CHARACTERIZATION AND SEQUENCE ANALYSIS OF THE F2 PROMOTER FROM CORYNEPHAGE BFK20

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Summary. – F2 promoter from corynephage BFK20 was isolated and characterized. It was functional in *Escherichia coli* and *Corynebacterium glutamicum*. Cloning of the F2 promoter into the pJUP05 promoter probe vector caused an increase of the neomycin phosphotransferase II specific activity. According to the Northern blot hybridization the *npt*II gene was expressed from the cloned F2 promoter. The apparent transcription start point in *E. coli* and *C. glutamicum* was determined. The -35 region of F2 promoter showed high similarity to that of *E. coli* promoter consensus sequence, but its -10 region was G+C rich and had no significant homology to that.

Key words: corynebacteria; corynephage; promoter region; S1 mapping

Introduction

Bacteria of the genera *Brevibacterium* and *Corynebacterium* are widely used for industrial production of amino acids and bioconversions. Whereas their physiology is relatively well understood, especially under the conditions of amino acid production, very little information exists about the regulation of their gene expression. A thorough study of these processes requires characterization of the signals involved in transcription. To our knowledge, only a few corynebacteria promoters have been isolated so far (Yamaguchi *et al.*, 1986; Sano and Matsui, 1987; Bardonnet and Blanco , 1991). Morinaga *et al.* (1987) have shown that *E. coli* P_{trp}, P_{lac} and P_{tac} promoters are expressed in coryneform bacteria. On the other hand, it has been shown that some corynebacteria promoters do not function in *E. coli* (Cadenas *et al.*, 1991).

We have previously isolated and characterized the corynephage BFK20 which causes lysis of *Brevibacterium flavum* CCM 251 (Koptides *et al.*, 1992). Its genome consists of a linear double stranded DNA molecule of 44 – 45 kbp with cohesive ends.

In this paper we describe a detailed characterization of the F2 promoter region from the corynephage BFK20. This is the first time a DNA fragment from a corynephage exhibiting promoter activity has been isolated and characterized.

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Materials and Methods

Bacteria strains. The following strains have been used: *B. flavum* CCM 251 as a host strain for bacteriophage BFK20, *C. glutamicin* RM3 and *E. coli* HB101.

Preparation and purification of phage lysates and phage DNA were performed as previously described (Koptides *et al.*, 1992).

DNA hybridization analysis. The 0.408 kbp *Sal*I-*Eco*RI DNA fragment from plasmid pJUF2, carrying the F2 promoter, was labelled with a random primer (New England Biolabs) and [alpha-^{32}P]dCTP (3000 Ci/mmol, Amersham), and used as a probe for Southern blot hybridization. The phage BFK20 DNA was digested with restriction endonucleases and the separated fragments were transferred to a HYBOND-N filter (Amersham). Prehybridization, hybridization and stringent washing procedures were performed as described by Sambrook *et al.* (1989).

Neomycin phosphotransferase II (NPTII) specific activity was determined as previously described (Koptides *et al.*, 1992).

Total RNA was isolated from *E. coli* harboring plasmid pJUP05 or pJUF2t3 and *C. glutamicum* harboring pJUF2t3. Cells were grown to A_{570} 0.6 – 0.8, resp. Cell pellets were homogenized for 1 min (3 mins in the case of *C. glutamicum*) by vortexing with 1 g of glass beads (4 mm in diameter) in 2 ml of denaturing solution (4 mol/l guanidine thiocyanate, 25 mmol/l sodium citrate pH 7.0, 0.5% sarkosyl and 0.1 mol/l 2-mercaptoethanol). Homogenized cells were extracted once with 2 ml of phenol, 0.4 ml chloroform and 0.15 ml of 3 mol/l sodium acetate pH 4.2. The water phase was precipitated with an equal volume of isopropanol for 1 hr at -20 °C. The RNA pellet was dissolved in 0.6 ml of denaturing solution and once again precipitated as describe above. Total RNA

