Chromosomal Mutations That Increase the Production of a Plasmid-encoded Haemolysin in *Escherichia coli*

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Transposon mutagenesis was used to isolate two *Escherichia coli* mutants which express very large amounts of haemolysin when carrying the multicopy plasmid pANN202-312. *E. coli* strain Hha-2 was isolated by Mud1 mutagenesis, and strain Hha-3 by Tn5 mutagenesis. The transposon insertion was chromosomal in both mutants and could be demonstrated to be unrelated to the haemolytic region of the plasmid. The substantial increase in both extracellular and intracellular haemolysin production was dependent upon plasmid copy number and was drastically reduced when either mutant carried the low-copy-number haemolytic plasmid pHly152. In both mutants, the marked increase in extracellular production was dependent upon the specific haemolysin transport genes, *hlyB* and *hlyD*. The lack of either gene function resulted in no external haemolysin production. SDS-PAGE analysis showed no change in the pattern of outer-membrane proteins of the mutants, although changes (differing between the two mutants) were seen in their periplasmic proteins. The mutations of both strains (termed *hha-2* and *hha-3*) were mapped at minute 10.5 of the *E. coli* chromosome. No relation to any known gene affecting gene regulation in *E. coli* could be found.

INTRODUCTION

The production of haemolysins, exotoxins that disrupt the membranes of different mammalian cells, is a property of several bacterial species. In *Escherichia coli*, haemolysin production is especially frequent among strains isolated from extraintestinal infections (Mackman *et al.*, 1985*b*; Müller *et al.*, 1983), and recent data correlate production of haemolysin and Gal–Gal adhesins with uropathogenicity (O'Hanley *et al.*, 1985). Haemolysin is extremely labile, which is one of the reasons why the biochemistry and regulation of its synthesis are still poorly understood.

In *E. coli*, the haemolytic determinant can be carried either on conjugative plasmids or on the chromosome (Müller *et al.*, 1983). DNA hybridization studies have shown a high degree of homology between different *E. coli* haemolysin determinants, irrespective of their origin (Berger *et al.*, 1982; De la Cruz *et al.*, 1980). The genetic structure and organization of the haemolysin determinant from the haemolytic plasmid pHly152 has been extensively studied (Noegel *et al.*, 1981). A cluster of four genes is necessary for the synthesis and secretion of this toxin. *hlyC* and *hlyA* code for the synthesis of the active haemolysin. *hlyB* and *hlyD* gene products are responsible for the secretion of the haemolysin into the external medium (Bhakdi *et al.*, 1986; Wagner *et al.*, 1983). By using Mud1 directed *hly*–*lacZ* fusions, it was shown that two promoters (p1 and p2) control the expression of the *hly* genes (Juárez *et al.*, 1984*b*). p1 controls the transcription of the sequentially ordered *hlyC*, *hlyA* and *hlyB* genes, whereas p2 controls the expression of *hlyD*. Under the control of p1, β -galactosidase is synthesized constitutively during the exponential growth phase, regardless of the composition of the culture medium (Juárez *et al.*, 1984*b*). An intriguing problem arose when the haemolysin determinant of pHly152 was cloned in pACYC184. The recombinant plasmid obtained, pANN202-312, did not confer the

production of larger amounts of haemolysin, although it should be a high-copy-number plasmid (Goebel & Hedgpeth, 1982). On the other hand, González-Carrero *et al.* (1985) reported a similar cloning experiment using pBR322 as vector. Cells harbouring the resulting haemolytic plasmid, pSU157, produced about 20 times more haemolysin than those harbouring the parental plasmid pHly152. In pANN202-312, insertions of *lacZ* in either *hlyC* or *hlyA* showed that β -galactosidase was synthesized commensurate with the copy number of the plasmid (Juárez *et al.*, 1984a). Thus, a transcriptional repression did not appear to be responsible for that effect. In order to obtain more information about the mechanisms that seem to repress haemolysin expression in cells harbouring pANN202-312, we decided to mutagenize an *E. coli* strain harbouring this plasmid in the hope that chromosomal mutations could restore the expected high level of haemolysin production due to the high copy number of the plasmid. Here we report the isolation and partial characterization of two such mutants.

