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Study of Regulation and Transport of Hemolysin by Using Fusion of the β-Galactosidase Gene (*lacZ*) to Hemolysin Genes

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Operon and gene fusions between *lacZ* and the hemolysin genes, *hlyC* and *hlyA*, were performed. These two genes are essential for the synthesis of active hemolysin and are transcribed from a common promoter (p_1). Whereas the amount of hemolysin produced in *Escherichia coli* is not changed by altering the *hly* gene dose, β-galactosidase activity follows the gene dosage in both types of fusions when *lacZ* comes under the control of p_1. This indicates that hemolysin is not negatively regulated on the transcription or translation level. The products of the gene fusions *hlyC*::*lacZ* and *hlyA*::*lacZ* were identified in maxicells as stable proteins of 146,000 and 220,000 daltons, respectively. Both fusion proteins possess β-galactosidase activity indicating that the performed fusions of *lacZ* to the *hly* genes do not destroy the reading frame of *hlyC* and *hlyA*. The fusion proteins HlyC-β-gal and HlyA-β-gal were predominantly detected in the cytoplasm, confirming previous data which suggested that the primary gene products of *hlyC* and *hlyA* are not transported across the cytoplasmic membrane.

Certain strains of *Escherichia coli* produce hemolysin, a toxic extracellular protein (18, 21). The percentage of hemolytic *E. coli* strains among those isolated from urinary tract infections is especially high (35 to 60%). The role of hemolysin production as a virulence property of these bacteria has been recently studied and results obtained with in vivo model systems indicate that hemolysin acts as a virulence factor (23, 25). The information for hemolysin synthesis can be carried either on the chromosome or on transmissible plasmids. Plasmid and chromosomal hemolysin (*hly*) determinants exhibit a high degree of homology (1, 8, 15) and consist of a cluster of four genes necessary for the synthesis and excretion of this toxin (16, 24). Some biochemical and regulatory aspects of its synthesis still remain obscure, and their clarification is hampered by its extreme instability (22).

By using Mu dl-directed *hly-lacZ* fusions, which allowed us to study the expression of *hly* genes by measuring β-galactosidase activity, we demonstrated that two promoters (p_1 and p_2) control the expression of the four *hly* genes. The left-hand (*hlyC*-proximal) promoter p_1 regulates the transcription of *hlyC*, *hlyA*, and $hlyB_a$, whereas promoter p_2 ($hlyB_b$ proximal) regulates the transcription of $hlyB_b$. Moreover, these studies show that transcription of the *lacZ* gene brought under the control of these promoters is constitutive and is unaffected by the quality of the culture medium (rich or minimal). Hemolysin, on the other hand, is expressed at a much higher efficiency in rich media (22), and its synthesis and excretion is highest during the exponential growth phase (22). The amount of hemolysin is not increased in *E. coli* K12 carrying the multicopy plasmid pANN202-312 (9) as compared with *E. coli* harboring the single-copy plasmid pHly152 (24). Insertions of Mud1 in pANN202-312 proved to be rather unstable (A. Juarez and W. Goebel, submitted for publication) and did not allow us to determine the reason for the lack of the copy effect of the *hly* genes on hemolysin production. This difficulty was overcome in the present study by constructing in vitro hybrid plasmids in which the *lacZ* gene was placed under the control of the left-hand *hly* promoter p_1 by using *lacZ*-containing fragments of the plasmids pMC871 and pMC874 (3). This approach enabled us to alter the copy number of the plasmids carrying the fused genes which led to the generation of HlyC-β-gal and HlyA-β-gal fusion proteins with functional β-galactosidase activity. This allowed us to precisely measure the expression of the *hly-lacZ* fusion genes under the control of the *hly* promoter p_1. Furthermore, the compartmentation of the fusion proteins HlyC-β-gal and HlyA-β-gal within the cell was analysed.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids employed are listed in Table 1.

Media and growth conditions. LB and M9 minimal media have been described previously (6). For the liquid assay of hemolysin, brain heart infusion (Difco Laboratories) medium (9) was used. When required, the media were supplemented with chloramphenicol (50 μg/ml), ampicillin (100 μg/ml), and kanamycin (20 μg/ml). Screening for Lac^+ clones was done on MacConkey plates, and blood agar of the following composition was used for the detection of hemolytic activity on solid medium: peptone, 10g; beef extract, 10 g; NaCl, 5 g; Bacto-Agar (Difco), 10 g; and distilled water to 1 liter.

Hemolysin and β-galactosidase assays. Hemolysin was assayed as described previously (24). For the β-galactosidase assay, overnight cultures were diluted (1:50) in fresh medium, and β-galactosidase was assayed in l-h intervals during the exponential and the stationary phases. β-galactosidase activity was measured as described by Miller (14), with sodium dodecyl sulfate-chloroform–treated cells.

Isolation of plasmid DNA. Plasmid DNA was isolated by the method of Birnboim et al. (2), which was adapted for 50-ml cultures. By this method the plasmid preparations were extracted once with phenol, once with phenol-chloroform, and once with chloroform. After ethanol precipitation, each pellet was suspended in 200 μl of distilled water and treated with RNase (100 μg/ml). Preparation of mini and maxi cells, labeling of the proteins with [^{35}S]methionine, cleavage with restriction enzymes, in vitro ligation of DNA, and transformation were performed as described previously (9, 16).

