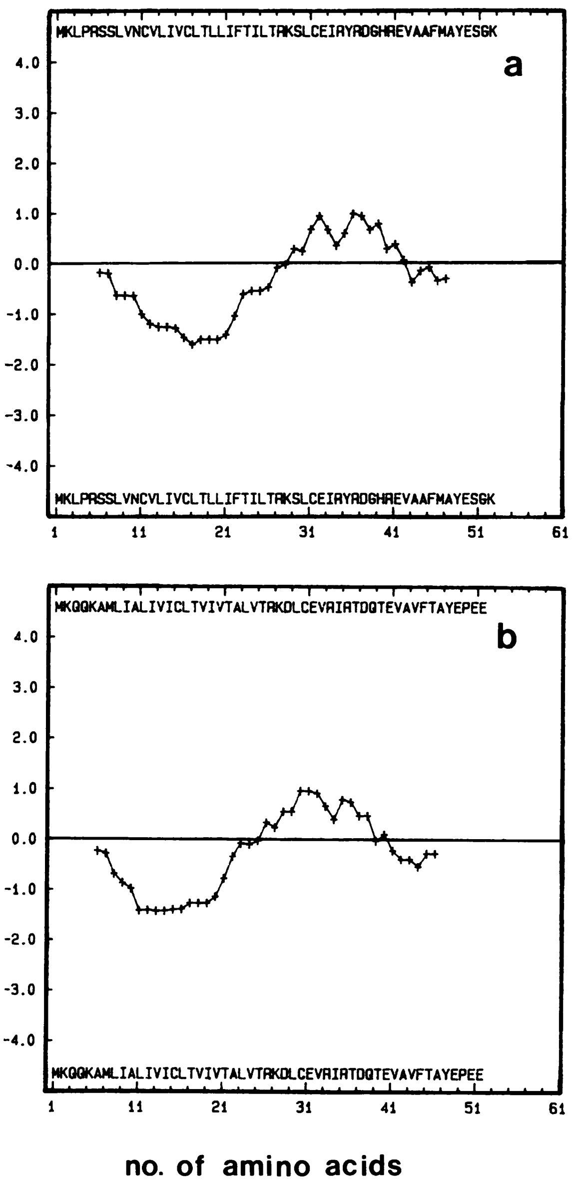

Fig. 5. Hydrophilicity plot of the *hok* (**a**) and *relF* (**b**) proteins according to the method of Hopp and Woods (1981). Values are averaged over 11 amino acids.

Hok and RelF proteins interfere with a vital function in the cell membrane

The apparent association of the *Hok* and *RelF* proteins with the cell membrane indicated that the proteins might affect a function of the membrane. As the proton gradient-dependent energy-generating machinery is tightly coupled to the cell membrane, we considered it important to measure any changes in the cell membrane potential ($\Delta\psi$) after induction of synthesis of the *hok* and *relF* gene products. We chose to determine $\Delta\psi$ by