METHODS

Bacterial strains and plasmids. The bacterial strains used are listed in Table 1. The low-copy-number haemolytic plasmid pHly152 (Noegel *et al.*, 1981), the multicopy haemolytic plasmid pANN202-312 (Goebel & Hedgpeth, 1982), its derivative pANN202-312::Mud1-2, which lacks the *hlyD* function (Juárez & Goebel, 1984), and plasmids pANN202-312::Tn5-10 (*hlyB*) (Wagner *et al.*, 1983) and pANN202-812 (Vogel *et al.*, 1988) have been previously described. Plasmid pANN202-312R was constructed identically to pANN202-812, using pACYC184 as vector instead of pBR322.

Media and genetic manipulations. LB medium (Miller, 1972) and blood agar (Wagner *et al.*, 1983) were routinely used for the maintenance and growth of the strains. When required, ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline were added at the concentrations indicated by Maniatis *et al.* (1982). Generalized transduction by *P1*cm_{ts} phage, and conjugation, were done as described by Miller (1972). For the mapping

Table 1. *E. coli* strains used

Strain	Relevant genotype	Source or reference
5K	F ⁻ r _K ⁻ m _K ⁻ rpsL thr thi leu lacZ	Juárez <i>et al.</i> (1984a)
SG932	F ⁻ lac trp pho supC Δ lon-100	Bishai <i>et al.</i> (1987)
Hha-2	5K hha-2::Mud1	This study
Hha-3	5K hha-3::Tn5	This study
BW5659	Hfr(PO53) lacY1 zdh-57::Tn10 mglP1 xyl-7	B. Bachmann
BW6163	Hfr(PO45) λ ⁻ zed-977::Tn10 relA spoT1 thi-1	B. Bachmann
BW6169	Hfr(PO13) leuB6 supE gal-6 λ ⁻ argA::Tn10 thi	B. Bachmann
BW6166	Hfr(PO18) supE λ ⁻ zhf-721::Tn10 thi malB	B. Bachmann
BW6156	Hfr(PO3) λ ⁻ relA spoT1 metB zje::Tn10	B. Bachmann
BW7261	Hfr(PO2A) leu-63::Tn10 tonA λ ⁻ Δ (argF-lacU169) ompF relA spoT	B. Bachmann
BW7623	Hfr(PO43) purE79::Tn10	B. Bachmann
BW7622	Hfr(PO44) λ ⁻ trpB::Tn10 relA spoT thi	B. Bachmann
BW6160	Hfr(PO118) λ ⁻ zdh::Tn10 relA spoT metB	B. Bachmann
BW7620	Hfr(PO42) λ ⁻ zed::Tn10 relA spoT thi	B. Bachmann
BW5660	Hfr(PO66) Δ (gpt-lac) supE λ ⁻ srlC::Tn10 thi	B. Bachmann
BW6175	Hfr(PO131) thr leu azi tonA lacY supE λ ⁻ argE::Tn10 thi	B. Bachmann
BW6159	Hfr(PO68) λ ⁻ relA spoF ilv-691::Tn10	B. Bachmann
BW6164	Hfr(PO48) thr-43::Tn10 supE malB sfa	B. Bachmann
BW6165	Hfr(PO120) ara lacY xyl mtl argE::Tn10	B. Bachmann
NK6051	Hfr(PO1) Δ (gpt-lac) purE::Tn10 relA spoT thi λ ⁻	B. Bachmann
JW353	F ⁻ thr leuB zae-502::Tn10 thyA met thi deoC lacY rpsL tonA λ ⁻ supE	B. Bachmann
NK5525	F ⁻ pro-81::Tn10 λ ⁻	B. Bachmann
JP3123	F ⁻ lacY aroL478::Tn10 purE tyrR rpsL xyl mtl	B. Bachmann
N3033	F ⁻ lacZ::Tn10 λ ⁻	B. Bachmann
NK5148	F ⁻ thr-34::Tn10 λ ⁻	B. Bachmann
NK6034	Hfr(PO1) car-96::Tn10 Δ (gpt-lac)5 relA spoT thi λ ⁻	B. Bachmann
YYC100	F ⁻ leuB Δ (aroP-aceF) zac-284::Tn10 proC purE trpE lysA metE thi ara lacZ xyl mtl tsx supE λ ⁻	B. Bachmann
SJ2	Hfr(PO1) panB zad-220::Tn10 relA spoT thi λ ⁻	B. Bachmann
2844	F ⁻ tsx-462::Tn10 λ ⁻	B. Bachmann
KL743	zba-300::Tn10	B. Bachmann
K450	nusB5 rpsL200	B. Bachmann

