Changes in Protein Synthesis on Mitomycin C Induction of Wild-Type and Mutant CloDF13 Plasmids

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Mitomycin C treatment of *Escherichia coli* K-12 cells containing the nonconjugative plasmid CloDF13 resulted in inhibition of host chromosome protein synthesis and a high rate of synthesis of two CloDF13-specified proteins whose molecular weights correspond to cloacin and immunity protein. Five molecules of immunity protein were synthesized for each cloacin DF13 molecule. Mitomycin C-treated cells containing a copy mutant of CloDF13 made three to four times as much of each protein as cells containing wild-type CloDF13. CloDF13 plasmids that contained the transposon TnI were isolated. Two did not induce after mitomycin C treatment, failing both to inhibit host cell synthesis and to produce the two new proteins. In minicells, they showed reduced CloDF13-specified protein synthesis and produced three TnI-specified proteins.

Many small multiple-copy nonconjugative plasmids, for example ColE1, ColE2, and CloDF13, code for the production of antibacterial proteins called bacteriocins. In exponential growth at 37°C, only a small fraction of a colicinogenic population produce measurable amounts of colicin spontaneously. If, however, the cells are treated with agents like ultraviolet light or mitomycin C, that interfere with deoxyribonucleic acid (DNA) metabolism, a large fraction of the cells produce colicin (5, 12) and, at least in some cases, cells that become committed to colicin production become nonviable (13). Colicins kill closely related sensitive bacteria, but strains that contain the homologous Col plasmid are immune to the action of the colicin due to the production of immunity substances that prevent the bacteriocidal lesions.

Tyler and Sherratt studied the kinetics of protein synthesis at various times after treatment with mitomycin C of cells containing plasmids ColE1, ColE2, and ColE3 (17). They found that host protein synthesis decreased rapidly after mitomycin C treatment of ColE2- and ColE3-containing cells, while at the same time new species of possible plasmid encoded proteins were synthesized in large amounts. At the concentration of mitomycin C used there was no effect on protein synthesis in plasmid-free cells. We extended these studies to include the small plasmid CloDF13 (molecular weight, 6 × 10⁶). Cloacin DF13 is the bacteriocidal protein produced by CloDF13-containing cells: it kills bacteria such as *Klebsiella edwardsii* but does not kill *Escherichia coli*.

Recently, the transposable segment of DNA known as TnI was used to study ColE1 plasmid function (16). If TnI, which confers ampicillin resistance, is inserted into a plasmid, it can interfere with the expression of the gene into which it goes and also exert polar effects on neighboring genes. We isolated several independent insertions of TnI into CloDF13 and observed the effects, if any, on induction. Kool et al. (10, 11) have already initiated a study of gene expression of CloDF13 in chromosomless minicells and have characterized some of the proteins produced.

**MATERIALS AND METHODS**

**Bacterial strains.** The strains used in our experiments are listed in Table 1.

**Media.** Strains were maintained on nutrient agar or in nutrient broth. Brain heart infusion medium (3.7%; Difco Laboratories) was used for minicell growth. Minimal medium was M9 salts (15) plus 0.5% glucose and 0.025% MgSO₄ (with or without 1% Casamino Acids). BSG buffer was as described by Kool et al. (10, 11). Supplements were threonine (40 μg/ml), proline (20 μg/ml), methionine (40 μg/ml), leucine (20 μg/ml), and thiamine (1 μg/ml). Concentrations of antibiotics were (micrograms per milliliter): ampicillin (Ap), 100; streptomycin, 150; spectinomycin, 60; chloramphenicol, 50; kanamycin (Km), 30; and tetracycline (Te), 10.

**Growth, labeling, colicin induction, and SDS-polyacrylamide gel electrophoresis.** The conditions of Tyler and Sherratt (17) were used for growth, labeling, colicin induction, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

**Isolation of CloDF13-TnI plasmids.** Several independent strains were made RP4* CloDF13* (in
TABLE 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td>E. coli K-12</td>
<td>thi leu thr rpsL minA minB</td>
<td>M. H. L. Green (1)</td>
</tr>
<tr>
<td>P678-54</td>
<td></td>
<td>A. Kool et al. (10)</td>
</tr>
<tr>
<td>P678-54 (CloDF13)</td>
<td></td>
<td>A. Kool and H. J. J. Nijkamp (8)</td>
</tr>
<tr>
<td>P678-54 (CloDF13rep3)</td>
<td>pro met Km' Ap' Tr'</td>
<td>N. Datta</td>
</tr>
<tr>
<td>J53 (RP4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K. edwardsii</td>
<td>Cloacin-sensitive indicator strain</td>
<td>A. Kool and H. J. J. Nijkamp (8)</td>
</tr>
</tbody>
</table>

* Gene symbols as described by Bachmann et al. (2). Km', Ap', Tr', Resistance to kanamycin, ampicillin, and tetracycline, respectively. rpsL, Streptomycin resistance.