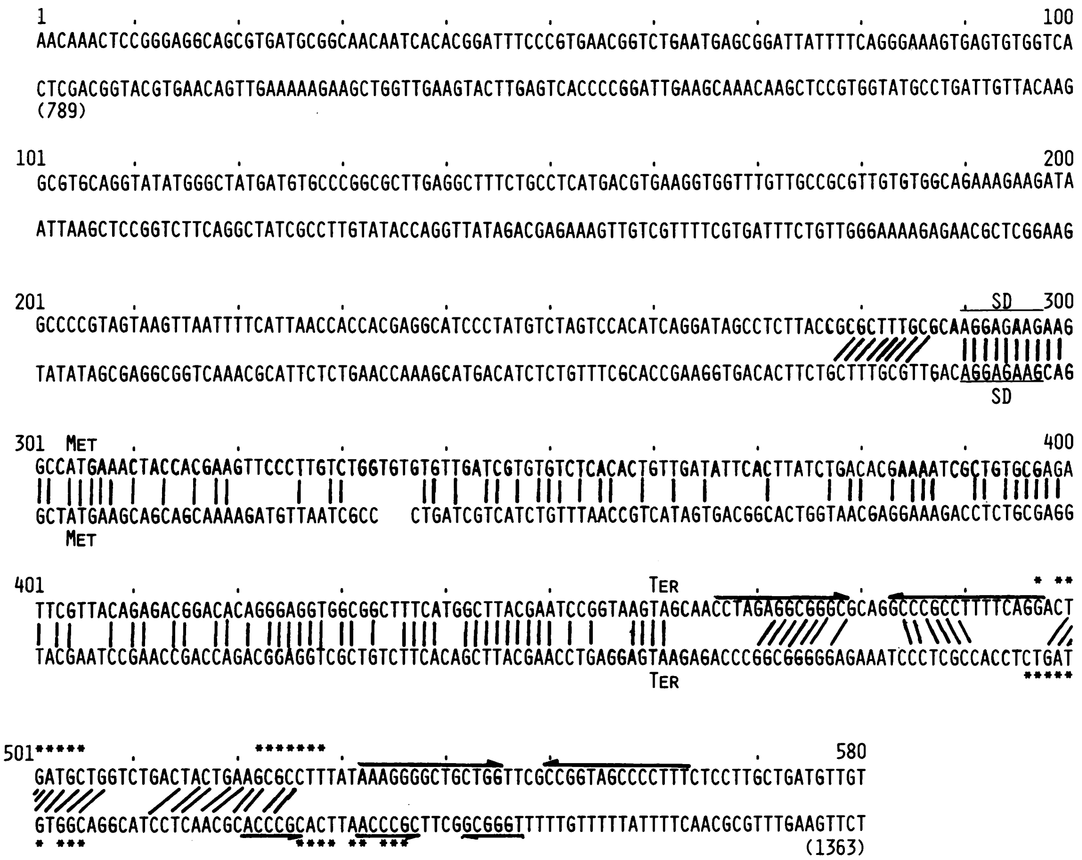

Fig. 6. Alignment of the nucleotide sequences of the *parB* region (upper line) and the part of the *relB* operon homologous to *parB* (lower line). Numbers are *parB* coordinates (as in P.B.Rasmussen, K.Gerdes and S.Molin, in preparation); the corresponding coordinates of the *relB* sequence are given in brackets. Identical bases are indicated with lines. Inverted repeats are indicated with arrows. The starts and stops of the *hok* and *relF* genes are indicated with 'Met' and 'Ter' respectively. Stars indicate stretches of bases conforming to the so-called 'EPU' consensus sequence (Gilson *et al.*, 1984) see text.

measuring uptake of the lipophilic cation tetraphenylphosphonium ([^{3}H]TPP$^+$; see Materials and methods). Plasmids pBD2941, pKG341, pKG634 and pBR322 were transferred into the permeable mutant strain CM12 of *E. coli* B, a derivative of strain AS19 (Sekiguchi and Iida, 1967), which was previously found to be permeable to TPP$^+$ (von Meyenburg *et al.*, 1985). The extent of uptake of [^{3}H]TPP$^+$ into growing cells of these plasmid-carrying strains at 30°C, and after different periods of temperature-induced synthesis of the *hok* and *relF* gene products, was determined (Figure 8a, b and c respectively). There is a rapid cessation of [^{3}H]TPP$^+$ uptake after the heat-induced transcription of both the *hok* and *relF* genes (Figure 8a and b). No effect was observed in the control strain (data not shown). Thus induction of either the *hok* or the *relF* gene leads to a collapse of the cell membrane potential.

The kinetics of reduction of the membrane potential is different for the different plasmids. $\Delta\psi$ is most rapidly reduced after induction of the *relF* gene expression from plasmid pBD2941 (down to zero within 5 min upon transfer to 41°C). When *hok* gene expression from pKG341 is induced it takes ~15 min before $\Delta\psi$ has reached its minimum. Since the *RelF* protein is expressed from the pL promoter, while the *Hok* protein is expressed under the control of the pR promoter, the differences in the kinetics of the membrane potential reduction are probably mainly due to differences in the level of gene expression of the two gene products (Remaut *et al.*, 1981).