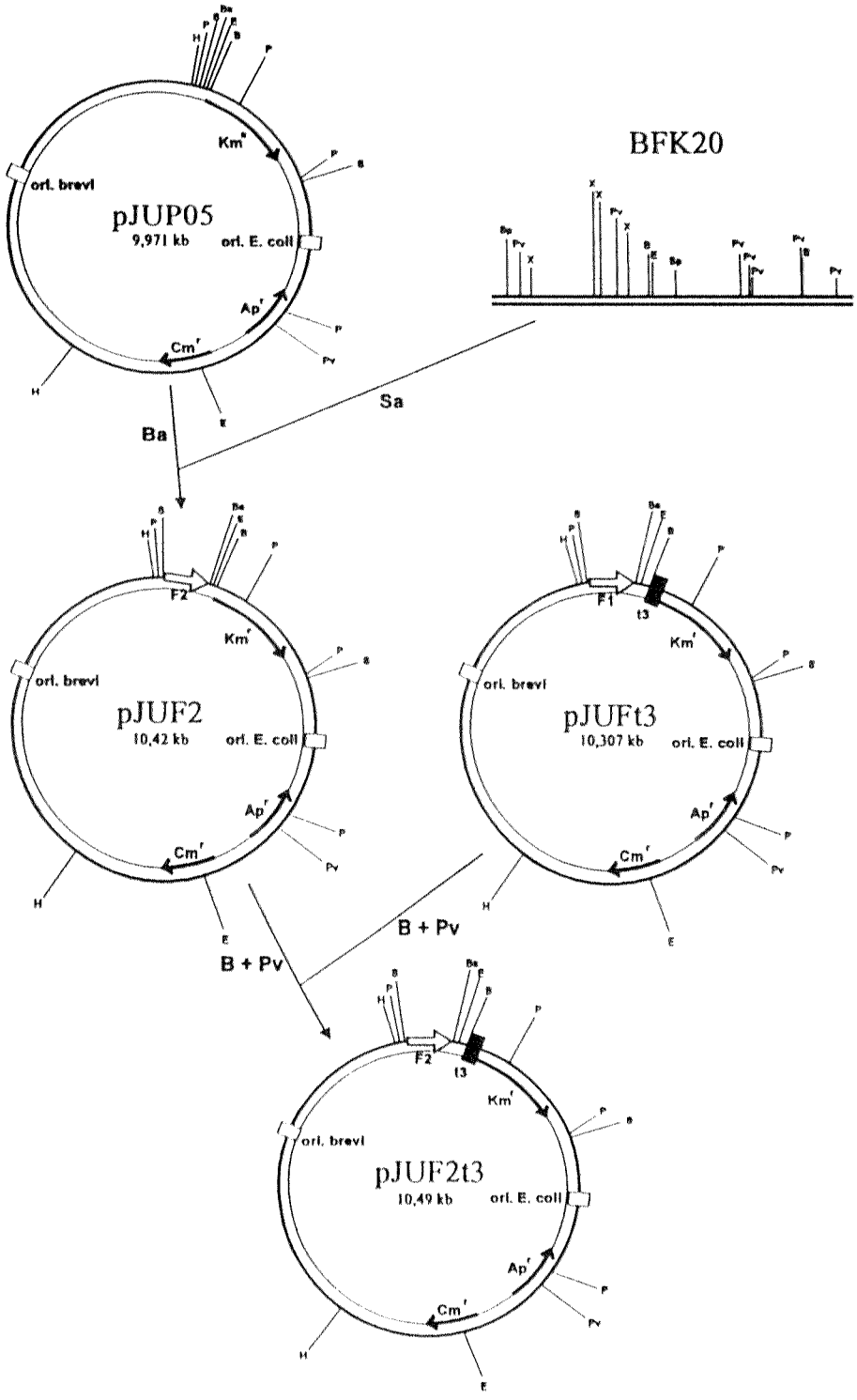

Fig. 1

Construction of the plasmids pJUF2 and pJUF2t3

Abbreviations used: H, *Hind*III; P, *Pst*I; S, *Sal*I; Ba, *Bam*HI; E, *Eco*RI; B, *Bgl*II; Pv, *Pvu*I; Sp, *Spe*I; X, *Xba*I; Sa, *Sau*3A. Km^s, sensitivity to kanamycin; Km^r, resistance to kanamycin; Cm^r, resistance to chloramphenicol; Ap^r, resistance to ampicillin.

was dissolved in 0.1 ml of diethyl pyrocarbonate (DEPC) treated water and used for S1 nuclease mapping or Northern blot analysis.

Northern blot analysis. Ten μg of total RNA isolated from *E. coli* harboring plasmid pJUP05, pJUF2 or pJUF2t3 were run on a formaldehyde agarose gel as described by Sambrook *et al.* (1989). After electrophoresis the RNA was transferred to a HYBOND-N filter. The blot was hybridized at 42 °C for 16 hrs in a hybridization solution (Sambrook *et al.*, 1989). The volume of hybridization solution was 0.1 ml/cm^2 of the blot. The 0.7 kbp *Pst*I fragment from pJUF2 (Fig. 1) carrying 0.19 kbp of the *npt*II gene was labelled with a random primer (New England Biolabs) and [alpha-^{32}P]dCTP (3000 Ci/mmol, Amersham). This probe, with a specific activity of approx. 10^9 cpm/μg, was used directly for hybridization. The filter was washed as described (Sambrook *et al.*, 1989).

