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Analysis of the Products of the *Myxococcus xanthus frz* Genes

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The frizzy (*frz*) genes of *Myxococcus xanthus* control the ability of cells to reverse direction of gliding motility. The orientation of the *frz* genes was studied by isolating transcriptional fusions with the transposon derivative Tn*5-lac*. The *frz* genes were then cloned in the proper orientation in an expression vector. By using maxicell experiments, we were able to identify several labeled bands which were plasmid encoded. To identify the labeled proteins and their respective genes, we constructed deletion plasmids in which various regions of the insert DNA had been removed. The plasmid-encoded proteins were then labeled in maxicell experiments, and the bands which correspond to the *frzCD*, *frzE*, and *frzF* gene products were identified. The sizes of the gene products agreed with the genetic and physical map of the cloned DNA.

Myxococcus xanthus, a gram-negative gliding bacterium, possesses a complex life cycle consisting of a vegetative phase and a developmental phase (fruiting body formation) (5, 17, 21). Control of cell motility is important throughout the life cycle, since thousands of cells must move in a coordinated manner to form and maintain vegetative swarms (hunting and food-gathering groups) and to aggregate during fruiting body formation. Hodgkin and Kaiser (7–9) discovered two multigene systems that control the movements of *M. xanthus*: the A system affects the movements of single cells, and the S system controls groups of two or more cells. Recently, we discovered a third multigene motility system in *M. xanthus*, the frizzy (*frz*) system, which appears to control the ability of cells to reverse the direction of gliding motility (1, 2, 21). Mutations in the *frz* genes (except *frzD*) change the pattern of colony growth from uniform spreading to the formation of filamentous swirls, composed of thousands of cells. This morphology is observed under both vegetative and developmental conditions (the cells cannot aggregate into raised mounds but are able to sporulate [20]). Observations of cell movements by time-lapse video microscopy showed that mutations in any of the *frz* genes, except *frzD*, cause the cells to reverse their direction of movement very rarely, about once every 2 h (2). In contrast, wild-type cells reverse their direction of gliding about every 6.8 min; net movement occurs since the interval between reversals can vary widely. *frzD* mutants reverse their direction three times as often as do wild-type cells, about once every 2.2 min; individual cells show little net movement and form smooth-edged colonies that appear nonmotile. The region of DNA carrying the *frz* genes was cloned in *Escherichia coli* and analyzed by isolating and characterizing new Tn*5* insertions at frequent intervals within the *M. xanthus* DNA and transducing the mutated DNA into *M. xanthus* for recombinational and complementation analysis (1). In these studies, five to six *frz* complementation groups were identified on 7.5 kilobases (kb) of cloned DNA (Fig. 1). All of the complementation groups are contiguous except the last, *frzF*, which is separated from the others by 1.4 kb of DNA. The different *frz* loci behave as separate transcriptional units, and mutations in all the loci are recessive, except for those in *frzD* which are dominant to wild-type in mero dipoids (1).

To determine the orientation of the *frz* genes, we used Tn*5-lac*, a derivative of Tn*5* which creates transcriptional fusions when it transposes to new DNA sites (11). Tn*5-lac* was constructed by inserting promoterless *lac* DNA near one end of Tn*5* such that expression of the *lac* genes (*lacZ* and *lacY*) is dependent upon transcription initiation from an exogenous promoter (Fig. 1c). Tn*5-lac* insertions in the plasmid pBB15 (Fig. 2) were isolated, and the sites of insertion and their orientation were determined by restriction enzyme mapping. Tn*5-lac* insertions in both orientations were isolated in *frzA*, *frzC*, *frzE*, and *frzF* (Fig. 1b). To study the expression of β-galactosidase activity under the control of the *frz* gene regulatory elements, the plasmids containing Tn*5-lac* insertions were transduced into *M. xanthus* DZF1 (15) by using the generalized transducing phage P1 (10, 16). The results of these experiments are presented in Table 1. Transductions were also performed with marked strains containing mutations in *frzA*, *frzB*, *frzC*, *frzE*, or *frzF* to confirm that the sites of insertion were in the correct complementation groups (data not shown). All of the *M. xanthus* strains tested showed positive β-galactosidase activity, as indicated by a blue or green color when grown on CYE agar (Casitone-yeast extract [4], a rich medium similar to L-broth [14]) supplemented with X-gal (Sigma Chemical Co.; 40 μg/ml) regardless of the orientation of the transposon. However, the level of activity was consistently higher with insertions that were oriented left to right (Fig. 1). Measurement of enzyme levels showed that the color on X-gal plates was not linear with β-galactosidase activity, so that the transposon insertions that were oriented right to left were actually very low in activity (R. Weinberg and D. Zusman, unpublished data). The same strains were also tested on 1/2 CTT agar (a poor growth medium used for motility studies [7]) and on CF agar (a low-nutrition medium which induces development [6]) supplemented with X-gal. Expression of the *lacZ* gene on these media was only detected with strains which had Tn*5-lac* insertions with an orientation of left to right. From these results, we conclude that the direction of transcription of the *frzA*, *frzC*, *frzE*, and *frzF* genes is left to right.