experiments, an Hfr collection containing transpositions of Tn10 around the *E. coli* chromosome was used (Wanner, 1986). Fine mapping was performed by transducing Tn10 insertions near the region where the mutations were localized. Both transconjugants and transductants were selected on LB agar containing tetracycline and chloramphenicol. Their haemolytic phenotype was tested subsequently on blood agar. Mating of plasmid pHly152, transformation, and amplification of pANN202-312 with spectinomycin, were performed as previously described (Juárez *et al.*, 1984a).

Transposon mutagenesis. Tn5 mutagenesis was performed using $\lambda::\text{Tn5}$ as described by Shaw & Berg (1979). Infections with Mud1 were done as described by Casadaban & Cohen (1979).

Haemolysin assay. Bovine 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. Then 600 μl of this solution was mixed with 10–100 μl of the corresponding sample to be assayed, and incubated at 37 °C. At different times, the Eppendorf tubes were centrifuged (Eppendorf centrifuge) for 30 s, and the release of haemoglobin was measured by the increase in A_{543} . In order to compare the haemolytic activity in the supernatants of different cultures, or that remaining associated with the external cell surface, the following units were used: 1 haemolytic unit = A_{543} per OD₆₀₀ unit min⁻¹ ml⁻¹ (OD₆₀₀ being used as a measure of culture density). For the periplasmic and cytoplasmic fractions, haemolysin activity was calculated as specific activity [units (mg protein)⁻¹].

Cell fractionation. This was done as described previously (Nieto *et al.*, 1987).

Outer-membrane isolation and protein analysis. The outer membranes of the different strains analysed were isolated by the method of Filip *et al.* (1973). SDS-PAGE was done by a modification (Ames *et al.*, 1973) of the method of Laemmli (1970).

Isolation and manipulation of plasmid DNA. Plasmid DNA was isolated by the method of Birnboim & Doly (1979). DNA electrophoresis and restriction analysis were done by standard techniques (Maniatis *et al.*, 1982).

RESULTS

Isolation of the superhaemolytic mutants Hha-2 and Hha-3

E. coli 5K(pANN202-312), which produced very narrow zones of haemolysis, was initially mutagenized with Mud1. Afterwards, cells were plated on blood agar medium supplemented with ampicillin and chloramphenicol. In five separate experiments, we screened more than 20 000 colonies for large haemolysis haloes. One clone was found that apparently produced much more external haemolysin than the parental strain. Tn5 was also used as a mutagenizing agent. A similar number of colonies was screened, and one was found that produced a much larger haemolysis halo. The mutants from each experiment, named Hha-2 and Hha-3 respectively, were purified and further characterized. Their cell and colony morphology, and also their growth characteristics in minimal or nutrient media, were identical to those of the parental strain.

The mutations in strains Hha-2 and Hha-3 are located on the chromosome, not on the haemolytic plasmid

Evidence indicating that the Mud1 phage was inserted in the chromosome and not in pANN202-312 was obtained when strains Hha-2 and Hha-3 were cured of plasmid DNA. The cured clones were ampicillin or kanamycin resistant and chloramphenicol sensitive. As the Ap^r or Km^r phenotypes could have been due to secondary transpositional events, plasmid DNA from strains Hha-2 and Hha-3 was also isolated and cleaved with *Hind*III. The restriction pattern obtained was identical to that of pANN202-312 (data not shown).

Haemolysin production of strains Hha-2 and Hha-3

The haemolysin production of both mutants harbouring pANN202-312 was quantitatively evaluated in the different cellular compartments and compared to the haemolysin production of the parental 5K strain (Table 2). As suspected from the size of the zones of haemolysis obtained on blood agar, external haemolysin activities were much higher in both mutants than in the parental strain. Periplasmic and cytoplasmic haemolysin activities were also higher in the mutant strains. Strain Hha-2 showed higher external haemolytic activity than strain Hha-3. When harbouring pANN202-312, strain 5K had a very low external activity, much lower than when it harboured pHly152. These results suggest that cells of strain 5K carrying pANN202-312 are not able to properly secrete this toxin into the external medium. In contrast, strains Hha-2

Table 2. *Haemolysin production of strains Hha-2 and Hha-3 compared to the parental 5K strain*

Values are means of at least three determinations made on independent cultures.