After induction of *hok* gene expression from the plasmid pKG634, [^{3}H]TPP$^+$ uptake decreases more slowly and is not completely eliminated (Figure 8c). In this case the pR promoter is inserted upstream of the region encoding the regulator of *hok* gene expression (the *sok* gene, cf. Figure 1). Thus the presence of the *sok* gene appears to partially counteract *hok* gene ex-

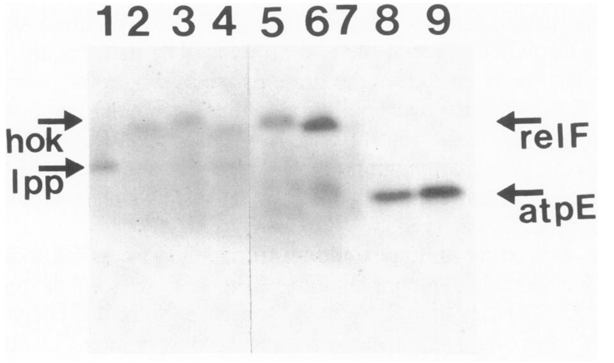

Fig. 7. Autoradiogram of a polyacrylamide gel showing the *hok* and *relF* gene products labelled with [^{35}S]methionine by the maxicell technique. Only the lower part of the gel containing proteins of interest is shown. **Lane 1:** *lpp* (lipoprotein, 6 kd) labelled by labelling total cells (von Meyenburg *et al.*, 1982); **lane 2:** membrane fraction from cells containing pKG341; **lane 3:** total fraction of cells containing pKG341; **lane 4:** cytoplasmic fraction from cells containing pKG341; **lane 5:** membrane fraction from cells containing pBD2941; **lane 6:** total fraction of cells containing pBD2941; **lane 7:** cytoplasmic fraction from cells containing pBD2941; **lane 8:** the *atpE* gene product (the c protein, 7 kd), membrane fraction; **lane 9:** the *atpE* gene product, total fraction. Labelled *atpE* gene product was obtained from an overproducer strain, see text.