Data on the orientation of the genes allowed us to subclone the *frz* genes into pDR720 (18), a plasmid vector suitable for foreign gene expression in *E. coli*. This vector contains the *E. coli trp* operator and promoter sequences

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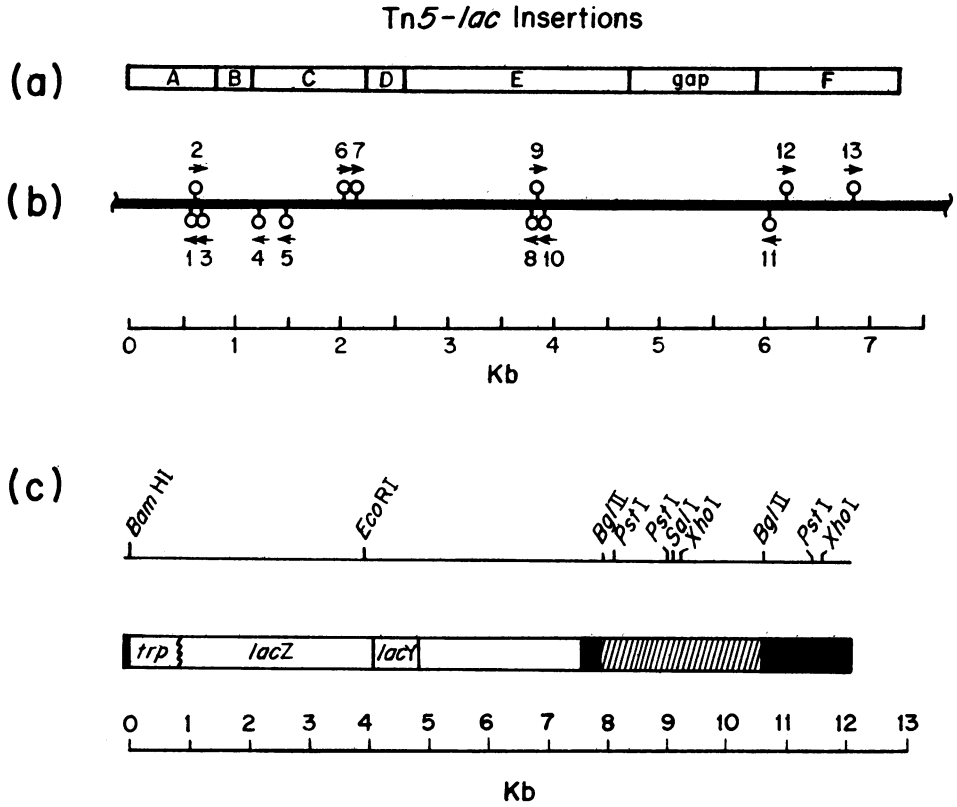