Strain	Haemolysin activity			
	External*	Cell-associated*	Periplasmic†	Cytoplasmic†
5K(pHly152)	0.054	0.3	0.3	0.2
5K(pANN202-312)	0.004	1.0	0.4	0.2
5K(pANN202-312R)	18.5	19.0	2.4	1.5
Hha-2(pHly152)	0.2	0.32	0.8	0.9
Hha-2(pANN202-312)	15.7	17.0	5.0	1.3
Hha-2(pANN202-312R)	34.2	18.5	2.2	1.4
Hha-3(pHly152)	0.18	0.40	0.6	1.1
Hha-3(pANN202-312)	11.2	16.0	5.2	1.8
Hha-3(pANN202-312R)	27.0	16.2	2.3	1.7

* Values are given as haemolytic units (see Methods).

† Values are given as specific activity (see Methods).

and Hha-3 seem to release haemolysin properly into the culture medium. We also measured the haemolysin production of both mutants, and the parental strain harbouring pANN202-312R (Table 2). This plasmid is identical to pANN202-312, but includes sequences upstream of *hlyC* which seem to be essential for correct expression and excretion of haemolysin. The presence of pANN202-312R in strain 5K clearly increased the production of active haemolysin, as well as its excretion to the external medium. Compared to strain 5K(pANN202-312R), both mutants carrying pANN202-312R showed a similar amount of active haemolysin in the different cellular compartments, but a slightly higher amount in the external medium. When pANN202-812 was used instead of pANN202-312R, the results obtained were the same. Thus, the main effect of the mutations in strains Hha-2 and Hha-3 appears to be to increase haemolysin expression and excretion in cells harbouring pANN202-312

The superhaemolytic phenotype depends upon the presence of the multicopy plasmid pANN202-312

If mutations causing the superhaemolytic phenotype act by releasing the mechanism that blocks high levels of expression of haemolysin in cells harbouring pANN202-312, strains Hha-2 and Hha-3 will be overproducers of haemolysin only when they harbour such a multicopy plasmid. To test this, plasmid pHly152 was transferred to strains Hha-2 and Hha-3 which had been cured of pANN202-312. Hly⁺Ap^rCm^s or Hly⁺Km^rCm^s transconjugants were obtained and their haemolytic activity was tested (Table 2). When compared to clones harbouring pANN202-312, clones harbouring pHly152 showed a very significant decrease in the amount of haemolytic activity in all cellular compartments assayed, except the cytoplasm. To further test the correlation between haemolysin production and the copy number of pANN202-312, amplification of this plasmid was performed in strain 5K and in both mutant derivatives. To increase the plasmid copy number, cells were incubated overnight with spectinomycin. Afterwards, cells were washed and resuspended in fresh medium. Once growth was restored, samples were removed for haemolysin assay and for plasmid DNA isolation. The results showed, for both mutants, a proportional increase in the amount of plasmid DNA and in the external haemolytic activity (Table 2). Thus, in strains Hha-2 and Hha-3 an increase in the copy number of pANN202-312 results in a corresponding increase in the amount of haemolysin produced.

Both the hlyB and hlyD gene products are needed for the secretion of haemolysin in mutants Hha-2 and Hha-3

The parental strain 5K(pANN202-312) seems to be unable to properly release haemolysin into the external medium. We tested both mutants to ascertain whether their increased external haemolytic activity was due to their ability to secrete haemolysin by using an alternative

Table 3. External haemolytic activity of strains 5K, Hha-2 and Hha-3 upon amplification of pANN202-312

Values shown are the means of two experiments.