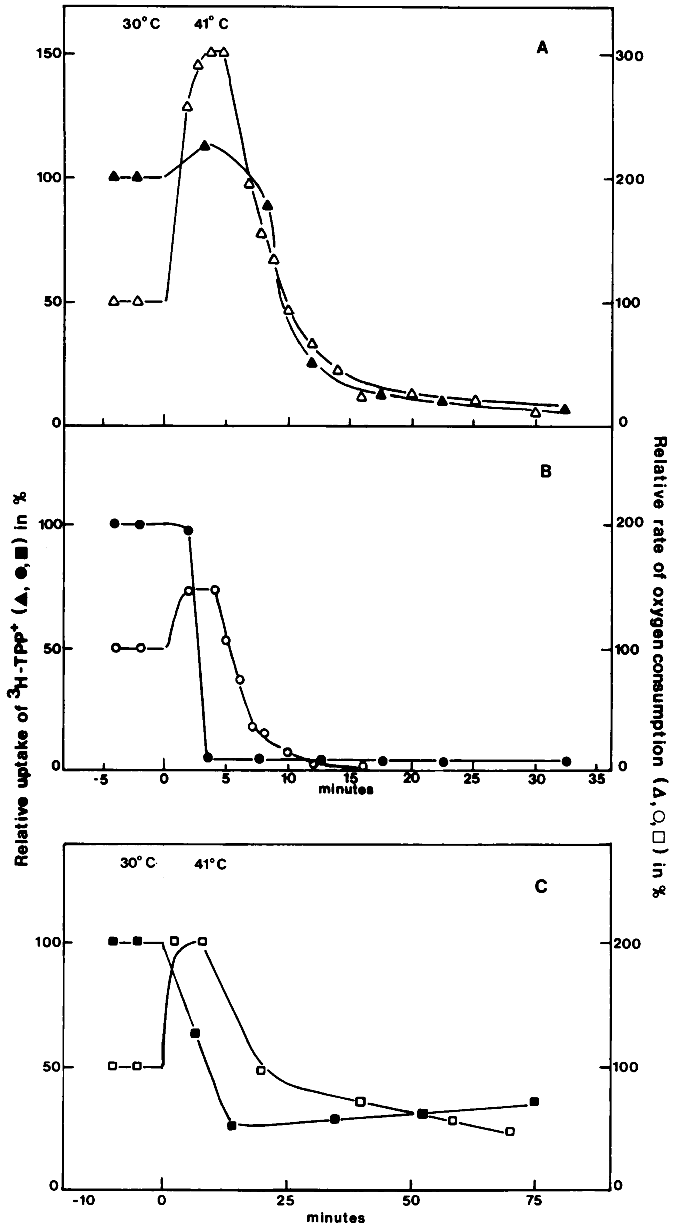

Fig. 8. Membrane potential (uptake of [^{3}H]TPP$^+$) and oxygen consumption of strain CM12 containing either pKG341 (**a**), pBD2941 (**b**) or pKG634 (**c**). Closed symbols show the membrane potential, open symbols show oxygen consumption. Note the difference in time scale between the **upper** (**a** and **b**) and **lower** (**c**) part of the figure.

pression, even when the *hok* gene is transcribed from the full de-repressed promoter pR of phage λ.

The rates of oxygen consumption of the same strains were measured in parallel with the TPP$^+$ uptake after a shift from 30 C to 41°C. As a reference level we used the rate of oxygen consumption at 2 min after the shift to 41°C (after the initial increase in oxygen consumption due to the temperature shift). In the strains with the *hok* and *relF* gene expression plasmids, respiration ceases after the initial increase upon the temperature shift (Figure 8). The kinetics of respiration cessation is similar to the kinetics of loss of membrane potential in the same three different cases. In two cases (Figure 8b and c) it appears that the loss of the membrane potential preceded the inhibition of respiration.

Table I. Loss frequencies/cell/cell cycle of *relB* and *parB*-carrying *oriC* minichromosomes in strains CM987 and JS115[a]

Plasmid	CM987 (*relB*$^+$)	JS115 (Δ*relB*)
pAL022	1.3×10^{-2}	2.9×10^{-2}
pAL041 (*parB*$^+$)	$<5.0 \times 10^{-4}$	$<5.0 \times 10^{-4}$
pAL042 (*relB*$^+$)	1.3×10^{-2}	2.5×10^{-2}

[a] Measured as described in Gerdes *et al.*, 1985.

Table II. Plasmids used and constructed

Plasmid	Relevant genotype	Source/reference
pKG341	pr *hok*$^+$ cI857	Gerdes *et al.*, 1986
pKG634	pR *hok*$^+$ *sok*$^+$ cI857	Gerdes *et al.*, 1986
pBD2430	pL *relB*$^+$ *relF*$^+$	Bech *et al.*, 1985
pBD2941	pL *relF*$^+$	This work
pLc28	contains pL	Remaut *et al.*, 1981
pNF2690	cI857	N.Fiil
pAL022	*oriC*$^+$	This work
pAL037	*relB*$^+$ *relF*$^+$	This work
pAL041	*oriC*$^+$ *parB*$^+$	This work
pAL042	*oriC*$^+$ *relB*$^+$	This work
pPR95	miniR1 *parB*$^+$	P.B.Rasmussen, K.Gerdes and S.Molin in preparation
pFHC271	*oriC*$^+$	Hansen *et al.*, 1981
pBR322		Bolivar *et al.*, 1977
pCMC1072	pR *atpE*$^+$ cI857	von Meyenburg *et al.*, 1985

Functional tests of the relB and parB regions: effects on the genetic stability of oriC minichromosomes

