Complementation of the *hha* mutation in *Escherichia coli* by the *ymoA* gene from *Yersinia enterocolitica*: dependence on the gene dosage

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The Hha protein from *Escherichia coli* is highly similar (82 %) to the YmoA protein from *Yersinia enterocolitica*. Both are members of a new class of proteins that modulates gene expression, probably by influencing DNA topology. In this paper, complementation of the *hha* mutation in *E. coli* by the *ymoA* gene from *Y. enterocolitica* has been studied. We show that the *ymoA* gene complements one of the phenotypic properties of *hha* mutants (high level of haemolysin production when they carry the recombinant plasmid pANN202-312) when cloned in a medium-copy-number plasmid but not when carried in a low-copy-number plasmid. Western blot analysis of the expression of YmoA in *E. coli* rules out inefficient expression of the protein. Surprisingly, the *hha* gene itself fails to complement the *hha* mutation when cloned in a medium-copy-number vector and causes genetic rearrangements of the *E. coli* chromosome as a consequence of insertion sequences mobilization.

Keywords: *hha* gene, *ymoA* gene, gene complementation, gene dosage, insertion element mobilization

INTRODUCTION

The *hha* gene has been identified as modulating the expression of haemolysin in *Escherichia coli* (Godessart *et al.*, 1988). Its product, the Hha protein, is an 8.6 kDa protein that shows 82 % similarity to the YmoA protein of *Yersinia enterocolitica* (De la Cruz *et al.*, 1992), a temperature-dependent modulator of the expression of virulence factors in this micro-organism (Cornelis *et al.*, 1991). Factors that alter the degree of DNA supercoiling mimic the effect of the *hha* mutation on the expression of haemolysin in cells harbouring the recombinant haemolytic plasmid pANN202-312. As *hha* mutants also show alterations in the degree of plasmid DNA supercoiling, it was suggested that the mechanism by which the Hha protein modulates gene expression is through changes of DNA topology (Carmona *et al.*, 1993). Both *hha* and *ymoA* mutants display a pleiotropic phenotype (Blanco *et al.*, 1991; Cornelis *et al.*, 1991; Nieto *et al.*, 1987). It has been suggested that both proteins are members of the same class of proteins that modulates

bacterial gene expression, probably by affecting DNA topology (De la Cruz *et al.*, 1992; Mikulskis & Cornelis, 1994).

Recently, complementation of the *ymoA* mutation by the *hha* gene has been studied (Mikulskis & Cornelis, 1994). The authors showed that some selection pressure occurred at the stage of introduction of the *hha*-gene-containing plasmid into *Y. enterocolitica* either by conjugation from *E. coli* or by electroporation. Transconjugants and transformants occurred at very low frequencies and carried mutations either in the promoter region or in the coding sequence of the *hha* gene (Mikulskis & Cornelis, 1994). Despite the sequence alterations, the modified recombinant plasmids did complement the *ymoA* mutation. The authors could also observe a significant mobilization of insertion sequences (IS) from the chromosome of the *E. coli* strain used as donor in the conjugation experiment. The authors interpreted this as being a consequence of the increased dosage of the *hha* gene, which was carried in a medium-copy-number plasmid.

In this paper, we have addressed the question of whether or not the *ymoA* gene, cloned in vectors with different copy number, complements the *hha* mutation in *E. coli*.

Abbreviation: IS, insertion sequence(s).

METHODS

Bacterial strains, plasmids and media. *E. coli* strains and plasmids are listed in Table 1. pLG338-30 is a 5 kb, low-copy-number (one to five copies per cell) plasmid containing the replicon from plasmid pSC101. Plasmid pANN202-312 carries the *hlyCABD* gene cluster from the low-copy-number haemolytic plasmid pHly152, and was previously described by Goebel & Hedgepeth (1982). Strain UBM1 of *Y. enterocolitica*, used for amplification of the *ymoA* gene, was isolated in a food-quality-control laboratory.

Bacterial cultures were grown in LB supplemented with antibiotics at the following concentrations (mg l⁻¹): ampicillin, 0.025 for plasmids derived from pLG338-30 and 0.1 for plasmids derived from pBR322; chloramphenicol, 0.05; and kanamycin, 0.025.