P678-54, and the plasmid DNA was isolated from them after chloramphenicol amplification (14) was transformed into P678-54 recipients, selecting for ampicillin resistance. The Ap' isolates were tested for tetracycline and kanamycin resistance, and Tr' Km' Ap' isolates were tested further for plasmid DNA size on neutral 5 to 20% sucrose gradients (3).

Minicells. Minicells were isolated by a modified form of the Kool et al. procedure (10, 11). The cells were grown, with aeration, overnight in 200 ml of brain heart infusion medium at 37°C. Most of the whole cells were removed by spinning in a rotor (6 by 250 ml) for 2.5 min at 3,000 rpm on a Measuring and Scientific Equipment, Ltd. (MSE) no. 18 centrifuge. The remaining cells were then pelleted at 15,000 rpm for 15 min, suspended in 1 ml of BSG, and run on a 15-ml 5 to 20% sucrose gradient in BSG for 20 min at 2,200 rpm in an MSE 6L centrifuge. The minicells banded at the top, were extracted, and were run through two more gradients. They were then washed and resuspended in 1 ml of supplemented minimal medium and left shaking for 30 min at 37°C. A 4-μCi amount of [35S]methionine (>100 Ci/mmol) per ml was then added, and labeling continued for 30 min. The minicells were then chased with L-broth and methionine (40 μg/ml) for 45 min, spun down, and suspended in the final sample buffer (17).

RESULTS

Kinetics of protein synthesis of CloDF13- and CloDF13rep-3-containing cells after mitomycin C treatment. We previously examined protein synthesis in mitomycin C-treated cells containing CloDF13 and the copy mutant (CloDF13rep-3) of CloDF13 isolated by Kool and Nijkamp (8). Cells were pulse-labeled with [14C]-amino acids at different times after mitomycin C treatment, and their labeled proteins were run on SDS-polyacrylamide gels. Examination of the gel autoradiograms showed that host protein synthesis rapidly decreased within 60 min of the treatment and was almost undetectable after 300 min. Moreover, two new protein bands began to increase in their rate of synthesis shortly after induction. In the case of CloDF13+ cells, the proteins were at their maximum rates of synthesis 150 min after induction. The molecular weights of the bands, as calculated from their mobility in gels, were about 75,000 and 10,000, which is in close agreement with the published values of Kool and Nijkamp for cloacin DF13 and its corresponding immunity protein (8). Densitometer tracings were made of the autoradiograms, and the relative increase in intensity of each of the two depressed bands was plotted against time after mitomycin C treatment (Fig. 1). About five times as much immunity protein was made as cloacin, taking into account the molecular weight difference. In the case of CloDF13rep3+ cells, three to four times as much of each of the two proteins was synthesized as compared with wild-type CloDF13-containing cells, which is not surprising since CloDF13rep-3 has a seven-times-higher plasmid copy number (8). Protein synthesis in CloDF13rep-3+ cells reached a maximal level at 100 min, which was earlier than when CloDF13 was present. It was shown previously that at least three proteins are synthesized from ColE2 and ColE3 on induction, two of which seem to be colicin and immunity protein (17). ColE2 and ColE3 also make four times the molar quantity of immunity protein as compared with colicin. Inhibition of synthesis of most, if not all, of the chromosomal proteins in induced CloDF13+ cells showed the same kinetics (a representative curve is shown in Fig. 1). As for ColE2 and ColE3 (17), inhibition seemed to be initiated about 20 min after addition of mitomycin C.