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TABLE 1. Bacterial strains and plasmids used in this study

E. coli carrying plasmid:	Genotypes	Source of reference
None (strain 5K)	*hsdR hsdM thi thr rpsL lacZ*	(7)
pANN202-312	*cat*$^+$ *hlyC*$^+$ *hlyA*$^+$ *hlyB*$_a$$^+$ *hlyB*$_b$$^+$	(9)
pMC871	*aac*$^+$ *lacZ*$^+$ *lacY*$^+$	Derived from pACYC177 (3)
pMC874	*aac*$^+$ *lacZ' lacY*$^+$	Derived from pACYC177 (3)
pANN651	*cat*$^+$ *hlyC-lacZ*$^+$ *lacY*$^+$	This study
pANN652	*cat*$^+$ *hlyC*$^+$ *hlyA-lacZ*$^+$ *lacY*$^+$	This study
pANN653	*cat*$^+$ *hlyC::lacZ' lacY*$^+$	This study
pANN654	*cat*$^+$ *hlyC*$^+$ *hlyA::lacZ' lacY*$^+$	This study
pSC101	*tet*$^+$	(6)
pANN655	*hlyC-lacZ' lacY*$^+$	This study

Plasmid DNA amplification. Since all plasmids used were derivatives of pACYC184, amplification was performed with spectinomycin (5). Overnight cultures were diluted (1:50) in fresh LB. When a cell density of 120 Klett units (3×10^8 cells per ml) was reached, spectinomycin (200 μl/ml) was added, and cells were incubated overnight. Cells were washed twice with fresh LB, suspended, and incubated at 37°C with agitation. Samples were taken in 1-h intervals, and β-galactosidase and hemolysin activities were assayed.

Cell fractionation. Cells were grown in brain heart infusion broth at 37°C to a density of 3×10^8 cells per ml and were pelleted by centrifugation. The supernatant was used for the determination of the external β-galactosidase activity. The cells were washed twice with 10 mM Tris hydrochloride (pH 7.0) and then suspended in 1/10 of the original culture volume of buffer containing Tris hydrochloride (10 mM [pH 7.4]), sucrose (25%), sodium EDTA (40 mM), and lysozyme (100 μg/ml; Sigma Chemical Co.) as described by Koshland and Botstein (11). After 30 min on ice, the cells were pelleted, and the supernatant (designated periplasmic) was taken for the determination of β-galactosidase activity.

Cells were suspended in 10 mM Tris hydrochloride (pH 7.0) and lysed by several (8 to 12) 15-s bursts with a Branson Ultrasonifier. Unlysed cells and large cell debris were removed by low-speed centrifugation. The supernatant was centrifuged at 35,000 rpm in a 50 Ti rotor for 2 h at 4°C. The supernatant (designated cytoplasmic fraction) was carefully removed and taken for the determination of the cytoplasmic β-galactosidase activity. The membrane pellet was washed with Tris hydrochloride (10 mM [pH 7.0]), resuspended in the same buffer, and used for the determination of the membrane-bound β-galactosidase activity.

RESULTS

In vitro construction of *hly*C-*lac*Z and *hly*A-*lac*Z fusions. Plasmid pANN202-312 contains a single *Bam*HI site located close to the C-terminal end of *hlyC*, one *Bgl*II site in *hlyA*, and one *Sal*I site outside of the *hly* genes (Fig. 1). The

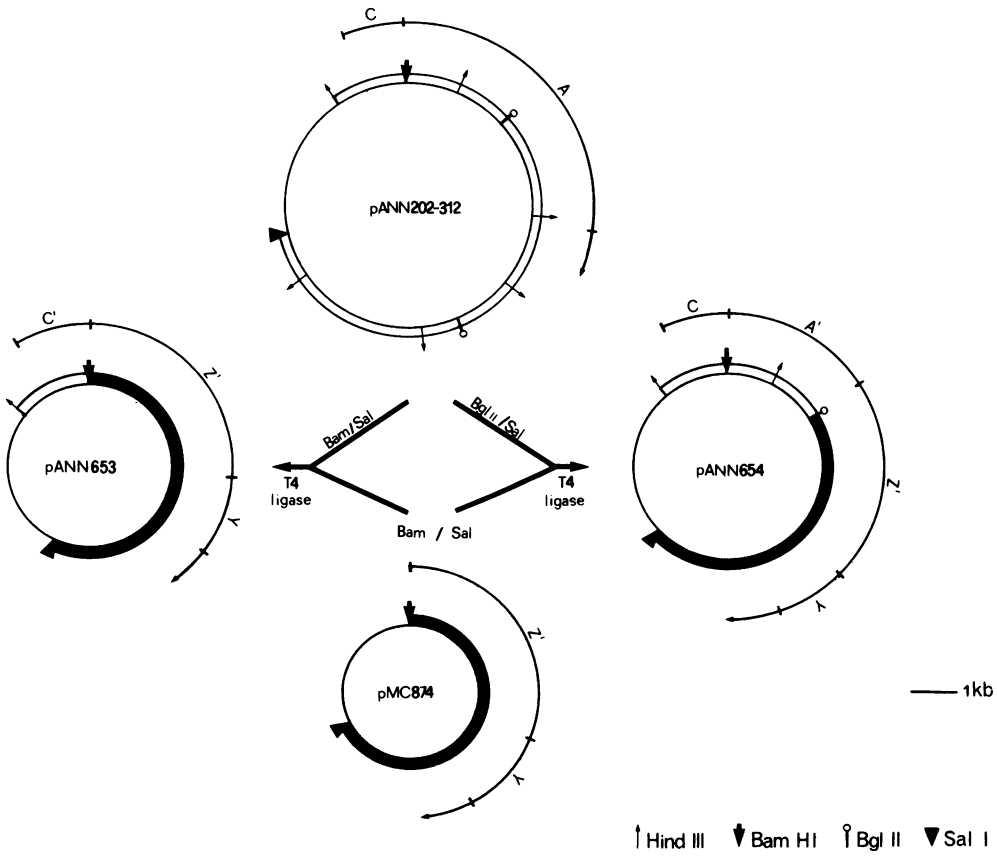

FIG. 1. Strategy for the construction of *hlyC*::*lacZ* and *hlyA*::*lacZ* fusion genes. The *Bam*HI-*Sal*I fragment of pMC874 containing *lacZ* (thick black line) was recombined in vitro with pANN202-312 cleaved with *Bam*HI-*Sal*I, generating pANN653 (*hlyC*::*lacZ* fusion). If pANN202-312 was cleaved with *Bgl*II-*Sal*I, the recombinant plasmid obtained was pANN654 (*hlyA*::*lacZ* fusion).