Strain	Physiological state*	Growth (OD ₆₀₀)	External haemolytic activity (units)	Plasmid DNA concn (mg ml ⁻¹)†
5K	A	0.40	0.004	0.57
5K	B	0.60	0.005	1.76
Hha-2	A	0.36	11.0	0.6
Hha-2	B	0.5	34.0	1.8
Hha-3	A	0.40	9.1	0.6
Hha-3	B	0.50	27.5	1.85

* A, Exponential growth phase, before addition of spectinomycin; B, amplification performed, cells washed and resuspended in fresh medium. Growth restored.

† Samples were removed, and the OD₆₀₀ was adjusted to 0.4 with LB. Plasmid DNA was isolated from 2 ml samples, and resuspended in 0.1 ml water.

Table 4. Effect of *hlyB* or *hlyD* mutations on haemolysin production of strains Hha-2 and Hha-3

Values are means of at least three determinations made on independent cultures.

Strain	Haemolysin activity*	
	Periplasmic	Cytoplasmic
5K(pANN202-312::Mud1-2)	0.5	0.4
Hha-2(pANN202-312::Mud1-2)	4.3	5.0
Hha-3(pANN202-312::Mud1-2)	2.5	6.5
5K(pANN202-312::Tn5-10)	0.4	0.3
Hha-2(pANN202-312::Tn5-10)	4.1	5.5
Hha-3(pANN202-312::Tn5-10)	5.2	6.0

* Values are given as specific activity. External and cell-associated haemolysin activities were undetectable in all the strains shown.

mechanism that does not need *hlyB* or *hlyD* gene products. Strains 5K, Hha-2 and Hha-3 were transformed with plasmid pANN202-312::Mud1-2, a derivative of pANN202-312 which contains a deletion in *hlyD* (Juárez & Goebel, 1984). Strains Hha-2 and Hha-3 harbouring this plasmid did not show external haemolysin activity. Therefore, *hlyD* is essential in strains Hha-2 and Hha-3 for the excretion of haemolysin to the medium. We also tested the haemolytic activity in the other cellular compartments of the mutant strains harbouring pANN202-312::Mud1-2 (Table 4). Both mutants showed several-fold higher values of haemolytic activity in the periplasmic and cytoplasmic compartments, as compared to the parental strain. Similar results were obtained when both mutants were transformed with plasmid pANN202-312::Tn5-10 (*hlyB*) (Table 4). Thus, haemolysin export in both mutants also requires the specific haemolysin export genes *hlyB* and *hlyD*.

Genetic analysis of the mutations in strains Hha-2 and Hha-3

We attempted to use phage P1 to transduce both the Hha-2 and Hha-3 mutations to either *E. coli* 5K or *E. coli* C600. Since strain Hha-2 harbours Mud1 and therefore is temperature sensitive, P1 vir was initially used. Surprisingly, no Ap^r transductant clones could be obtained. A temperature-resistant derivative of strain Hha-2 was obtained as previously described (Nieto *et al.*, 1987), and P1 *cm cr1 100* transduction was attempted, but again transduction of the Ap^r marker of strain Hha-2 could not be achieved. In contrast, transduction of the Km^r marker of strain Hha-3 could easily be accomplished. Interestingly, only 10% of the Km^r transductants harbouring pANN202-312 showed the large haemolytic halo and demonstrated an *in vitro*

Table 5. *Frequency of coinheritance of tetracycline resistance and Hha⁺ phenotype in the matings of Hfr::Tn10 strains with mutants Hha-2 and Hha-3*

Values shown are the means of two experiments.

Donor strain	Tn10 map position (min)	Frequency of coinheritance using receptor strain:	
		Hha-2	Hha-3
BW6164 (<i>thr-43::Tn10</i>)	0	0.08	0.04
BW7261 (<i>leu-63::Tn10</i>)	2	0.47	0.52
BW7623 (<i>purE79::Tn10</i>)	12	0.65	0.60

Table 6. *Cotransduction frequency of tetracycline resistance and Hha⁺ phenotype*

Values shown are the means of two experiments.

Donor strain	Tn10 map position (min)	Frequency of cotransduction	
		Recipient:	
		Hha-2	Hha-3
NK5525 (<i>pro-81::Tn10</i>)	6	0	0
2844 (<i>tsx-462::Tn10</i>)	9	0.013	0.015
JP3123 (<i>aroL478::Tn10</i>)	9.5	0.045	0.088
KL743 (<i>zba-300::Tn10</i>)	10	0.35	0.45

haemolytic activity equivalent to strain Hha-3. Successive retransduction of the Km^r marker did not increase the frequency of coinheritance of the superhaemolytic phenotype.