Since the *hok* gene expression is instrumental in rendering plasmid R1 genetically stable (through postsegregational killing of plasmidless cells), we asked whether the homologous chromosomal gene *relF* might have a similar 'stabilizing' function with respect to the chromosome by postsegregational killing of chromosomeless cells. In order to test this inference we constructed *oriC* minichromosomes (Messer *et al.*, 1978; von Meyenburg *et al.*, 1979) carrying the *parB* region with the *hok* gene of plasmid R1 on the one hand (pAL041), and the complete *relB* operon with the *relF* gene on the other hand (pAL042). Since the *parB* system has been found to exhibit allele-specific incompatibility (Gerdes *et al.*, 1985) we tested the *relB*$^+$ (*relF*$^+$) minichromosome in the *relB* deletion strain JS115 (Table I). The stability of the *oriC* minichromosomes was also tested in a wild-type *E. coli* K-12 strain (CM987).

The minichromosome carrying the *parB* region was very stably inherited in both strains compared with the *oriC* minichromosome without *parB*, which is very unstably inherited (von Meyenburg *et al.*, 1979). On the other hand, the presence of the *relB* operon did not increase the stability of the minichromosomes in either of the two strains. It is not known why the minichromosome itself (pAL022) is slightly more stable in strain CM987 than in JS115 (Table I).

Discussion

The genetic stability conferred by the partitioning locus *parB* to the antibiotic resistance plasmid R1, on which it naturally resides, or to other replicons, into which *parB* has been inserted, has been shown to be due to postsegregational killing of those cells in the population which at cell division have lost (or are born without) a *parB*-carrying replicon (Gerdes *et al.*, 1986).

The 'killing' could be assigned to the expression of a small gene, *hok*, whose expression is appropriately controlled by the neighbouring *sok* gene. Other plasmids (e.g. the broad host range plasmid RK2) have been found to contain genes whose expression is lethal to the host cell, the killing effects of these genes, however, have not been ascribed to any known physiological function (Figurski *et al.*, 1982. Young *et al.*, 1985). Recently, Jaffé *et al.* (1985) showed that the *ccdB* gene of plasmid F encodes a product which, under certain circumstances, is lethal to the host cell. More specifically, the expression of the killing factor seems to be regulated such that a dividing cell containing only one copy of a ccd^+ plasmid produces one viable plasmid-containing cell and one non-viable plasmid-free cell. Thus the *ccd* system of plasmid F seems to express its killing effect only in situations where the ccd^+ plasmid is lost due to an abnormal low copy number of the plasmid, whereas the *parB* system of R1 expresses its killing effect in the plasmid-free cell whatever the cause of plasmid loss. The mechanism of host cell killing by the *ccdB* gene product and the regulation of the *ccd* system has not yet been elucidated (Jaffé *et al.*, 1985).

In the present analysis the mode of action of the *hok* gene product has been investigated. The findings may be summarized as follows: (i) the *hok* gene encodes a small hydrophobic 52 amino acid long polypeptide which associates with the cellular membrane; (ii) a closely related gene, *relF*, is present in the *E. coli* chromosome as part of the *relB* operon (Bech *et al.*, 1985) the product of which is 40% homologous with the Hok protein and which also associates with the cell membrane; (iii) induced synthesis of the RelF polypeptide also leads to killing of the host cells and to the characteristic Hok-induced change in cell morphology (Figure 3); (iv) the rate of killing is dependent on the level of expression of the *relF* and *hok* genes respectively, and (v) shortly after induction of synthesis of the Hok or RelF proteins the electrochemical potential, $\Delta\psi$, and the rate of oxygen consumption decrease, the rapidity of the decrease in $\Delta\psi$ and respiration again corresponding to the degree of expression of those genes.