plasmids pMC871 and pMC874 constructed by Casadaban et al. (3) contain the *lacZ* gene on *Bam*HI-*Sal*I restriction fragments. In plasmid pMC871, the *lacZ* gene is intact, whereas in pMC874, the first eight codons are missing. Thus, ligation of the *Bam*HI-*Sal*I fragment from pMC871 to pANN202-312 cleaved by *Bam*HI-*Sal*I or *Bgl*II-*Sal*I results in an operon fusion in which the *lacZ* gene is transcribed under the control of the left-hand *hly* promoter p_1. Fusion of the *Bam*HI-*Sal*I fragment from pMC874 into the same sites of pANN202-312 leads to the production of fused proteins with the amino termini corresponding to proteins HlyC and HlyA, respectively, and the carboxy terminus to β-galactosidase (Fig. 1). Transcription of these two fused genes is again under the control of p_1, and translation of the transcripts should be regulated by possible *hly*-specific regulatory sites on these transcripts (ribosome binding site, attenuator, etc.). Such ligation mixtures were transformed into *E. coli* 5K *lacZ*, and transformants were selected on MacConkey plates containing chloramphenicol (Cm). The Cm^r Lac^+ transformants were tested for the hemolytic phenotype. With all four ligation mixtures, colonies with the expected phenotype, Hly^- Cm^r Lac^+, were obtained, indicating that the desired *Bam*HI-*Sal*I fragments were inserted into plasmid pANN202-312, which caused the Lac^+ phenotype and abolished the Hly^+ phenotype. This was further confirmed by isolating plasmid DNA of each of the four types of transformants obtained. Plasmid DNA was cleaved with appropriate combinations of restriction enzymes to demonstrate the correct insertions. Figure 2 shows the recombinant plasmids obtained, i.e., pANN651 (*Bam*HI-*Sal*I fragment from pMC871 fused to *Bam*HI-*Sal*I cleaved pANN202-312), pANN652 (same fragment fused into pANN202-312 cleaved *Bgl*II-*Sal*I), pANN653 (fusion of *Bam*HI-*Sal*I fragment from pMC874 into pANN202-312 cleaved by *Bam*HI-*Sal*I), and pANN654 (fusion of the same fragment from pMC874 into pANN202-312 cleaved by *Bgl*II-*Sal*I).

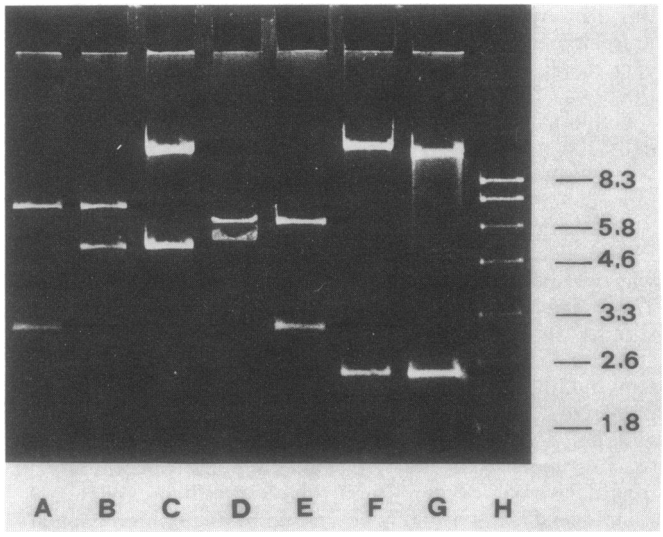

FIG. 2. Agarose gel analysis of DNAs of the fusion plasmids. *Bam*HI-*Sal*I double cleavage of pMC871 (A), pANN651 (B), pANN202-312 (C), pANN653 (D), and pMC874 (E). *Hin*dIII cleavage of pANN652 (F), and pANN654 (G). Molecular size standard (H), *Eco*RI-digested SPP1 DNA. Plasmids pANN651 and pANN653 were cut with *Bam*HI and *Sal*I, since they represent insertions of a *Bam*HI-*Sal*I fragment from either pMC871 or pMC874 into pANN202-312 cleaved by *Bam*HI-*Sal*I. Recombination of pANN202-312 cut by *Bam*HI-*Sal*I with a *Bgl*II-*Sal*I fragment of pMC871 or pMC874 destroys both the *Bam*HI and *Bgl*II sites. Plasmids pANN652 and pANN654 are therefore shown as the *Hin*dIII restriction pattern. Insertion of the *Bam*HI-*Sal*I fragment from either pMC871 or pMC874 (both lack *Hin*dIII sites) into pANN202-312 cleaved by *Bgl*II-*Sal*I yields recombinant plasmids with two *Hin*dIII restriction sites (Fig. 1).