S1 nuclease mapping of the transcription start point and sequencing. Determination of the 5' transcription start point (*tsp*) was performed as described by Farkašovský *et al.* (1990) with some modifications.

The plasmid pJUF2 was BamHI digested, dephosphorylated, 5'end-labelled with T4 polynucleotide kinase and [gamma-^{32}P]ATP (3000 Ci/mmol), and *Sal*I-digested. The resulting 0.462 kbp labelled *Sal*I-*Bam*HI fragment carrying the F2 promoter (Fig. 1) was gel purified. Fifty μg of total RNA isolated from *E.coli* HB101 or *C. glutamicum* (see Total RNA isolation) was added to 10 ng of the labelled fragment (approx. 60000 cpm) and the mixture was ethanol precipitated. The pellet was dissolved in 20 μl of DEPC-treated water, lyophilized and dissolved in 10 μl of hybridization buffer (3 mol/l Sodium trichloroacetate, 50 mmol/l PIPES pH 7.0, 5 mmol/l EDTA). After heating to 60 °C for 5 mins, samples were incubated for 4 hrs at 40 °C and further treated with 100 units of S1 nuclease. Protected DNA fragments were analyzed on DNA sequencing gels. Sequencing was performed as described by Maxam and Gilbert (1980).

Results and Discussion

Isolation of the F2 promoter from BFK20 DNA

The BFK20 DNA was *Sau*3A digested and the resulting fragments were cloned into the *Bam*HI linearized pJUP05 promoter probe vector (Barák *et al.*, 1990) (Fig. 1). Transformants were selected on LB plates supplemented with chloramphenicol (Cm) (10 μg/ml). From positive clones which grew on LB plates supplemented with kanamycin (Km) (100 μg/ml) we isolated plasmid pJUF2. Restriction analysis showed that pJUF2 carries a 0.445 kbp *Sau*3A fragment from BFK20 DNA. The minimal inhibition concentration (MIC) of Km for clone *E. coli* [pJUF2] was 600 μg/ml, whereas for *E. coli* [pJUP05] the MIC was 20 μg/ml. The specific activity of NPTII in *E. coli* [pJUF2] was 45-fold higher than that in *E. coli* [pJUP05] (Table 1).

The increased levels of NPTII activity in *E. coli* [pJUF2] was probably a result of more efficient transcription of the *npt*II gene. Northern blot hybridization (Fig. 2) revealed a stronger band, corresponding to *npt*II reporter gene mRNA in the pJUF2 sample (lane 2), as compared to pJUP05 (lane 1). The F2 promoter was localized on the BFK20 restriction map by Southern blot hybridization (Fig. 3).

Table 1. NPTII production in E. coli and C. glutamicum

Plasmid	NPTII specific activity (pmol/min/mg protein)	
	E. coli	C. glutamicum[a]
none	0	0
pJUP05	2	0
pJUF2	90	–
pJUF2t3	12	4
pJUF2D	59	–

[a]No clones harboring pJUF2 plasmid were detected.

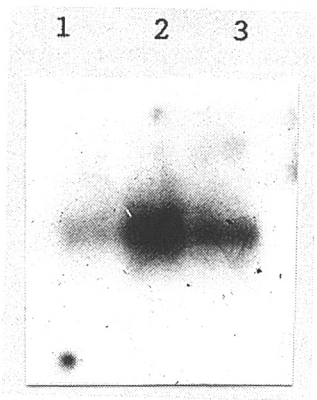

Fig 2
Northern blot hybridization of the 0.7 kbp PstI fragment from plasmid pJUF2 with total RNA isolated from E. coli HB101 harboring plasmid pJUP05 (lane 1), pJUF2 (lane 2), and pJUF2t3 (lane 3)

Construction of the plasmid pJUF2t3. Introduction of the F2 promoter into C. glutamicum

C. glutamicum cells were transformed with plasmid pJUF2. We isolated only deleted derivatives from the transformed clones. From this observation we conclude that the F2 promoter is too strong in *C. glutamicum* and therefore causes plasmid instability in this strain. As we have shown previously, transformation of *C. glutamicum* with pJUF1 plasmid was possible only when we introduced the t3 transcription terminator downstream from the strong F1 promoter (Koptides *et al.*, 1992).