The mechanism of postsegregational killing by expression of the *hok* gene, or the killing due to induced expression of the *relF* gene, thus appears to be due to the inhibition of a vital function in the cell membrane through the insertion of the respective polypeptide in the cytoplasmic membrane. How can such small polypeptides have such a dramatic deleterious effect on both the membrane potential ($\Delta\psi$) and the respiratory activity? It seems that both are affected simultaneously, $\Delta\psi$ decreasing marginally before respiration (Figure 8). It is not the collapse of $\Delta\psi$ *per se* which is the cause of the decrease in respiration as shortcircuiting of the membrane potential by carbonyl-cyanide-m-chlorophenylhydrazone (CCCP, a prodonophore) or by the overproduction of the subunit 'a' of the ATP synthase does not lead to inhibition of respiration, rather the rate of oxygen consumption increases (von Meyenburg *et al.*, 1985; unpublished data). On the other hand, inhibition of respiration by cyanide does not lead to a collapse of $\Delta\psi$ either (von Meyenburg, unpublished data).

We are therefore led to propose that the Hok and RelF polypeptides interact with a membrane function, the inactivation of which simultaneously leads to inhibition of oxygen consumption and a shortcircuiting/uncoupling of the membrane potential. An obvious target therefore could be a component of the respiratory chain. Interference with this function by Hok or RelF should lead to an inhibition of electron transport and proton pumping; the interference with the proton pumping must be of the kind leading to an actual proton leak such that $\Delta\psi$ could not be maintained by alternative proton pumps such as H^+-ATPase.

A possible explanation of the morphological changes apparent in *Hok*-killed cells (Figure 3) and of these cells lower buoyant density (Gerdes *et al.*, 1986), both effects which only appear later after induction and therefore must be considered secondary effects, is that due to the permeabilization of the cell membrane, water accumulates in the cell due to osmotic pressure and a simultaneous loosening of the rigid peptidoglycan layer; only the latter effectively allowing for an expansion of the cell volume and dilution of the intracellular macromolecules.

In the light of the high toxicity of the *hok* and *relF* gene products it is not surprising that both genes appear to be tightly regulated. The *parB* region of plasmid R1 encodes another gene, *sok*, the product of which inhibits the expression of the *hok* gene in *trans* (Gerdes *et al.*, 1986). It is transcribed in the opposite direction (Figure 1A) and presumably yields a non-translated RNA (K.Gerdes, unpublished data). We therefore think that the *sok* gene product controls *hok* gene expression post-transcriptionally, probably inhibiting the translation of the *hok* mRNA to which it is complementary. An extensive elaboration of this control model will be published elsewhere. With respect to the expression of the *relF* gene, the third gene in the *relB* operon, it appears that its transcription is under negative control of the *relB* gene product (Bech *et al.*, 1985; unpublished results). The *relF* gene also seems to be subject to post-transcriptional control (unpublished results) and it is interesting to note that there is a high degree of homology of its translation start region with the one of the *hok* mRNA (Figure 5).

The physiological role of the *relF* gene is unknown: one possibility is that the *relF* gene could be involved in chromosome maintenance. We were able to test this proposal by cloning both the *relB* operon (including *relF*) and the *parB* region separately into unstably inherited *oriC* minichromosomes. The data presented in Table I show that the *parB* locus but not the *relB* operon, stabilized *oriC* minichromosomes. Thus the function of the *relF* gene is probably not one of postsegregational killing of chromosomeless cells, although it shares structural and functional homology with the *hok* gene. Maybe the *relF* gene product is involved in control of respiratory activity under extreme physiological growth conditions.

The close relationship between the *hok* and *relF* genes and their regulatory sequences implies that the two genes have evolved from a common ancestral gene. It is tempting to speculate that an ancient plasmid R1 has, by illegitimate recombination, picked up the chromosomal *hok* homologous gene, which then evolved into the present plasmid maintenance system.

Materials and methods

Bacterial strains and plasmids

The *E. coli* K-12 strain CSH50 (*Δlac pro rpsL*; Miller, 1972) was used as recipient of ligated DNA in the plasmid construction procedures, whereas the K-12 strain JC411 (*metB leu his argG lacY malA xyl mtl gal rpsl*; Bachmann, 1972) was used in the physiological experiments. Strains CM987 ($relB^+$; Hansen *et al.*, 1981) and JS115 (*ΔrelB*) (donated by J.-P.Bouche) were used to test the genetic stability of *oriC* minichromosomes carrying the *parB* or *relB* genes. Strains CSR603 (Sancar *et al.*, 1979) was used in the maxicell experiments. The plasmids used and constructed are listed in Table II.