The map location of both insertions was determined by using the Hfr::Tn10 kit (Wanner, 1986). For strain Hha-2 both Hfr matings and P1 transductions are easier to carry out with the Tn10 transposon than with the larger Mud1 insertions. In both mutants, we obtained the highest frequency of Tc^r transconjugants that recovered the haemolytic phenotype of the parent strain when strains BW7261 and BW7623 were used as donors (Table 5). The frequency decreased when the donor strains had the transposon insertion outside the region between minutes 2 and 12 in the *E. coli* chromosome. For detailed mapping we used Tn10 insertions between minutes 2 and 12, and transduced Tn10 to both mutants, selecting for Tc^r transductants which had lost the ability to produce large haemolysis haloes. With the Tn10 insertions, the highest cotransduction frequency between Tc^r and parental haemolytic phenotype was 35% for Hha-2 and 45% for Hha-3, using strain KL743 (*zba-300::Tn10*) (Table 6). We located both mutations (which we termed *hha-2* and *hha-3*), at minute 10.5 (*nusB*, *tinA*, *acrA*) of the *E. coli* genetic map.

The *zba-300::Tn10* insertion has been reported to be closely linked to classical *lon* mutations with coinheritance frequencies of 60% (Trisler & Gottesman, 1984) or 76% (Maurizi *et al.*, 1985). Although the coinheritance frequencies between Tc^r and the haemolytic phenotype in our mutants were always lower, and neither of the mutants showed the typical phenotype of *lon* mutants (presence of filaments, UV sensitivity, altered λ or P1 lysogeny, etc.), we tested whether the *lon* mutation could be related to the superhaemolytic phenotype. Strain SG932 ($\Delta lon-100$) was transformed with pANN202-312, and the haemolytic transformants were tested for external haemolysin production. No increase in the external haemolytic activity could be detected with respect to strain 5K. As the *nusB* gene product is also implicated in gene expression, we tested the expression of pANN202-312 in strain K450 (*nusB5*). Clones harbouring this haemolytic plasmid showed very narrow haemolysis zones and their *in vitro* external haemolytic activity was equivalent to that of strain 5K (data not shown).

For both mutants, the transductants which had recovered the parental haemolytic phenotype were tested for loss of the transposon antibiotic resistance marker. The Hha-3 Tc^r transductant clones which recovered the parental haemolytic phenotype were Km^s. In contrast, the Tc^r transductants of strain Hha-2 were still Ap^r.

Characterization of outer-membrane and periplasmic proteins from strains Hha-2 and Hha-3

To test whether the haemolytic phenotype of both mutants was at least partly due to alterations in the cell envelopes, their outer-membrane and periplasmic proteins were analysed and compared to those of the parental 5K strain. The SDS-PAGE protein pattern of the outer-membrane fraction was identical in all three strains (data not shown). In contrast, SDS-PAGE analysis of the periplasmic contents of both mutants showed some, as yet uncharacterized, minor differences when compared to strain 5K. These differences were more apparent in strain Hha-2; they may be correlated with the Hha-2 mutation, because they could not be detected in the periplasmic protein pattern of the Tc^r transductants which recovered the parental haemolytic phenotype.

DISCUSSION

We report here the isolation of two mutants that overproduce haemolysin when harbouring the multicopy plasmid pANN202-312. In both mutants, Hha-2 and Hha-3, the transposon is inserted into the chromosome. Thus, chromosomal genes are involved in the expression of haemolysin encoded by pANN202-312. We have also shown that such high levels of production of haemolysin depend on the presence of pANN202-312 and not on the parental plasmid pHly152. Thus, the expected multicopy effect for haemolysin production with pANN202-312 is obtained with the mutants we report here. For strain Hha-3, the genetic experiments correlate a single Tn5 insertion with an increase in haemolysin synthesis. The observation that only 10% of the Km^r transductants showed high haemolysin production suggests that the region where Tn5 is inserted may be flanked by sequences which are repeated in other locations of the *E. coli* chromosome. For strain Hha-2, it is remarkable that P1-mediated transduction of the Ap^r marker does not appear possible. The presence of the *Mud1* genome may reduce the frequency with which the region of interest is encapsidated into P1 particles. Because of the failure in transduction, some combination of two different *Mud1* insertions cannot be ruled out as a cause of the haemolytic phenotype of strain Hha-2. Nevertheless, the similar results obtained from both mutants in the genetic exchange experiments performed in mapping the *hha-2* and *hha-3* loci suggest that a single *Mud1* insertion may also be responsible for the haemolytic phenotype of strain Hha-2. The fact that the Hha-2 Tc^r transductants which recover the parental haemolytic phenotype still remain Ap^r may be due to secondary *Mud1* transpositions which are not related to the haemolytic phenotype. Even when the bacteria are cultured at 30 °C, secondary transpositions of *Mud1* are frequent events (Mulligan *et al.*, 1982).