FIG. 1. Physical and genetic map of the *frz* region and sites of Tn*5-lac* insertion. (a) Map of the *frz* region, from data published previously (1). (b) Tn*5-lac* insertions in pBB15 were isolated by transposition of Tn*5-lac* from P1 to the plasmid (11). Independent insertions were isolated, and their location and orientation were determined by restriction mapping. Plasmids with insertions in *frz* genes are shown. Insertions above the line are oriented as in panel c to detect transcription from left to right, and insertions below the line are in the opposite orientation and detect transcription from right to left. (c) Map of Tn*5-lac*. The sturcture of Tn*5-lac* and the restriction sites used to determine the orientation of the Tn*5-lac* insertions are shown (the information was provided by L Kroos). Symbols: ▨, area of Tn*5* between the IS*50* elements; ▬, IS*50* regions; ▭, regions of *trp* and *lac* DNA inserted in Tn*5*.

inserted into the *Sma*I site upstream of a promoter less *galK* gene. When we induced *E. coli* cells harboring pDR720 by adding 3-β-indoleacrylic acid to the medium, at least 10% of total cellular protein, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was the *galK* gene product. When we induced the same cells containing the plasmid pBB20 (this plasmid contains the *Xho*I-*Bam*HI fragment of pBB12 containing the *frz* genes cloned into the *Sal*I and *Bam*HI sites of pDR720; Fig. 2 and 3b), neither the *galK* gene product nor the *frz* gene products were expressed at readily detectable levels. This result indicates that the *frz* genes are poorly expressed in *E. coli* even though transcription is initiated at a strong *E. coli* promoter. The poor expression of the *galK* gene in pBB20 is not surprising, considering the large size of the insert DNA (10 kb). However, we were able to detect expression from pBB20 by using the maxicell procedure of Sancar et al. (19). Figure 3a shows the labeling pattern obtained. It should be noted that the *trp* promoter was necessary for expression of the cloned DNA since maxicell experiments with pBB15, which lacks the trp promoter, failed to show any labeled polypeptides except for the 28,000-molecular-weight product of the ampicillin gene. From the autoradiograms in Fig. 3, we were able to identify the *galK* gene product (M_r, 42,000) since it is present in the vector plasmid pDR720. To identify the other labeled proteins and their corresponding genes, we constructed deletion plasmids in which various regions of the insert DNA had been removed (Fig. 3b). The plasmid-encoded proteins were then labeled in maxicell experiments and analyzed as follows (Fig. 3a). A modification of the method of Sancar et al. (19) was used. *E. coli* DH1 (13), a *recA* strain which can be transformed with high efficiency, was grown exponentially in L-broth (14) at 34-37°C. A 5-ml aliquot of 5×10^8 cells per ml was pipetted into a petri plate (100 by 15 mm) and exposed to 4 J/m^2 of UV light per s for 40 s. The irradiated cells were immediately placed in a foil-wrapped test tube (17 by 100 mm) and incubated for 2 h at 34°C with agitation. Cycloserine (Sigma Chemical Co.) was then added (final concentration, 200 μg/ml), and incubation was continued overnight. The cells were collected by centrifugation (5000 × *g* for 10 min), washed with 5 ml of sulfate-free minimal medium, and then suspended in 5 ml of sulfate-free minimal medium containing 200 μg of cycloserine per ml and 50 μg of indole-acrylic acid (Sigma Chemical Co.) per ml. The cells were incubated for 1 h at 34°C, at which time 8 to 100 μCi of [^{35}S]methionine (New England Nuclear Corp.) was added to the culture, and incubation was continued for an additional 2 h. The cells were then collected by centrifugation and suspended in 150 μl of protein solubilization buffer (12). A portion (25 to 40 μl) of the whole-cell extract was boiled and then analyzed by electrophoresis on 10 or 15% sodium dodecyl sulfate–polyacrylamide gels. The gels were fixed with 10% acetic acid and treated with En^{3}Hance (New England Nuclear