Biochemical methods

Large scale preparation of plasmid DNA was as described by Stougaard and Molin (1981), small scale preparation of plasmid DNA was according to Birnboim and Doly (1979). Digestion with restriction endonucleases and ligation of restricted DNA was accomplished as described previously (Gerdes *et al.*, 1985). Transformation of bacterial cells was done by the method of Cohen *et al.* (1972).

Detection of plasmid encoded proteins

The maxicell method of Sancar *et al.* (1979) was used to label plasmid-encoded polypeptides. Cycloserine (100 μg/ml) was added to the maxicell culture after u.v.-irradiation. The maxicells were labelled for 10 min with [^{35}S]methionine, 2 min after the temperature was raised to 42°C to induce the *hok* and *relF* genes, and then chased for another 10 min with non-radioactive methionine.

Preparation of crude membranes

Crude membrane fractions were prepared from maxicells labelled with [^{35}S]methionine as described above using the NaOH precipitation method of Russel and Model (1982).

Plasmids

pKG634 and pKG341. The construction of these plasmids is described elsewhere (Gerdes *et al.*, 1986). Plasmid pKG634 is a pBR322 derivative containing the λ pR promoter inserted in front of the *hok* gene. The plasmid carries the cI857 allele of the λ repressor gene. The plasmid also carries the *sok* gene (Figure 1). Plasmid pKG341 is an analogous plasmid containing the pR promoter in front of the *hok* gene but does not carry the *sok* gene (Figure 1).

pBD2941. Plasmid pBD2941 contains a fragment of the *relB* operon beginning 16 bp before the start codon of the *relF* gene and ending 45 bp after its stop codon. This fragment was brought into the polylinker region of pUC8 and from there moved (using the *Eco*RI and *Hin*dIII sites of the polylinker region) to a position just downstream of λ pL of pLc28 (Remaut *et al.*, 1981).

pAL037. Plasmid pBD2430 is a pUC8 derivative containing the entire *E. coli relB* operon on an *Eco*RI-*Hin*dIII restriction fragment (Bech *et al.*, 1985). The plasmid was restricted with *Hin*dIII, the overhanging ends filled by treatment with the Klenow fragment of DNA polymerase I, and *Eco*RI linkers added. One plasmid which had taken up an *Eco*RI linker was designated pAL037. This plasmid contains the *relB* operon on an *Eco*RI restriction fragment, thus facilitating transfer of the fragment in further plasmid constructions.

pAL022. The *Mst*I fragment of the minichromosome pFHC271 (Hansen *et al.*, 1981) extending from position 1230L to 2499R in the *oriC* region was ligated to the *Xmn*I fragment of pBR322 carrying the tetracycline reistance gene. Thus pAL022 is a minichromosome derivative of 6160 bp containing a unique *Eco*RI restriction site and conferring tetracycline resistance on plasmid-bearing cells.

pAL041. The 900 bp *parB*-carrying *Eco*RI fragment of pPR95 (P.B.Rasmussen, K.Gerdes and S.Molin, in preparation) was inserted into the *Eco*RI site of pAL022.

pAL042. The *relB*-carrying fragment of pAL037 was inserted into the *Eco*RI site of pAL022.

Measurement of the cell membrane potential

The membrane potential was measured by the method of Hirota *et al.* (1981). The lipophilic cation tetraphenylphosphonium (TPP$^+$) is taken up intracellularly in proportion to the size of the cell membrane potential. Thus the membrane potential can be measured indirectly by determining the extent of uptake of [^{3}H]TPP$^+$ into growing cells.

Rate of oxygen consumption

The rate of oxygen consumption was determined using a Clark type electrode (Kier *et al.*, 1976) by measuring the decrease in dissolved oxygen concentration in aliquots of the cultures at the respective temperatures (von Meyenburg *et al.*, 1985).

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