Cloning of the *ymoA* and *hha* genes. Synthesis of the *ymoA* gene was carried out by the PCR technique. A colony of the strain of *Y. enterocolitica* used in this study was resuspended in 100 µl distilled water, 1 µl of the suspension being used directly as template in the PCR reaction. Amplimers YMOA1 (5'-GGGATCCGAGCGTTTATTCAGACTTTTC-3') and YMOA2 (5'-GGTCGACCGTTGTGTCTTAACGACG-3') were designed according to the *ymoA* sequence published by Cornelis *et al.* (1991), except for the seven nucleotides on both 5' ends that were included to generate targets for *Bam*HI (YMOA1) and *Sal*I (YMOA2), thus allowing easy cloning after amplification. The 351 bp product was then cloned into the pGEM-T vector system (Promega, A3600), its nucleotide composition being determined by DNA sequencing with Sequenase (United States Biochemical), and was finally cloned as a *Bam*HI-*Sal*I fragment into plasmids pLG338-30 and pBR322. Gene *hha* from *E. coli* was cloned by the same procedure described for *ymoA*. Amplimers HHA1 (5'-GGGATCCAACCTCGCATCAACCTCCAG-3') and HHA2 (5'-GGTCGACTTCATGGCGAAAAAGTATAAA-3') were designed according to the sequence published by Nieto *et al.* (1991). *E. coli* 5K was used as template for amplification of the *hha* gene, which was then sequenced and cloned following the same procedure described for *ymoA*.

Haemolysin assay. Sheep erythrocytes (6 ml) were washed several times with 0.9% NaCl and resuspended in 130 ml of the same saline solution to which 20 ml 0.2 M CaCl₂ was added; 1.2 ml of this solution was then mixed with 200 µl of the corresponding sample to be assayed, and incubated at 37 °C. At

different times, the microfuge tubes were centrifuged for 30 s, and the release of haemoglobin was measured by monitoring the increase in A_{543} using a Shimadzu model UV-265FW spectrophotometer. To calculate the haemolytic units we used the following formula: units = $1000 \times A_{543}$ per OD₆₀₀ unit min⁻¹ ml⁻¹ (OD₆₀₀ being used as a measure of culture density).

Haemolysin Western blot analysis. Samples were taken when cells entered the stationary phase (OD₆₀₀ = 1.0). Upon centrifugation, 1 ml supernatant samples were concentrated by acetone precipitation and used without further manipulation for the detection of HlyA by Western blot analysis as previously described by Nieto *et al.* (1991), using rabbit polyclonal antiserum raised against HlyA.

Hha- and YmoA-specific antibodies. A peptide that included the 17 amino-terminal residues of the Hha protein was synthesized and injected into rabbits as a conjugate with keyhole limpet haemocyanin by standard procedures. Testing the extracted serum allowed us to realize that it was able to recognize YmoA as well as Hha. Western blot analysis of the expression of Hha and YmoA was done by standard procedures.

RESULTS

Amplification, sequencing and cloning of the *ymoA* gene from *Y. enterocolitica* and the *hha* gene from *E. coli*

We amplified *ymoA* with its own promoter from *Y. enterocolitica* chromosomal DNA. A single 0.35 kb DNA fragment was synthesized and its sequence was determined in both directions. The base composition of the amplified sequence turned out to be exactly as previously described by Cornelis *et al.* (1991). The *ymoA* gene was then cloned with its own promoter into the *Bam*HI-*Sal*I sites of plasmids pLG338-30 and pBR322, forming plasmids pCB1Y and pCB2Y, respectively. The *hha* gene was also amplified, sequenced and cloned with its own promoter exactly as described for *ymoA*. A 0.57 kb fragment was the result of the amplification procedure. The nucleotide sequence of the fragment turned out to be as previously described by us (Nieto *et al.*, 1991). Finally, the *hha* gene was cloned into the *Bam*HI-*Sal*I sites of plasmids pLG338-30 and pBR322, forming plasmids pCB1H and pCB2H, respectively.