In order to introduce the F2 promoter into the *C. glutamicum* cells, we inserted this transcription terminator downstream from the F2 promoter (Fig. 1). The 7.312 kbp

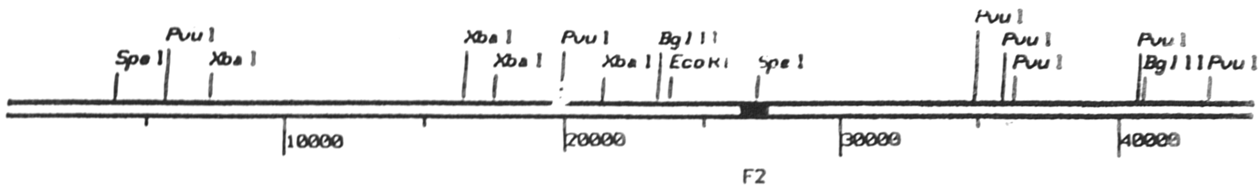

Fig. 3
Localization of the F2 promoter on the BFK20 DNA restriction map
Southern blot hybridization was performed according to Sambrook *et al.* (1989). The *Sal*I-*Bgl*II 0.487 kbp fragment from plasmid pJUF2, carrying the F2 promoter was used as a probe. F2 black box: F2 promoter.

*Bgl*II-*Pvu*I fragment from pJUF2 carrying the F2 promoter was ligated with the 3.23 kbp *Bgl*II-*Pvu*I fragment from the pJUFt3 plasmid (Koptides *et al.*, 1992) carrying the t3 transcription terminator (Fig. 1). The resulting plasmid pJUF2t3 was used to transform *E. coli* and *C. glutamicum* cells. This plasmid was stable in these microorganisms in spite of the presence of the pJUF2 sequences. Therefore, we assume that the F2 promoter was efficiently expressed in *E. coli* and corynebacteria.

Presence of the inserted transcription terminator between the F2 promoter and the *npt*II gene caused a decrease of both *npt*II mRNA transcription (Fig. 2) and NPTII specific activity in *E. coli* cells (Table 1). The specific activity of the enzyme in *E. coli* [pJUF2t3] was 7.5-fold lower than that in *E. coli* [pJUF2]. Our measurements showed that the specific activity of NPTII in C. *glutamicum* was three times lower than that in *E. coli* (Table 1). This result is in agreement with data of Ozaki *et al.* (1984) showing that the level of enzymes conferring antibiotic resistance in *C. glutamicum* is four to ten times lower than that in *E. coli*.

Nucleotide analysis of the F2 promoter

The nucleotide sequence of the 0.445 kbp DNA fragment carrying the F2 promoter was determined (Fig. 4). Two *Xho*I sites were identified in this sequence. Deletion of the 0.198 kbp *Xho*I fragment caused a 1.5-fold decrease in

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  1 GGATCAACAAGGTTTTGTCTATAAGAACGTCGTGTTCTCTACGTTCGTGTCCGAGGTGAC
                                                         XhoI
 61 GCTCACGTGGACGGTCGATAAAGGGTGGCGTGAGGCTATTGTCCTGGGCGACCCTCGAGC
                                   SpeI
121 CCGTGAGTCACGTATCGAAAACCTCAAGCGCACTAGTCAGTCCATTAAAGAAGCGATAGA

181 CCGCGTGAAAAGCACAGTACTCTAGGAGGTTGACCATGACCGATATTTACCCGTATCCCG

241 TCGATAACCAAGACGACGTAGACCCTATGGCGTGGCTATTTTTCAACACACCCGGCGCGC
               XhoI
301 CTAAAGACACGCTCGAGGTTGCTAACGCTCGGTGCGCTCACCTACGCGCGCTAGGTGTTG
                                       ATbox   -35
361 ACCTCACTCCTGAGGTCACCGAGCCGTCAACGCTAAAAT [ATGACG] CGCTCGGTGGTACGG
                                             ------->         <---
       -10          +1         BamHI
421 GCG [CGCCGT] GGGGAACACGGCCGGTGGATCC
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Fig. 4
Nucleotide sequence of the F2 promoter region
The promoter consensus elements are marked -35 and -10. The apparent transcription start point (nt +1) is marked by an asterisk. Convergent arrows indicate the inverted repeats. The A+T rich region is marked as ATbox. The GenBank accession No. is L13364.