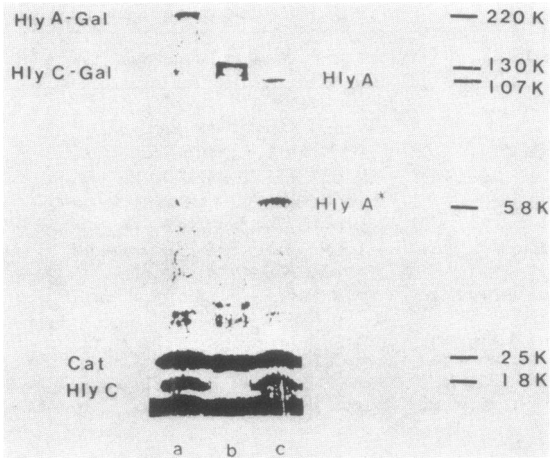

FIG. 3. Gene products expressed in maxicells by plasmids pANN654 (a), pANN653 (b), and pANN202-3128 (c). UV-irradiated cells of *E. coli* CGSC5830 carrying these plasmids were labeled with [^{35}S]-methionine, and proteins were separated on a sodium dodecyl sulfate-polyacrylamide gel.

Detection of the fusion proteins HlyC-β-gal and HlyA-β-gal in maxicells of *E. coli*. The products of the fused *hlyC*::*lacZ* and *hlyA*::*lacZ* genes were analyzed in maxicells or minicells by introducing the recombinant plasmids pANN653 and pANN654 into *E. coli* CGSC5830 (17) or *E. coli* P678-54. After UV irradiation CGSC5830, cells were labeled with [^{35}S]methionine, and proteins were separated on sodium dodecyl sulfate-polyacrylamide gels. The results are shown in Fig. 3. Cells carrying pANN653 express, in addition to the 26,000-dalton vector protein Cat (chloramphenicol-acetyltransferase), a large protein of 146,000 daltons. The original HlyC protein of 18,000 daltons is absent. Since fusion of *lacZ* occurs close to the C-terminal end of *hlyC* in pANN653 (M. Vogel and W. Goebel, unpublished data), the experimental value of this protein corresponds well to the expected molecular weight of the fused HlyC-βgal protein (130,000 daltons for the β-galactosidase part and about 16,000 daltons for the truncated HlyC). Cells harboring plasmid pANN654 express intact HlyC, Cat, and a new large protein of 220,000 daltons. We have previously shown (9) that a *Bgl*II deletion mutant of pANN202-312 expresses a truncated HlyA of 96,000 daltons. Since fusion of *lacZ* in pANN654 occurs into this *Bgl*II site, the expected molecular weight for the fusion HlyA-β-gal protein is again in good agreement with the experimental value. It has been further demonstrated (Härtlein, Ph.D. thesis, University of Wurzburg, 1984) that these new proteins indeed possess β-galactosidase activity. Lane C in Fig. 3 shows as a control, CGSC5830 cells containing plasmid pANN202-3128, which, as previously reported (7), carries only *hlyC* and *hlyA* and leads to production of periplasmic hemolysin but not of extracellular hemolysin. The same results were obtained with minicells from E. coli P678-54 which were carrying these plasmids. The labeled proteins correspond to HlyC, Cat, a 107,000-dalton protein which is the primary product of *hlyA*, and a 58,000-dalton protein which is the major processing product of HlyA. We

TABLE 2. Operon and gene fusions obtained between pANN202-312 and pMC871 or pMC874

Plasmid	Source of derivation	Fusion type	Protein formed	β-galactosidase activity (U)
pANN651	pANN202-312 (*Bam*HI-*Sal*I) and pMC871 (*Bam*HI-*Sal*I)	operon fusion *hlyC-lacZ*	native β-galactosidase	3,500
pANN652	pANN202-312 (*Bgl*II-*Sal*I) and pMC871 (*Bam*HI-*Sal*I)	operon fusion *hlyA-lacZ*	native β-galactosidase	1,700
pANN653	pANN202-312 (*Bam*HI-*SAl*I) and pMC874 (*Bam*HI-*Sal*I)	gene fusion *hlyC::lacZ*	HlyC-βgal	700
pANN654	pANN202-312 (*Bgl*II-*Sal*I) and pMC874 (*Bam*HI-*Sal*I)	gene fusion *hlyA::lacZ*	HlyA-βgal	250
pHly152-Mu d1A[a]	Mu d1 insertion into *hlyC* of pHly152	operon fusion of *lacZ* in *hlyC*	native β-galactosidase	175
pHly152-Mu d1B[a]	Mu d1 insertion into *hlyA* of pHly152	operon fusion of *lacZ* in *hlyA*	native β-galactosidase	40–75

[a] These Mu d1 insertions are described in A. Juarez, C. Hughes, and W. Goebel, submitted for publication.

suggest that this protein, modified by HlyC, is the active hemolysin, as was recently reported (10). It is interesting to notice that the fused HlyA-β-gal protein is not altered in its size (even after extensive incubation of the maxicells at 37°C), which suggests that no proteolytic processing of this HlyA-β-gal fusion protein occurs.