Table 1. *E. coli* strains and plasmids

Strain/plasmid	Genotype/description	Source/reference
Strains		
5K	F ⁻ <i>hsdR hsdM rpsL thr thi leu lacZ</i>	Godessart <i>et al.</i> (1988)
Hha3	5K <i>hha::Tn5phoA</i>	Godessart <i>et al.</i> (1988)
Plasmids		
pANN202-312	<i>hlyC HlyA HlyB HlyD</i> ; Cm ^r	Goebel & Hedgepeth (1982)
pSUP104- <i>sac</i>	Cm ^r Km ^r ; <i>sacB</i> cassette	Simon <i>et al.</i> (1991)
pLG338-30	<i>ori</i> _{PSC101} ; Ap ^r	Cunningham <i>et al.</i> (1993)
pBR322	<i>ori</i> _{PMB1} ; Ap ^r Tc ^r	Bolívar <i>et al.</i> (1977)
pCB1Y	pLG338-30 + PCR-synthesized <i>ymoA</i> ; Ap ^r	This study
pCB2Y	pBR322 + PCR-synthesized <i>ymoA</i> ; Ap ^r	This study
pCB1H	pLG338-30 + PCR-synthesized <i>hha</i> ; Ap ^r	This study
pCB2H	pBR322 + PCR-synthesized <i>hha</i> ; Ap ^r	This study

Table 2. Haemolytic phenotype on blood-agar plates of transformants obtained with recombinant plasmids carrying either *ymoA* or *hha*

Strain*	Recombinant plasmid	Replicon	Copy number	Gene cloned	Haemolytic haloes
5K	None				Narrow (parental phenotype)
Hha3	None				Large (mutant phenotype)
Hha3	pCB1Y	pLG330	Low	<i>ymoA</i>	Large
Hha3	pCB1H	pLG330	Low	<i>hha</i>	Narrow
Hha3	pCB2Y	pBR322	Medium	<i>ymoA</i>	Narrow
Hha3	pCB2H	pBR322	Medium	<i>hha</i>	Variable†

* All strains also contained the haemolytic plasmid pANN202-312.

† The colonies were basically poorly haemolytic but displayed heterogeneity in the size of the individual haloes.

Transformation of *E. coli* with the recombinant plasmids harbouring either *hha* or *ymoA*

Plasmids pCB1Y, pCB2Y, pCB1H and pCB2H were transformed into *E. coli* strain Hha3(pANN202-312). This strain forms large haemolytic haloes on blood-agar plates (highly haemolytic phenotype) as a consequence of the *hha* mutation. Transformants were plated on blood-agar plates containing the relevant selective agents for the plasmids and Tn5 and incubated for 24 h. Observation of the haemolytic haloes obtained for each recombinant plasmid gave an initial picture of the haemolytic phenotype of the recombinant clones (Table 2). Whereas all the transformants obtained with plasmid pCB1Y showed a highly haemolytic phenotype, transformants obtained with plasmid pCB1H showed a haemolytic phenotype similar to that of the parental 5K(pANN202-312) strain (narrow haemolytic haloes). As in both cases the colonies were homogeneous in size and in haemolytic phenotype, only one colony from each transformation was purified to carry out the complementation analysis. All transformants obtained with plasmid pCB2Y showed the parental haemolytic phenotype. One colony was purified to carry out the complementation analysis. Finally, transformants obtained with pCB2H gave rise to colonies that were basically poorly haemolytic but which displayed heterogeneity in size. Haloes were also heterogeneously sized. Two colonies, Hha3(pANN202-312)(pCB2H)A and Hha3(pANN202-312)(pCB2H)B, were studied. No differences in the transformation frequencies were apparent for any of the plasmids used.