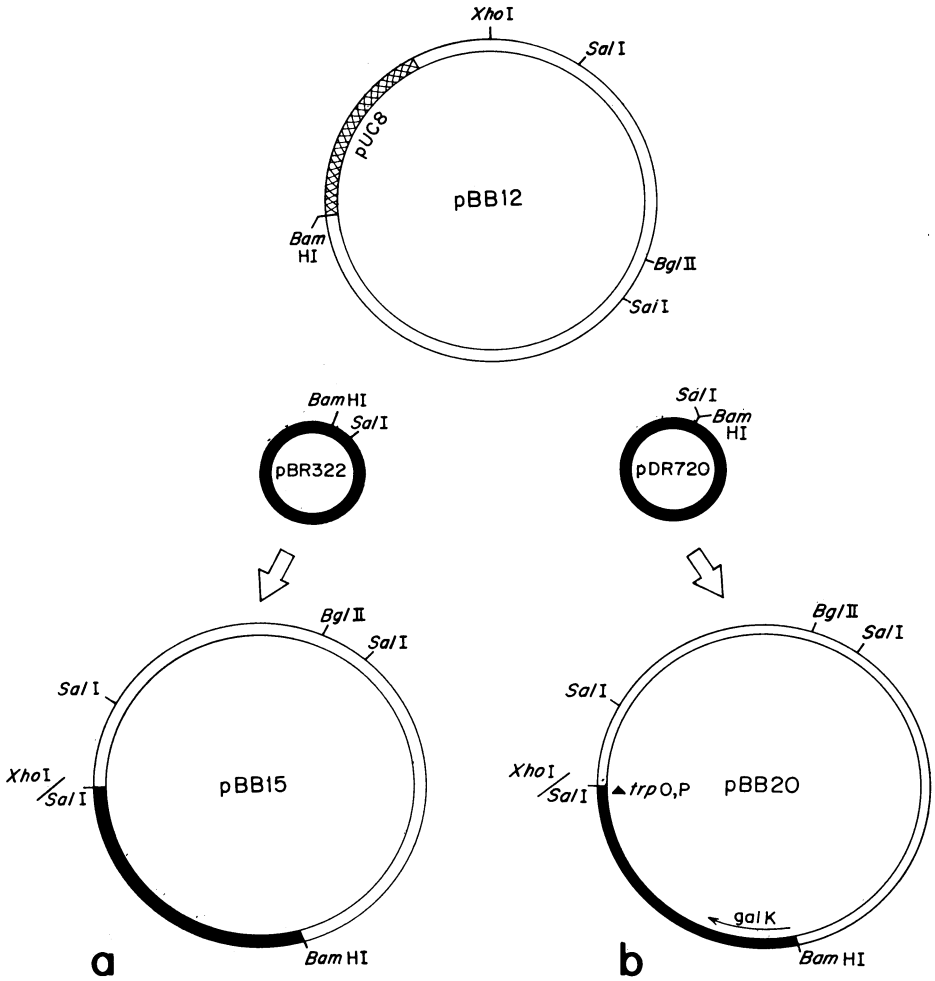

FIG. 2. Derivation of clones. (a) Construction of pBB15. The recombinant plasmid pBB15 was constructed by inserting the 10-kb fragment of pBB12 (1) containing the *frz* genes (obtained by cutting the plasmid with *Xho*I and *Bam*HI) into pBR322 (3) cleaved with *Sal*I and *Bam*HI. After transformation, plasmids from ampicillin-resistant colonies were screened for the desired genotype. (b) Construction of the expression plasmid pBB20. The plasmid was constructed in the same manner as was pBB15 except pDR720 was used instead of pBR322. Symbols: ▨, pUC8 DNA; ▬, pBR322 in panel a and pDR720 in panel b; ▭, *M. xanthus* DNA. The locations of the *galK* gene and the *trp* promoter are also shown.

Corp.) or 1 M sodium salicylate (Sigma Chemical Co.). Kodak X-ray film (XAR-5) was preflashed and exposed for 2 to 4 days at −76°C. The following experiments identify the gene products of *frzE*, *frzC-D*, and *frzF*.

(i) *frzE* gene product. The complementation data (Fig. 1) showed that *frzE* is the largest complementation group, coding for polypeptide(s) with a maximum molecular weight of 80,000 to 100,000. The deletion in pBB21 (Fig. 3b) removed the DNA between the *Sal*I sites at 1.3 and 4.8 kb, eliminating essentially all of *frzC*, *frzD*, and *frzE* DNA. This deletion resulted in the loss of three bands with molecular weights of about 90,000, 47,000, and 45,000 (Fig. 3a). The deletion in pBB22 extended from the *Bgl*II site at 4.3 kb to the *Bam*HI site at the end of the insert, eliminating part of the *frzE* gene and all of the *frzF* gene. This deletion resulted in the loss of the band at M_r 90,000 and the appearance of a faint new band at an M_r of 75,000. Since the truncated *frzE* gene in pBB22 is large enough to code for a polypeptide of molecular weight 75,000, the band at 90,000 is likely to be the *frzE* gene product. Additionally, pBB25 contains the *frzE* gene and codes for the 90,000-molecular-weight protein while pBB24 lacks the *frzE* gene and does not produce the protein. We therefore conclude that the *frzE* complementation group codes for a large polypeptide with molecular weight of about 90,000.