Determination of the β-galactosidase activity of *E. coli* carrying the recombinant plasmids pANN651 to pANN654. *E. coli* 5K strains containing the four recombinant plasmids described above (pANN651 to pANN654) were grown in LB to log phase (110 Klett units), and β-galactosidase activity was determined as described (14) (Table 2). It is evident that in both cases in which *lacZ* is operon fused (pANN651 and pANN652), the β-galactosidase activity is considerably higher than in the corresponding gene fusions (pANN653 and pANN654). Compared with the β-galactosidase activity which was described previously (Juarez and Goebel, submitted for publication), with Mu *d*1 (*Mu lacZ bla*) (3) insertions into similar sites (i.e., in *hlyC* or *hlyA*) of the low-copy plasmid pHly152 (three to four copies per cell) (16), these values are considerably higher and suggest that the higher copy number of the plasmids pANN651 and pANN652 (about 20 to 25 copies per cell, as measured by the intensity of the plasmid and chromosomal bands on agarose gels from cell lysates, with *E. coli* 5K(pBR322) as standard) is responsible for the increased β-galactosidase activity. The decrease in the β-galactosidase activities of the *hlyA-lacZ* fusions compared with those of the *hlyC-lacZ* fusions, which is observed in both sets of recombinant plasmids, reflects the expected polarity in transcription from *hlyC* to *hlyA* (24). Comparison of the β-galactosidase activities of the new recombinant plasmids (pANN651 to pANN654) with those of previous Mu *d*1-pHly152 constructions suggests that β-galactosidase activity is plasmid copy number dependent when *lacZ* is under the control of the left-hand *hly* promoter p_1, in contrast to hemolysin expression, which is not affected by plasmid copy number (1, 24). This indicates that the observed lack of increase in the level of hemolysin in response to increasing gene dose does not result from negative control of the *hly* genes *C*, *A*, and B_a at the transcriptional level or a negative regulatory effect on the transcripts at the level of translation.

One should keep in mind, however, that Mu d1 represents a rather complicated system in which transcription proceeds through the S gene of the Mu phage and other sequences (part of the *trp* operon and part of the *lac* operator) before it reaches the *lacZ* gene. Direct comparisons of the β-galactosidase activities of the two systems may therefore lead to incorrect conclusions. We therefore tried to alter the copy number of the recombinant plasmids pANN653 and pANN654 (carrying the fused *hlyC::lacZ* and *hlyA::lacZ* genes, respectively) by the following two different approaches.

Amplification of plasmids pANN653 and pANN654 with spectinomycin. If the high β-galactosidase activity determined by the multicopy hybrid plasmids pANN653 and pANN654 reflects the copy number, further amplification of the copy number of these plasmids should yield higher levels of the enzyme. Both plasmids contain a basic replicon which allows the continuation of replication in the absence of protein synthesis. Since both plasmids carry the *cat* (chloramphenicol-acetyltransferase) gene, amplification was performed with spectinomycin by treating logarithmically grown cultures of *E. coli* 5K carrying these plasmids with this drug overnight. Cells were then washed and suspended in fresh medium without spectinomycin. Cell density and β-galactosidase activity were measured at l-h intervals during the next 7 h. The results (Fig. 4) show that after addition of spectinomycin, the β-galactosidase activity decreased gradually due to the stop of protein synthesis. After the cells were washed free of spectinomycin, cell growth did not start immediately, and during this lag phase, β-galactosidase activity remained at the same low level. However, when cell growth started again, a sharp increase in enzyme activity was detected in strains carrying either of the two plasmids. The highest β-galactosidase activity was twice that measured before addition of spectinomycin and remained constant for the next 2 h. This indicates that the increase in the copy number of the plasmids correlates with an increase in β-galactosidase activity, although β-galactosidase activity was not colinear to the increase in copy number, which reaches more than 100 per cell after the spectinomycin treatment. We assume that this lower-than-expected β-galactosidase activity may be caused by the limited capacity of the cell to synthesize the hybrid proteins (HlyC-βgal and HlyA-βgal).

Similar amplification experiments with spectinomycin were performed with *E. coli* 5K harboring the hemolytic plasmid pANN202-312, and external and internal hemolysin activity was measured. In contrast to the β-galactosidase activity, no significant increase in the amount of hemolysin synthesis was observed, although the copy number of pANN202-312 was likewise increased to over 100 per cell, as determined by the intensity of the plasmid band on agarose gels from cell lysates. Control experiments indicate that spectinomycin does not exert an inhibitory effect on hemolysin activity.

Construction of a low-copy-number plasmid expressing

HlyC-βgal fusion protein. To study the effect of low plasmid copy number on β-galactosidase activity when *lacZ* is under the control of the *hly* promoter p_1, we cloned the *Hin*dIII-*Sal*I fragment of pANN653 in the low-copy-number plasmid pSC101, which exists in the cell in 3 to 5 copies (6). This fragment contains the complete regulatory region for the expression of the *hly* genes *C*, *A*, and *B* (A. Juarez, C. Hughes, M. Vogel, and W. Goebel, Mol. Gen. Genet., in press), most of the *hlyC*, and the *lacZ* gene fused to *hlyC* in frame (see above).

Insertion of the *Hin*dIII-*Sal*I fragment of pANN653 into cleaved pSC101 cleaved by *Hin*dIII-*Sal*I yields a recombinant plasmid which is under pSC101 replication control, since the inserted fragment does not carry any replication functions. By the strategy which is indicated in Fig. 5, plasmid pANN655 was constructed in vitro and transformed in *E. coli* 5K. Since the Tet^r gene of pSC101 is destroyed by this insertion, selection was accomplished on lactose minimal agar plates (*E. coli* 5K is *lacZ*). Clones able to grow on this medium were isolated and purified. Plasmid DNAs from these clones were cleaved with a combination of *Hin*dIII and *Sal*I. Fig. 6 shows that the clones carry the expected plasmid termed pANN655 with the *Hin*dIII-*Sal*I fragment of pANN653 inserted in pSC101. *E. coli* 5K containing pANN655 was grown in LB to 110 Klett units, and the β-galactosidase activity was determined to be 100 U. The copy number in *E. coli* 5K of pANN653 is 20 to 25, that of pANN655 is 3 to 5, and the β-galactosidase activities expressed by these plasmids are 700 and 100 U, respectively. Thus, there is a good correlation between the β-galactosidase activity and the copy number exhibited by plasmids carrying the fused *hlyC*::*lacZ* gene under the control of the *hly*-p_1 regulatory region. When the complete *hly* determinant extending from the *Hin*dIII site in front of *hlyC* to the *Sal*I site behind *hlyB*$_b$ (24) was introduced into pSC101 by a strategy similar to that described previously (9) and the hemolysin activity expressed by this low-copy-number Hly plasmid, pANN101-312, was determined, it was again found