Complementation of the *hha* mutation by the *ymoA* and *hha* genes

Complementation analysis was carried out by measuring the external haemolytic activity (active haemolysin) during growth and also by detecting the HlyA polypeptide in the external medium by Western blot analysis of the supernatant of the cultures (total secreted haemolysin) of the selected transformants, strain Hha3(pANN202-312)

and the parental strain 5K(pANN202-312). The results obtained are shown in Fig. 1. Differences in growth kinetics were only detected for the strains carrying plasmids pCB2HA and pCB2HB, which respectively reached a final OD₆₀₀ of 1.0 1 and 2 h after the other strains studied (data not shown). As can be seen, strains carrying the *hha* gene cloned on the low-copy-number plasmid pCB1H and the *ymoA* gene cloned on the medium-copy-number plasmid pCB2Y clearly complemented the *hha* mutation and displayed haemolysin production kinetics and final levels of total and active haemolysin similar to those exhibited by the parental strain 5K(pANN212-312). On the other hand, the *ymoA* gene cloned in pCB1Y did not complement the *hha* mutation at all. Strain Hha3(pANN202-312)(pCB1Y) showed similar kinetics and levels of haemolysin expression to those shown by strain Hha3(pANN202-312). Finally, the two strains harbouring the *hha* gene cloned in pCB2H did not complement the haemolytic phenotype, with both haemolysin production kinetics and final levels being different to those of the 5K(pANN302-312) strain.

Recent data reported by Mikulskis & Cornelis (1994) indicated that the *hha* gene induced IS jumping and other genetic rearrangements in the *E. coli* genome when cloned in a pACYC184 plasmid derivative. The recombinant plasmid itself was also affected and presented alterations in the sequence of the cloned *hha* gene. Thus, following the complementation analysis, we decided to test if the sequences of the *hha* and *ymoA* genes cloned in plasmids pCB1H, pCB1Y, pCB2H and pCB2Y had remained unaltered. With this aim, recombinant plasmids were isolated from strains Hha3(pCB1Y), Hha3(pCB2Y), Hha3(pCB1H), Hha3(pCB2H)A and Hha3(pCB2H)B, digested with *Bam*HI-*Sal*I and electrophoretically analysed. No differences in size were detected for any of the five plasmids analysed, either in the insert or in the vector sequences. Thus, none of them contained any IS element. Four inserts [the one obtained from strain Hha3(pCB2H)B was not used further] were then purified from the gel and cloned again into new vector molecules

Fig. 1. Analysis of the haemolytic phenotype of the *E. coli* strains harbouring different recombinant plasmids (all strains also contained the haemolytic plasmid pANN202-312). (a) Evaluation of the haemolytic activity during growth of strains 5K (●), Hha3 (○), Hha3(pCB1Y) (▲), Hha3(pCB1H) (△), Hha3(pCB2Y) (▼), Hha3(pCB2H)A (■) and Hha3(pCB2H)B (□). Measurements were taken until the OD₆₀₀ of each culture reached 1.0. (b) Western blot of HlyA. Lanes: 1, strain 5K; 2, strain Hha3; 3, strain Hha3(pCB1Y); 4, strain Hha3(pCB1H); 5, strain Hha3(pCB2H)A; 6, strain Hha3(pCB2H)B; 7, strain Hha3(pCB2Y). The amount of sample supernatant loaded was corrected depending upon the protein concentration of each culture. All samples loaded thus correspond to amounts of HlyA produced by a similar number of cells. Molecular mass markers (in kDa) are shown on the left.

that had not previously been used for cloning purposes. Plasmid pLG338-30 was used to clone the fragments carrying genes *bha* or *ymoA* isolated from pCB2H and pCB2Y, respectively, and plasmid pBR322 was used for cloning those fragments isolated from pCB1H and pCB1Y. With this procedure, inserts previously cloned in a low-copy-number plasmid (pLG338-30) were now carried in a medium-copy-number plasmid (pBR322) and vice versa. The new recombinant plasmids were transformed in strain Hha3(pANN202-312). Again transformants were homogeneous in the size of the haemolytic haloes, except for the clones containing plasmid pCB2H. An individual clone of each type was isolated to carry out another complementation analysis by measuring the active

and total haemolysin. The values obtained were the same as those already observed for the recombinant plasmids initially obtained (results not shown). The fact that a fragment containing either the *bha* or *ymoA* gene from plasmids that did not complement the *bha* mutation could then complement it when cloned in a different plasmid, showed that the *bha* and *ymoA* genes were functional and that modifications of their sequences were not at the origin of the differences in haemolysin production observed when those genes were cloned into two different-copy-number vectors.