The construction of pANN202-312 creates interesting questions regarding the control of haemolysin synthesis and transport. Cells harbouring this plasmid produce a very low amount of free external haemolysin, leaving a large amount attached to the external surface of the cell. It has been recently reported (Vogel *et al.*, 1988) that the haemolytic plasmid pHly152 contains a sequence of about 600 bp (*hlyR*) which is located about 1500 bp upstream of *hlyC*, and its acting in *cis* seems to be necessary for correct expression and secretion of haemolysin. When included in the haemolytic determinant of pHly152 which is cloned in a multicopy plasmid, *hlyR* enhances the expression of haemolysin more than 50-fold. Deletion of this entire *hlyR* sequence leads to the same low level of haemolysin synthesis and excretion as that expressed by pANN202-312. When this plasmid was constructed, such sequences were considered not to belong to the haemolysin determinant of pHly152, and were not included in the fragment cloned in pACYC184 (Goebel & Hedgpeth, 1982). Thus, the absence of *hlyR* in pANN202-312 seems to be the reason for the low haemolysin expression and excretion in cells harbouring this plasmid. Nevertheless, in strains carrying a *hha* mutation, the expression of haemolysin coded by pANN202-312, as well as its excretion, are equivalent to those obtained in other *E. coli* strains harbouring recombinant haemolytic plasmids which include *hlyR*. Thus the *hha* mutation seems to compensate for the lack of *hlyR* in pANN202-312.

The mechanisms that control expression of haemolysin in *E. coli* are unclear. Specifically, the role of the *hlyC* gene product and its mechanism of action have been controversial (Felmlee *et al.*, 1985; Härtlein *et al.*, 1983; Mackman & Holland, 1984; Mackman *et al.*, 1985a; Nicaud

al., 1985), and it is not known how this protein activates the precursor polypeptide and renders haemolysin active. The characterization of the mutants reported here may help us to understand better the mechanisms which regulate the synthesis and secretion of active haemolysin. The physiological properties of the mutants, and the location of the mutations in the *E. coli* genetic map, do not relate them to other previously known functions affecting gene expression, although the mutations do map very close to *lon*.

An interesting question is whether this effect is specific for *E. coli* haemolysin, or represents a pleiotropic mutation affecting the expression of other proteins. Recent data show that strain Hha-2 is able to secrete an *Aeromonas* cytotoxin with an external activity 20 times higher than that of the parental 5K strain (Nieto *et al.*, 1987). Additionally, both Hha-2 and Hha-3 mutants are able to secrete into the external medium both a *Vibrio cholerae* haemolysin (A. Juárez, unpublished) and an IL2-diphtheria toxin chimeric protein without the release of other periplasmic proteins (A. Juárez & J. R. Murphy, unpublished). The secretion of proteins by *E. coli* remains a major problem to be solved in both theoretical and practical aspects. With a few exceptions, most of the secretable proteins cloned in *E. coli* remain in the periplasmic space (Löfdahl *et al.*, 1983; Talmagde *et al.*, 1980; Tsukagoshi *et al.*, 1984). The mutants we describe here may represent an interesting approach to the secretion question in *E. coli*.

Although the initial research interest in haemolysin production by *E. coli* was its contribution to virulence, the fact that this protein is one of the very few proteins secreted to the external medium by *E. coli* makes the study of haemolysin very important in understanding gene expression and protein secretion in *E. coli*.

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