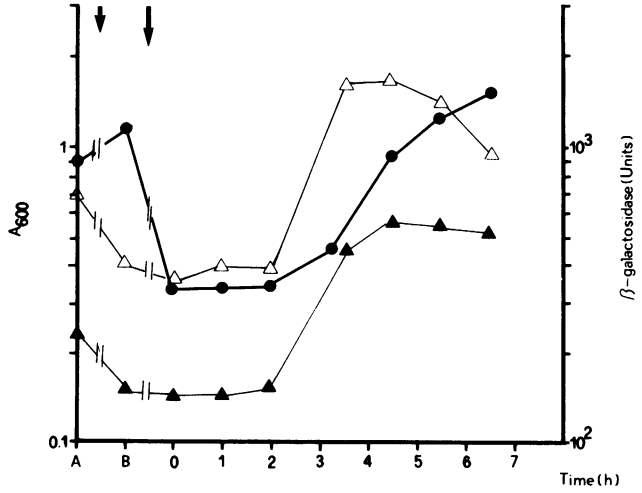

FIG. 4. β-galactosidase activity in *E. coli* 5K(pANN653) and 5K(pANN654) after plasmid amplification with spectinomycin. Spectinomycin was added to logarithmically growing cells (▼), which were then incubated overnight. Afterwards, cells were washed (⬇) and suspended in fresh medium. Symbols: ●, cell density; △, β-galactosidase activity of *E. coli* 5K(pANN653); and ▲, β-galactosidase activity of strain 5K(pANN654).

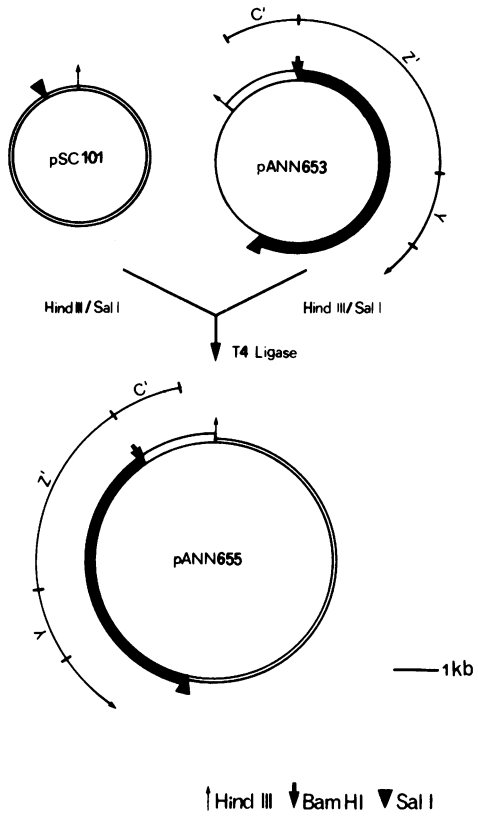

FIG. 5. Construction of plasmid pANN655. Both pSC101 and pANN653 were cleaved with *Hin*dIII and *Sal*I and ligated in vitro. After *E. coli* 5K was transformed with the mixture, colonies possessing a Cm^s phenotype that were able to grow on lactose minimal medium were selected.

that both internal and external hemolysin activity remained the same as that expressed by the multicopy plasmid pANN202-312. These data clearly indicate that the failure of hemolysin to demonstrate gene dosage effect can not be explained by a transcriptional or translational control.

The other products of *hly* genes do not affect the expression of the *hly*::*lacZ* fusion proteins. The results of the experiments described above, in which we studied the regulation of *hly*::*lacZ* fusions, do not rule out the possibility that other products determined by *hly* genes, particularly HlyB$_a$ and HlyB$_b$ (24), may act as negative regulators on the expression of hemolysin. In one type of *hly*::*lacZ* fusion, HlyC is present as native protein (plasmids pANN652, pANN654), HlyA is synthesized as a truncated (pANN652) or fused (pANN654) protein, and neither of the two recombinant plasmids expresses HlyB$_a$ or HlyB$_b$. A possible regulatory role for HlyB$_a$, HlyB$_b$, or both could therefore not be detected with these plasmids. To test this possibility, we transferred plasmid pANN205-222, a pBR322-derived recombinant plasmid which expresses HlyB$_a$ and HlyB$_b$ under the control of the *lac* promoter and is present in the cell in a similar copy number as pANN651, to pANN654, which derives from pACYC184 (24). In addition, the low-copy-number plasmid pHly152, which carries the complete hemolysin-determinant and thus expresses all *hly* gene products, was transferred to *E. coli* 5K containing either pANN651 or pANN653. β-Galactosidase activity was determined as described above. The results (Table 3) do not show