(ii) *frzC* gene product. The complementation and physical mapping data showed that *frzC* is large enough to code for a polypeptide(s) with a molecular weight of 50,000. The deletion in pBB21, which removed *frzC*, *frzD*, and *frzE* resulted in the loss of bands at molecular weights of 90,000, 47,000 and 45,000, as described above. The deletion in pBB23 removed the *frzE* genes but retained the *frzC* and *frzD* genes. This strain showed a loss of the band at an M_r of 90,000, but the other bands at 47,000 and 45,000 were still produced (Fig. 3a). On the other hand, the plasmid pBB27, which contains a truncated *frzC* gene, lacked these bands. (The plasmid pBB28 contains a similar deletion to that of pBB27 except that it retains the *frzC* gene; it also codes for the bands at M_rs of 47,000 and 45,000). Since the *frzD* locus is extremely small, we think that the polypeptide with a molecular weight of 47,000 is the gene product of *frzC*. The band at 45,000 is most likely a breakdown product of the

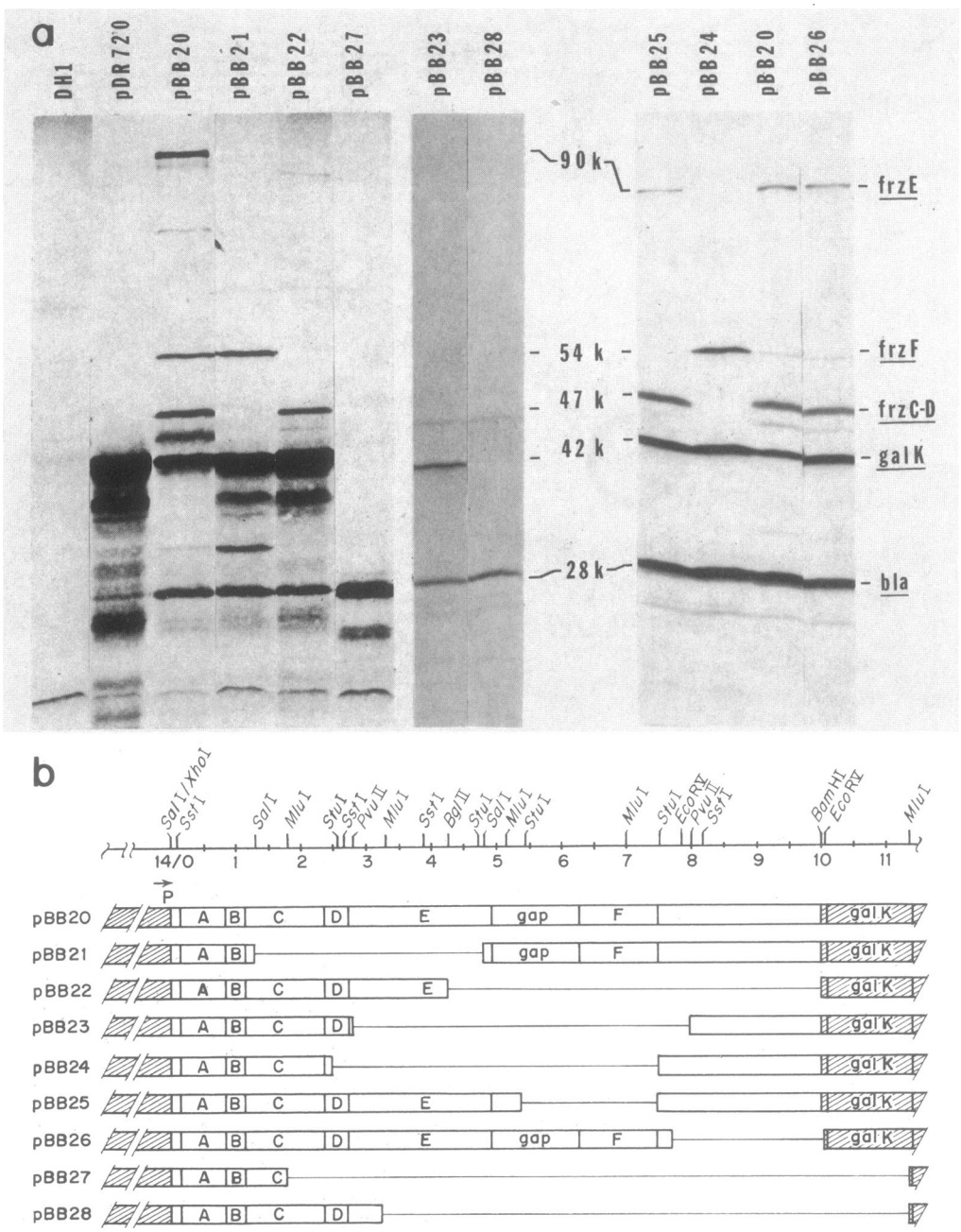

FIG. 3. (a) Maxicell experiments for labeling plasmid-encoded proteins. A modification of the method of Sancar et al. (19) was used. *bla*, β-Lactamase. (b) Deletions of the expression plasmid pBB20. The deletion plasmids were constructed by partial or complete digestion with restriction enzymes, followed by ligation and transformation of the digested plasmids. The enzymes used for each deletion are described in the text. Partial digests and religation of the DNA can result in inversion of one or more segments of DNA. In deletions in which inversions of fragments are possible, e.g., pBB25, orientation of the DNA was determined by restriction mapping. Symbols: ▨, vector DNA; ▭, *M. xanthus* insert DNA.

47,000-molecular-weight polypeptide since it is present only when the band at an M_r of 47,000 is present and not in all experiments.