As plasmid pCB2Y complemented the *bha* mutation but plasmid pCB1Y did not, we decided to test if the *ymoA* gene was poorly expressed in *E. coli*, only a high-gene dosage rendering sufficient levels of the YmoA protein to complement the *bha* mutation. To test this, we monitored the production of Hha and YmoA proteins by Western blot analysis of cell extracts obtained from strains harbouring the corresponding recombinant plasmids. Cell extracts from clones pCB1Y and pCB1H showed similar amounts of YmoA and Hha, respectively, as also did clones harbouring pCB2Y and pCB2H (data not show), thus indicating that YmoA was properly expressed in *E. coli*.

Mobilization of IS in the *E. coli* strains containing the recombinant plasmids

A surprising result from this study was that the 0.57 kb DNA fragment containing the *bha* gene failed to complement the highly haemolytic phenotype of strain Hha3(pANN202-312) when cloned in plasmid pBR322. The transformants obtained with plasmid pCB2H showed heterogeneity on blood-agar plates and the two purified isolates displayed haemolysin levels and production kinetics different from each other and from those shown by the control strain Hha3(pANN202-312). Considering the previously reported results of Mikulskis & Cornelis (1994) on the effect of a high-gene dosage of *bha* on IS element mobilization, we decided to test if, in our model, different levels of Hha and YmoA proteins could also account for IS mobilization. To do this, an IS element 'trap', a *sacB*-gene-containing plasmid, was used. *E. coli* cells containing the *Bacillus subtilis* *sacB* gene will not grow in medium containing 5% (w/v) sucrose unless inactivation of the *sacB* gene occurs. With this aim, strain Hha3(pSUP104-*sac*) was transformed with the same plasmids used in the complementation studies. Immediately after transformation, five colonies from each experiment were resuspended in liquid medium and grown overnight. Aliquots of each culture were plated in parallel on sucrose-containing and non-sucrose-containing agar. Table 3 shows the number of sucrose-resistant mutants among 10⁸ cells obtained for each culture. Significant differences were only detected for the clones containing the pBR322-related plasmids. Clones containing plasmid pCB2H clearly rendered higher numbers of colonies growing on sucrose plates than the control strain containing plasmid pBR322 and the strain containing the cloned *ymoA* gene. No significant differences were detected

Table 3. Influence of *hha* and *ymoA* gene dosage on transposition mutations: numbers of sucrose-resistant mutants among 10⁸ cells in five different clones

Strain	Number of mutants					Geometric mean
	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5	
Hha3(pBR322)	53	52	245	100	436	177
Hha3(pCB2H)	894	1083	603	578	357	703
Hha3(pCB2Y)	18	68	36	21	54	39
Hha3(pLG338-30)	25	19	23	93	17	35
Hha3(pCB1H)	10	123	12	27	32	41
Hha3(pCB1Y)	34	3	98	56	41	46

ted among the strains containing pLG338-30-related plasmids. Because *sacB* inactivation could be due not only to IS element transposition but also to deletions or point mutations, plasmid DNA from 10 clones of each type was extracted and electrophoretically analysed. Plasmids were considered to contain an IS element if their size was bigger than the original plasmid pSUP104-*sac*, a deletion if they were smaller, or a point mutation if no differences were detected (Simon *et al.*, 1991). The number of presumed IS-containing plasmids detected for pCB2H, pCB2Y and pBR322 was eight, seven and seven, respectively. As the number of resistant clones that contained IS insertions was similar, it is apparent that plasmid pCB2H enhances the frequency of transposition events. This effect, which was not observed when the gene was cloned in a low-copy-number plasmid such as pLG338-30, could be responsible for the heterogeneous haemolytic phenotype displayed on blood-agar plates by clones harbouring plasmid pCB2H. It could also account for the non-complementing haemolytic phenotype described for the *E. coli* strains Hha3(pANN202-312)(pCB2H)A and Hha3(pANN202-312)(pCB2H)B. On the other hand, strains containing *ymoA* either on a pBR322 derivative or on a pLG338-30 derivative showed no apparent difference in IS mobilization when compared with the control strain carrying only the vector.