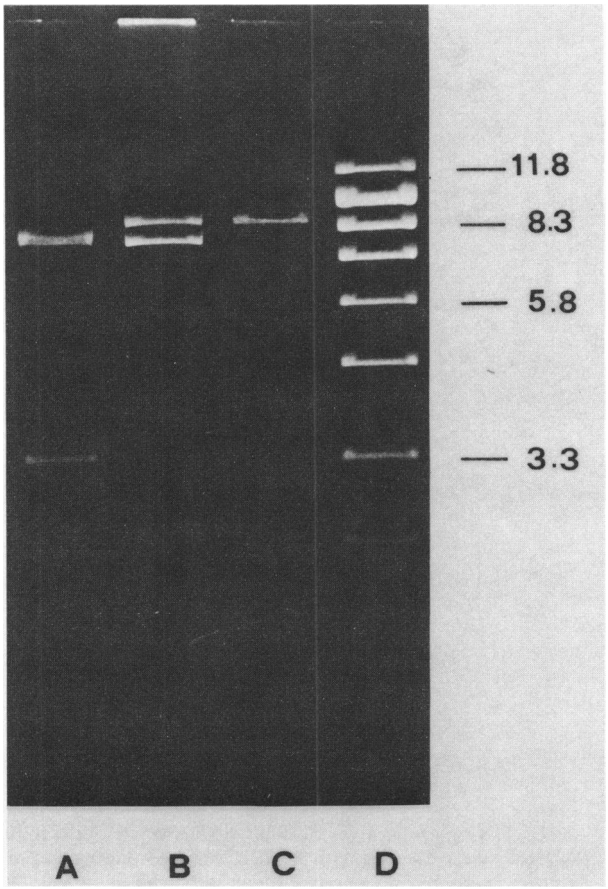

FIG. 6. Agarose gel analysis of plasmid pANN655. *Hin*dIII-*Sal*I cleavage of pANN653 (A), pANN655 (B), and pSC101 (C). Molecular size standards (D), *Eco*RI-digested SPP1 DNA plus two linearized plasmids of 11.8 and 9.8 kilobases.

any difference in β-galactosidase activity with and without additional plasmids. It can therefore be excluded that *hly* gene products $HlyB_a$ and $HlyB_b$ act in trans as negative controlling elements on the expression of the *hly* genes under the control of the left-hand *hly* promoter p_1, i.e., *hlyC*, *hlyA* and $hlyB_a$ (24). A negative transcriptional effect of *hly*A on p_1 is unlikely but can not be entirely ruled out by the present data since the copy number of pHly152 is six- to eight-fold lower than that of pANN651 or pANN653.

Compartmentation of the fusion proteins HlyC-βgal and HlyA-βgal. As shown previously (10, 24), the gene products of *hlyC* and *hlyA* are required for the synthesis of active hemolysin and its concomitant transport across the cytoplasmic membrane. It has been demonstrated in studies with *E. coli* minicells that the primary gene product of *hlyC* is a protein of 18,000 daltons, whereas that of *hlyA* is a protein of 107,000 daltons (9, 10). Neither of these two proteins appears to be transported across the cytoplasmic membrane in mini- or maxicells (10), and transport of active hemolytic protein seems to require a complicated proteolytic processing of HlyA and its modification by HlyC (10; M. Härtlein, unpublished data). The hybrid proteins, HlyC-βgal, contain the intact N-terminal ends of HlyC and HlyA and, hence, should carry possible signal sequences if present on the HlyC or HlyA polypeptide chains. In addition, both fusion proteins contain more than two-thirds of the amino acid sequences of these two Hly proteins. Both exhibit β-galactosidase activity, which can be used to monitor the fate and the localization of these fusion proteins in a much simpler and more sensitive way than can be done with hemolysin activity or the radioactively labeled Hly proteins in mini- or maxicells. Cells harboring pANN653 (*hlyC*::*lacZ* fusion) express β-galactosidase activity which is exclusively associated with a stable protein of 146,000 daltons (see above), and the β-galactosidase activity is primarily found in the cytoplasm, similar to native β-galactosidase (Table 4). Only a small amount of β-galactosidase activity is detectable in the cytoplasmic membrane fraction. No β-galactosidase activity is detectable in the periplasm. The β-galactosidase activity of cells carrying plasmid pANN654 (*hlyA*::*lacZ* fusion) is associated with a protein of 220,000 daltons, as shown above. In contrast to HlyA itself, this protein is perfectly stable, and no proteolytic degradation occurs in maxicells (Fig. 3). Most of the β-galactosidase activity of *E. coli* pANN654 is found in the cytoplasm, and no activity can be detected in the periplasm or the outer membrane. Again, only a relatively small amount of β-galactosidase activity is associated with the cytoplasmic membrane. The total amount of β-galactosidase activity of these cells is lower than that of cells harboring pANN653, as expected since pANN654 carries *hlyC* and part of *hlyA*, and both genes are expressed in this order from the common promoter p_1 located in front of *hlyC*.

DISCUSSION

Synthesis of hemolysin in *E. coli* exhibits several unusual regulatory features. (i) Active hemolysin is synthesized and secreted only during the active growth phase. Its synthesis decreases considerably in the late logarithmic phase and stops completely once the cells enter the stationary phase (22). (ii) The amount of hemolysin produced is not proportional to the gene dosage; e.g., amplification of the four genes which determine synthesis and secretion of hemolysin (10, 24) does not result in a higher amount of either internal or external hemolysin than that observed in cells harboring a single copy of the hemolysin (*hly*) determinant. (iii) Hemolysin synthesis is repressed under anaerobic conditions (A. Juarez, unpublished data). (iv) Hemolysin seems to be the product of a processing mechanism which involves proteolytic degradation of a primary gene product (HlyA) which is modified by another *hly*-specific protein (HlyC) to yield active hemolysin. This processing and modification seem to be a prerequisite for its transport across the cytoplasmic membrane (10). Excretion of hemolysin into the environment requires yet two other gene products ($HlyB_a$ and $HlyB_b$), both of which are predominantly found in the outer membrane fraction of hemolytic *E. coli* cells (10).