Insertion of Tnl into CloDF13. Since cloacin and immunity protein are coordinately derepressed after mitomycin C induction, we decided to try to interfere with the induction process by using the Tnl transposon. Plasmid RP4 was used as the "donor" of the Tnl sequence. We transferred RP4 into the CloDF13-containing minicell-producing strain, isolated the plasmid DNA after chloramphenicol amplification of CloDF13 DNA, and used it to transform the
minicell strain. Several ampicillin-resistant transformants were isolated, and the cells were tested for resistance to kanamycin and tetracycline. The Tc\(^+\) Km\(^+\) strains were assumed to lack RP4, and in fact all Ap\(^+\) cells tested were Tc\(^+\) Km\(^+\). This is not surprising since CloDF13 DNA (and presumably CloDF13::Tnl DNA), but not RP4 DNA, is amplifiable by chloramphenicol treatment. The plasmid DNA was then isolated from the Ap\(^+\) transformants and sedimented through 5 to 20% neutral sucrose gradients with \(^{14}\)C-labeled ColE1 DNA to check for the increase in molecular weight expected after Tnl1 insertion (Fig. 2). Four isolates with the expected increase in molecular weight were found and used for further studies. The new plasmids were named pC1302, pC1303, pC1304, and pC1305, and all showed wild-type plasmid DNA levels as measured by sucrose gradient analysis (6). All of the CloDF13::Tnl plasmids exhibited a broader peak than expected for a monodisperse DNA species. However, treatment of the cleared lysate with SDS (0.25%) converted this material to two sharp, symmetrically sedimenting species corresponding to monomer supercoils and open circles (SDS "relaxes" the "relaxation complex" present [3]; results not shown). Similarly, examination of plasmid DNA from cleared lysates either on agarose gels or in an electron microscope showed that greater than 80% of the circular DNA corresponded in size to CloDF13::Tnl monomers (molecular weight, 9.2 × 10\(^{6}\)). We also noticed similar broad peaks after sedimentation of cleared lysates containing ColE1::Tnl and ColE2::Tnl DNA, but not with the parental plasmids. We feel it possible that the high level of gene expression from Tnl1 (e.g., see Fig. 3) may make the CloDF13::Tnl DNA sediment faster and heterogeneously because much of the transcription machinery may remain attached to the sedimenting DNA.

Strains containing either pC1302 or pC1303 produce biologically active cloacin, whereas strains containing pC1304 or pC1305 do not. Strains containing any of the four plasmids maintained the plasmid stably, even in the absence of ampicillin selection.

Kinetics of mitomycin C-induced protein synthesis specified by CloDF13::Tnl plasmids. The patterns of protein synthesis after

![Fig. 1. Protein synthesis after mitomycin C treatment of CloDF13- and CloDF13rep-3-containing cells. Densitometer tracings were taken of autoradiograms of SDS-polyacrylamide gels (17), and the relative amounts of proteins synthesized at each time were calculated from the area under the relevant peaks. Samples for the gels were prepared as follows. Mitomycin C (2 μg/ml) was added to a growing culture at time zero, and 1-ml volumes were removed at the indicated times and pulse-labeled for 5 min at 37°C with 0.5 μCi of \(^{14}\)C-labeled amino acids. Cold L-broth (4 ml) was added, and the cells were pelleted and suspended in 100 μl of final sample buffer. Samples (10 μl each) were run on polyacrylamide gels and autoradiographed as described previously (17). Molecular weights were determined by comparison of the migration of known molecular weight standards run on the same slab gel. Symbols: ——, CloDF13 rep-3 cloacin; —×—, CloDF13 rep-3 immunity protein; ——, CloDF13 cloacin; —×—, CloDF13 immunity protein; —O—, typical chromosomal protein band.](image1)

![Fig. 2. Sucrose gradient profiles of \(^{3}H\)-labeled CloDF13 and CloDF13::Tnl plasmid DNAs. Plasmid DNA from cleared lysates was sedimented through 5 to 20% neutral sucrose gradients for 100 min at 45,000 rpm in an SW50.1 rotor on a Beckman L2 centrifuge. \(^{14}\)C-labeled ColE1 DNA was added to the layered sample as an internal size marker. (a) pC1302; (b) CloDF13. Symbols: ●, \(^{3}H\) counts per minute; ○, \(^{14}\)C counts per minute.](image2)
mitomycin C induction of cells containing the different CloDF13::Tnl plasmids are shown in Fig. 3A and B. The cloacin-producing strains containing plasmids pC1302 (Fig. 3B) and pC1303 induced normally and gave the two protein bands produced by CloDF13. Non-cloacin-producing strains carrying pC1304 (Fig. 3A) and pC1305, however, failed to show any change in protein synthesis. Host cell protein synthesis carried on as normal, and both of the new bands failed to appear. Insertion of Tnl into CloDF13 had thus produced a pleiotropic effect on induction, supporting the idea that immunity and cloacin are under coordinate control in the induction process.