Table 2. Comparison of the F2 promoter with some corynebacteria promoters

Promoter	–35[a]	–10	Source	Reference
F2	ATGACG	CCGCGT	BFK20	this work
P_{trp}	tacACa	aataaT	*B. lactofermentum*	Sano & Matsui (1987)
P1	tTGcgc	ttaaGa	pAM330	Yamaguchi *et al.* (1986)
P5	tTcACc	ttaata	pAM330	Yamaguchi *et al.* (1986)
P_{thr}	AaagCa	tataGT	*C. glutamicum*	Peoples *et al.* (1988)

[a]Capital letters refer to identical nucleotides.

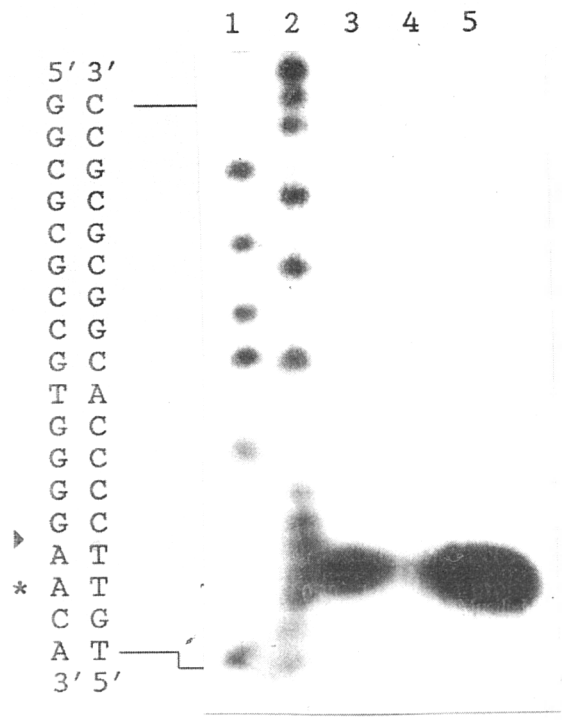

Fig. 5
Nuclease S1 mapping of the apparent tsp in the F2 promoter region
G+A sequence (lane 1); T+C sequence (lane 2); S1 protection experiment with total RNA isolated from *E. coli* [pJUF2t3] (lane 3), *E. coli* [pJUP05] (lane 4) and *C. glutamicum* [pJUF2t3] (lane 5). The arrow indicates the position of the DNA protected fragment and the asterisk indicates the apparent tsp after substraction of 1.5 nucleotide. Differences between the sequence (Fig. 4) and the ladder in this figure is caused by a compression.

NPTII specific activity (Table 1) as compared to the control [pJUF2]. This plasmid with the deletion was designated pJUF2D.

The promoter region is probably located on the 0.14 kbp *Xho*I-*Bam*HI fragment (Fig. 4). The decrease in NPTII activity could be caused by the deletion of a sequence influencing the promoter strength.

In order to determine the apparent *tsp* and thus precisely localize the -10 and -35 regions of the F2 promoter, the S1 mapping procedure was performed.

We found that in both *E. coli* and *C. glutamicum* RNA transcription starts at dA 0.435 kbp downstream from the 5' end of the cloned fragment (Fig. 5). The designated -35 region exhibited strong similarity with that of the *E. coli* consensus sequence. Conversely, the -10 region was G+C rich. Only the dT residue was conserved out of the CGCCGT hexamer. It has been suggested that dT plays an important role in RNA polymerase(RNAP)-promoter interaction (Rosenberg and Court, 1979). An inverted repeat sequence was found between the -35 and -10 region of the F2 promoter. Horwitz and Loeb (1990) proposed that such sequences could be involved in the regulation of RNA transcription. Examination of this possibility in the case of the F2 promoter is now under investigation. Upstream from the -35 region an A+T rich sequence (AT box) was identified. Nishi and Itoh (1986) found that A+T rich sequences accompany strong promoters and that such regions affect the promoter strength. We suggest that these AT boxes could bend the DNA so that the RNAP can more efficiently bind to the DNA molecule (Horwitz and Loeb, 1990).

The F2 promoter is compared with some corynebacteria promoters in Table 2.

According to our observations the F2 promoter is recognized by both *E. coli* and corynebacteria RNAP. On the other hand, this promoter seems to be stronger in corynebacteria than in *E. coli*, since the pJUF2 plasmid shows structural instability in *C. glutamicum*. This could be a result of structural differences between the RNAPs of corynebacteria and *E. coli*.

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