In a previous communication, we applied the technique of Mu d-1 (Mu *lacZ bla*) fusion to the *hly* genes to study the regulation of the four *hly* genes. These studies indicated that transcription of the four *hly* genes is achieved by two

TABLE 3. Effect of the *hly* gene products on the expression of the *hlyC*::*lacZ* and *hlyC-lacZ* genes

E. coli carrying *lac* fusion plasmid:	Additional plasmid in the cell	β-galactosidase (U)
pANN651	None	3,500
pANN651	pANN205-222	3,600
pANN651	pHly152	3,700
pANN653	None	700
pANN653	pANN205-222	685
pANN653	pHly152	750

TABLE 4. Cellular location of the hybrid HlyC-βgal and HlyA-βgal proteins

Plasmid	Protein formed	β-Galactosidase activity (U)			
		External	Periplasmic	Membrane bound	Cytoplasmic
None	None	22	25	30	28
pANN651	native β-galactosidase	20	30	82	3,300
pANN653	HlyC-βgal	30	25	122	560
pANN654	HlyA-βgal	18	22	76	189

promoters (p_1 and p_2). The left-hand promoter p_1 regulates transcription of the three genes *hlyC*, *A*, and B_a. Since *hlyC* and *hlyA* alone are sufficient for synthesis of active hemolysin and its comcomitant transport across the inner membrane (10, 24), and the regulatory effects described above can be already observed on this minimal *hly* system, we suspected that a possible regulation of the *hly* genes at the transcriptional level may result from modulation of the activity of promoter p_1. We have chosen in this investigation operon and gene fusions of *lacZ* (with the plasmids pMC871 and 874) with *hlyC* and *hlyA* to address two problems. (i) Is the failure of hemolysin synthesis to respond to gene dosage caused by a negative control mechanism at the transcriptional level, translational level, or both? (ii) Do the gene products of *hlyC*, *hlyA*, or both possess properties which allow their direct transport across the *E. coli* membranes? This question is of particular importance, since our previous data indicated that the primary product of *hlyA*, a protein of 107,000 daltons, has to be proteolytically processed and modified before being transported as active hemolysin. More recently, Mackman and Holland (12) have reported that a protein of 107,000 daltons can be isolated in relatively large amounts from the supernatant of hemolytic *E. coli* strains, a protein which the authors correlate with hemolysin. Both types of *lacZ* fusions can be readily achieved by inserting *lacZ*-carrying *Bam-Sal* fragments from pMC871 or pMC874 (4) into single *Bam*HI or *Bgl*II sites located close to the C-terminal ends of *hlyC* or *hlyA*, respectively, and the single *Sal*I site located outside of the *hly* determinant in the recombinant Hly plasmid pANN202-312. The gene fusions with the *lacZ* carrying *Bam*HI-*Sal*I fragments from pMC871 and pMC874 lead to the expected fusion proteins which possess β-galactosidase activity, indicating that the fusion into these sites does not destroy the reading frame of the two genes. Activity of β-galactosidase of these fusion proteins clearly follows the gene dosage when the fused genes *hlyC*::*lacZ* and *hlyA*::*lacZ* or the fused operons Δ(*hlyC-lacZ*) and *hlyC*, Δ(*hlyA-lacZ*) are under the control of promoter p_1. This is particularly seen when the copy number of the plasmid carrying the fused *hlyC*::*lacZ* gene is altered. We therefore conclude that the limitation in the amount of hemolysin in *E. coli* is not due to a negative transcription control of the essential genes (*hlyC* and *hlyA*) and also not due to a negative translational control but is caused by yet unknown mechanism(s) at the posttranslational level. Possible steps for this regulation are the proteolytic processing of *HlyA* or its modification by *HlyC*.

Our data further show that the dramatic decrease in hemolysin activity in the late exponential phase of the *E. coli* cultures is also not caused by transcriptional or translational effects, since the β-galactosidase activity of cells harboring the fused *hlyC*::*lacZ* or *hlyA*::*lacZ* genes increases with the cell mass, reaches a maximum in the early stationary phase, and remains constant upon further incubation of the cells independent of the media (rich or minimal) used.

Both fusion proteins, HlyC-βgal and HlyA-βgal, are predominantly found in the cytoplasm. No β-galactosidase activity can be detected in the supernatant or the periplasmic space. The somewhat higher portion of β-galactosidase activity, particularly of the HlyA-βgal protein, associated with the cytoplasmic membrane fraction is probably not caused by a partial penetration of the fusion protein into the membrane but rather by unspecific adsorption of this protein to the membrane fraction. This conclusion is based on the following observations. (i) Partial penetration into the cytoplasmic membrane by a β-galactosidase fusion protein carrying a portion of a secretory protein at the N-terminal end leads to its complete recovery in the cytoplasmic membrane, thereby rendering the cells phenotypically Lac^- (13). This effect is particularly pronounced when the fusion site of β-galactosidase is close to the C-terminal end of the secretory protein (13) as in both of our fusion proteins. Nevertheless, the phenotype of cells synthesizing these two fusion proteins is Lac^+, and little is found in the membrane. (ii) The membrane-bound portions of the HlyC-βgal and HlyA-βgal proteins can be almost completely removed from the membrane by extensive washing of the membrane fraction. We feel that these data reconfirm our previous conclusions (10) that the primary gene product of HlyC, a 18,000-dalton polypeptide, and that of HlyA, a 107,000-dalton protein, are not secretory proteins per se.

Finally, the analysis of the *HlyA*-βgal fusion protein shows that this large polypeptide of 230,000 daltons is perfectly stable in mini- and maxicells in the presence of functioning HlyC protein, in contrast to the HlyA product itself, which is proteolytically processed in both cell systems to a major form of 58,000 daltons. This is in agreement with recently described data (10) showing that the removal of a short sequence from the C-terminal end of the *hlyA* gene leads to production of a truncated 104,000-dalton protein which is also not proteolytically processed and is found predominantly in the cytoplasm. From both sets of data we conclude that the C-terminal end of the primary HlyA product is necessary for its subsequent proteolytic processing.

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