**CloDF13::Tnl**-specified proteins synthesized in minicells. In minicells containing wild-type CloDF13, we observed the synthesis of three major proteins of molecular weights approximately 70,000, 20,000, and 10,000 (Fig. 3C). This is in agreement with Kool et al. (9), who also showed the largest and smallest proteins to be cloacin and immunity protein, respectively. The two proteins derepressed by mitomycin C induction ran exactly together on gels with cloacin and immunity protein from minicells, giving extra support to their identity. We found a changed pattern of protein synthesis in minicells containing the CloDF13::Tnl plasmids. Minicells containing...
pC1302 and pC1303 (both cloacin·) synthesized all three wild-type proteins, but in addition we observed three new bands (molecular weights, 32,000, 30,000, and 28,000) (Fig. 3E). Since we observed these bands also when we inserted Tn1 into ColE1, ColE2, and ColK (G. Dougan, W. Tacon and D. J. Sherratt, unpublished data), it is likely that the three bands are specified by the transposon Tn1. We have shown the protein of molecular weight 30,000 to have β-lactamase activity (G. Dougan and D. J. Sherratt, unpublished data). Minicells containing the cloacin· plasmids pC1304 and pC1305 (Fig. 3F) also synthesized the three presumptive Tn1-specified proteins in the same relative amounts, but both had much reduced levels of presumptive immunity protein and cloacin, although the 20,000 molecular weight protein was still synthesized normally.

We do not know whether the CloDF13 proteins synthesized in minicells reflect basal repressed expression from the genome; whether there are two populations of minicells, one derepressed for cloacin synthesis (and possibly derived from spontaneously derepressed whole cells) and the other repressed; or whether expression in minicells is uncontrolled and shows little quantitative resemblance to expression in repressed or derepressed whole cells. Treatments of minicells with mitomycin C had no effect on the proteins that they synthesize (D. J. Sherratt, unpublished data).

DISCUSSION

It was reported previously that CloDF13-containing cultures produce large amounts of cloacin upon induction with mitomycin C (greater than a 40-fold increase [4]). However Kool et al. (9) were only able to detect about four times more immunity protein. Our data indicate that a large amount of immunity protein is produced on mitomycin C treatment. Jake et al. (7) found a large increase in E3 immunity protein after induction, and Tyler and Sherratt (17) supported their findings and also gave evidence that presumptive ColE2 immunity protein production increases by a similar amount. Our results suggest that cloacin and its immunity protein are both derepressed on induction. The fact that cells containing CloDF13rep-3 synthesize more of the two proteins supports the assumption that it is plasmid protein synthesis we are observing, since it has been reported that CloDF13rep-3· cell populations spontaneously produce seven times more cloacin than CloDF13· populations due to increased plasmid copy number in strains carrying this mutant plasmid. The reason for the coordinate induction of immunity protein and colicin is not yet known, although the cell may need to protect itself from its own colicin to induce successfully.

With transposon Tn1 we were able to interfere with the normal induction process. Strains carrying cloacin-producing CloDF13·Tn1 plasmids seemed to be normal in induction, and we assume that all genes involved in induction were functional. Strains containing the cloacin· CloDF13·Tn1 plasmids, however, behaved differently: after mitomycin C treatment, they failed either to inhibit host cell protein synthesis to a significant degree or to synthesize the two new protein bands.

In minicells, the CloDF13·Tn1 plasmids specify three proteins that we believe are Tn1 proteins. At least one of these proteins (molecular weight, 30,000) has β-lactamase activity (Dougan and Sherratt, unpublished data). The function of the other two proteins is now under investigation. Cloacin-producing strains appear normal otherwise. The cloacin· CloDF13·Tn1-containing strains, however, show reduced levels of cloacin in minicells. The level of immunity protein is also reduced but not as much. The level of the 20,000 molecular weight protein is near the normal value. Tn1 insertion into a gene or transcription unit responsible for normal induction would explain the observed phenotype of strains containing the cloacin· CloDF13·Tn1 plasmids.

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LITERATURE CITED