DISCUSSION

Mikulskis & Cornelis (1994) reported complementation of the *ymoA* mutation in *Y. enterocolitica* by the *hha* gene of *E. coli*. Nevertheless, complementation was only accomplished when the recombinant plasmids harbouring the *hha* gene contained mutations either in the promoter region of *hha* or in the *hha* gene coding region. This was interpreted as the Hha protein being present at levels toxic for *Y. enterocolitica*, thus selecting for mutations which reduced Hha expression or modified it, clones harbouring such modified plasmids being viable. In this study, we show that introduction of the *ymoA* gene in *E. coli*, either in low- or in medium-copy-number plasmids, did not result in the selection of clones containing a modified *ymoA* gene. These results agree with those reported by Mikulskis & Cornelis (1994), which also indicated that *E. coli* is more tolerant to *hha* or *ymoA* than *Y. enterocolitica*,

as both genes could be cloned and manipulated in the former host without accumulation of mutations. Nevertheless, *ymoA* complements the effect of the *hha* mutation on haemolysin expression only when cloned in the pBR322 derivative and not when plasmid pLG338-30 was used as vector. There are at least two possible explanations for these results. One of them could rely on the differences between proteins YmoA and Hha. Despite the 82% similarity that they exhibit, they are different in size. Both differences in size and in the amino acid sequence may account for an inefficient function of YmoA in *E. coli*, it only being able to substitute for Hha when overexpressed. Another possible explanation could rely on the fact that *ymoA* was cloned in *E. coli* under the control of its own promoter region, which would not necessarily be efficiently recognized by the transcriptional machinery of *E. coli*. Related to that, we should point out that Western blot analysis of the expression of the YmoA and Hha proteins in *E. coli* rules out an inefficient expression of YmoA and reinforces the hypothesis that structural differences between Hha and YmoA may account for the inefficiency in complementing the *hha* allele.

Usually, vectors derived from ColE1 or from pMB1, such as pBR322 itself, are present at 15–20 copies per cell in *E. coli* and are said to be medium- or even low-copy-number. However, several authors have already pointed out that their copy number may be considered too high when they are used for cloning prokaryotic regulatory or membrane protein genes (Wang & Kushner, 1991; Claverie-Martín *et al.*, 1989; Spratt *et al.*, 1980) or viral genomes (Cunningham *et al.*, 1993) in *E. coli*. Here we show that the 0.57 kb DNA fragment containing the *hha* gene is responsible for an increase in genetic instability as a result of IS mobilization from the *E. coli* genome when cloned in a medium-copy-number but not in a low-copy-number vector. This observation was also reported by Mikulskis & Cornelis (1994). We hypothesize that IS mobilization could be at the origin of the heterogeneous phenotype observed on blood-agar plates for the clones harbouring plasmid pCB2H. It could also explain why strains Hha3(pCB2H)A and Hha3(pCB2H)B, which contained this plasmid, exhibited a reduced growth rate in liquid culture compared to all other strains and showed aberrant haemolytic phenotypes when the complementation analysis was carried out. Finally, transposition of IS elements in

strains harbouring plasmid pCB2H could also indicate that Hha may have a role in IS stability in the *E. coli* genome, but this point must be studied further.

As changes in DNA topology may influence the expression of several different genes and thus affect the physiology of the bacterial cell in very different ways, one can recommend special care in the choice of the appropriate cloning vector when a protein that probably influences DNA topology, like Hha, is studied. A similar observation was made when the *osmZ* gene coding for the histone-like protein H-NS was cloned in a ColE1 derivative vector and used for complementation analysis. The gene accumulated mutations at high frequency, probably selected to down-regulate its expression or the activity of the protein (Hulton *et al.*, 1990). According to our results, cloning this class of proteins only into medium-copy-number plasmids like pBR322 is not an appropriate procedure for carrying out genetic complementation analysis, although it can help to obtain some additional information. Overproduction of proteins influencing DNA topology can be of interest, as it has been shown here and by some other authors (Spurio *et al.*, 1992). This is also the case in a study where the authors searched for genes that circumvented the export-defective phenotypes of *sec* mutations when cloned into a multicopy plasmid derived from pBR322 (Ueguchi & Ito, 1992). One of the clones that was active in suppression contained the gene for the histone-like protein H-NS, which had never been described as influencing secretion in *E. coli*, and, according to the authors, the study described a novel phenotype of H-NS.

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