(iii) Analysis of the *frzD* locus. Using the maxicell procedure, we did not detect any labeled polypeptides with a molecular weight within the range of a possible gene product of *frzD*. In our previous genetic experiments we were, in fact, unable to determine whether *frzD* is even a separate complementation group, since it is dominant (1). We were able to show that *frzD* is not part of *frzE*, but we did not determine whether *frzD* is separate from *frzC* (2). Since we have identified the band at M_r 47,000 as the likely *frzC* gene product, we prepared the deletion plasmid pBB24 which removes the C-terminal portion of *frzD* but leaves the *frzC* intact (Fig. 3b). If *frzD* and *frzC* are separate genes, then the *frzC* gene product should be unaffected by this deletion. The results show that *E. coli* harboring the plasmid pBB24 did not produce the 47,000-molecular-weight *frzC* protein (Fig. 3a). Thus, *frzD* and *frzC* probably constitute a single gene, *frzCD*. The dominant phenotype of the *frzD* mutants may be

TABLE 1. β-Galactosidase expression in *M. xanthus* Tn*5-lac* strains

Tn*5-lac* insertion	*frz* complementation group	Orientation[a]	Level of β-galactosidase activity in[b]:		
			CYE	1/2 CTT	CF
1	*frzA*	←	+ +	−	−
2	*frzA*	←	+ + +	+ + + + +	+ + + + +
3	*frzA*	→	+ +	+	+
4	*frzC*	←	+ +	−	−
5	*frzC*	←	+ +	−	−
6	*frzC*	→	+ + + +	+ + + + +	+ + + + + +
7	*frzC*	→	+ + + +	+ + + + +	+ + + + + +
8	*frzE*	←	+ +	−	−
9	*frzE*	→	+ + +	+ + +	+ + + + +
10	*frzE*	←	+ +	−	−
11	*frzF*	←	+ +	−	−
12	*frzF*	→	+ + +	+ + + +	+ + + +
13	*frzF*	→	+ + +	+ + + + +	+ + + +

[a] Arrow indicates transcriptional orientation of the Tn*5-lac*: →, Tn*5-lac* is oriented as in Fig. 1c and detects transcription from left to right; ←, Tn*5-lac* is in the opposite orientation.

[b] Estimates of the level of β-galactosidase activity on plates containing X-gal (14) were obtained by visual estimation of color intensity, from no activity (−) to the maximum activity detectable (+ + + + + +), after 48 h of growth.

caused by a protein with multiple domains: An N-terminal (*frzC*) moiety which can bind to an active site and a nonfunctional, C-terminal region (frzD) which prevents normal activity from being expressed. An alternate explanation of our data, that *frzD* is a separate gene necessary for the expression of *frzC*, is not favored since fusion proteins between *frzC* and downstream regions deleted for *frzD* have been observed (see below).

(iv) *frzF* gene product. The complementation and physical mapping data showed that *frzF* is large enough to code for a polypeptide(s) with a moleuclar weight of 54,000. The plasmid pBB25 has a deletion which extends from the *Stu*I site at 5.3 kb to the *Stu*I site at 7.5 kb (Fig. 3b), thus removing *frzF* and most of the gap region. Deletion of this DNA fragment results in the loss of the bands at M_rs of 54,000 and 33,000 (Fig. 3a). In contrast, pBB26, which contains a deletion in the terminal region of the insert (7.5 to 10.0 kb) but does not remove *frzF* or the gap region (Fig. 3b), produced these polypeptides (Fig. 3a). Thus, the *frzF* gene product corresponds to the bands at an M_r of 54,000 or 33,000. However, we estimate the maximum coding capacity of the gap region to be for a polypeptide with a molecular weight of less than 50,000. We therefore suggest that the band at 54,000 is the *frzF* gene product and the band at 33,000 is a gene product from the gap region. This hypothesis has since been supported by preparing deletions which remove only the gap region (they lack the band at 33,000) and deletions which retain the gap region but not the *frzF* gene (they lack the band at 54,000 [data not shown]).

The conclusion that the band at 54,000 is the *frzF* gene product appears at first examination to be inconsistent with the labeleing experiments in Fig. 3a for pBB24. The plasmid pBB24 has a deletion in *frzF* but appears to produce a large amount of a polypeptide with a molecular weight of 54,000. We therefore reexamined the labeled extracts on different gel systems to better resolve the bands and discovered that on a 10% polyacrylamide gel, this band has, indeed, a lower mobility and is clearly different from the band at 54,000 observed with the other extracts (data not shown). We think that the band in pBB24 is a chimeric protein created by the fusion of *frzC* to an open reading frame downstream of *frzF*. This fusion would support the notion that *frzD* is not necessary for the expression of *frzC*.

(v) *frzA* and *frzB* gene products. We were unable to detect any bands in the maxicell experiments which might correspond to the *frzA* or *frzB* gene products, despite their proximity to the *trp* promoter. We prepared deletion strains which lack *frzA* and *frzB*, but they showed labeling patterns identical to that of the control strain (data not shown). The small size of *frzB* may account for our inablity to detect its product since it can code for a gene product with a molecular weight not greater than 9,200 and may be much smaller then that. The *frzA* gene product should be large enough for detection since *frzA* has a coding capacity for polypeptide(s) with molecular weights of 18,500 to 31,500. The possibility that these gene products contain no methionine residues and therefore would not be labeled with [^{35}S]methionine was tested by using a mixture of ^{14}C-labeled amino acids to label the proteins by the maxicell procedure. However, the labeling pattern obtained was identical to the one shown here for [^{35}S]methionine (data not shown).

In this paper, we have been able to identify the gene products of *frzCD*, *frzE*, and *frzF* by using the *E. coli* maxicell procedure. In each case, the complementation groups (which were originally characterized as transcriptional groups [1]) appeared to code for a single polypeptide near the maximum size predicted from the genetic data. Thus, *frzCD* code for a polypeptide with a molecular weight of 47,000, *frzE* codes for a polypeptide with a molecular weight of about 90,000, and *frzF* codes for a polypeptide with a molecular weight of about 54,000. The identification of these *frz* gene products should allow further studies on the purification and characterization of the native proteins from *M. xanthus*.

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