Transfer and Expression of an *Erwinia chrysanthemi* Cellulase Gene in *Zymomonas mobilis*

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The cellulase gene from *Erwinia chrysanthemi* coding for endoglucanase Z was subcloned into a broad-host-range plasmid pGSS33 in *Escherichia coli*. The recombinant pNB20 was transferred into *Zymomonas mobilis* ATCC 10988 by mobilization using the helper plasmid RP4. Plasmid pNB20 was stably maintained in *E. coli* and *Z. mobilis* hosts. The endoglucanase gene *celZ* was expressed efficiently and the level of expression was higher in *Z. mobilis* than in *E. coli*. The specific activity of the enzyme was comparable to that of the parent strain of *Er. chrysanthemi*. The proteins produced by *Z. mobilis* and *Er. chrysanthemi* presented identical immunological and electrophoretic properties. Biosynthesis of endoglucanase occurred during the exponential growth phase of *Z. mobilis* and about 35% of the enzyme was released into the medium in the absence of detectable cell lysis. The endoglucanase appeared to be located in the periplasmic space in *Z. mobilis*.

INTRODUCTION

Zymomonas mobilis is a Gram-negative bacterium which presents high potential for ethanol production, showing higher ethanol yield and productivity than yeasts. However, this bacterium has a limited range of utilizable substrates since it can only ferment glucose, fructose or sucrose. Recombinant DNA techniques can be used to transfer the genes coding for hydrolysing enzymes such as β -galactosidase, α -amylase or cellulases into *Z. mobilis*. Assuming expression of these hydrolase genes in *Z. mobilis* and secretion of the enzymes into the culture medium, it may be expected that recombinant *Z. mobilis* strains would directly ferment starch or cellulose into ethanol.

There are only few reports of heterologous gene expression in *Z. mobilis*. Resistances to the antibiotics chloramphenicol and tetracycline were expressed and used as selectable markers in the bacterium (Afendra & Drainas, 1987; Brestic-Goachet *et al.*, 1987; Conway *et al.*, 1987*a*). The β -galactosidase gene (*lacZ*), transferred using transposon Tn951, was poorly expressed in *Z. mobilis* even in the presence of inducer (Carey *et al.*, 1983). In contrast, high expression was obtained using gene fusions between *lacZ* and DNA fragments containing promoters from *Z. mobilis* as demonstrated by β -galactosidase activities (Byun *et al.*, 1986; Yanase *et al.*, 1986). In this study we report the transfer and expression of an *Erwinia chrysanthemi* cellulase gene (*celZ*) in *Z. mobilis*. This gene was selected for two main reasons: (a) its product, endoglucanase Z, accounts for almost all (96–97%) the endoglucanase activity of *Er. chrysanthemi*; (b) the enzyme is secreted into the culture medium during the late exponential growth phase (Boyer *et al.*, 1984*a*).

Other preliminary reports on expression of cellulase genes in *Z. mobilis* have recently been published (Yoon *et al.*, 1988; Lejeune *et al.*, 1988).

Abbreviation: CMCase, carboxymethylcellulase.

Table 1. *Plasmids and strains used*

Organism	Relevant genotype and description*	Reference
<i>Z. mobilis</i>		
ZM1	Wild-type	ATCC 10988
ZM1-3	A spontaneous mutant derived from ATCC 10988 resistant to 50 µg rifampicin ml ⁻¹	This paper
<i>E. coli</i>		
HB101	<i>recA13 hsr hsm rpsL20</i> (Sm ^r)	Boyer <i>et al.</i> (1969)
RR1ΔM15	<i>hsr hsm lacZΔM15, F'lacI^a lac ΔM15 proAB</i>	Ulrich (1982)
Plasmids		
pGSS33	Ap ^r Cm ^r Sm ^r Tc ^r ; broad-host-range replicon <i>mob</i> site	Sharpe (1984)
RP4	Ap ^r Km ^r Tc ^r ; mobilizing plasmid	Datta <i>et al.</i> (1971)
pUC18	Ap ^r	Norrandar <i>et al.</i> (1983)
pMH13	Ap ^r <i>celZ</i> ⁺ derivative of pUC18	Boyer <i>et al.</i> (1987)
pMH14	Ap ^r <i>celZ</i> ⁺ deletion derivative of pMH13	Boyer <i>et al.</i> (1987)
pNB20	Ap ^r Cm ^r Sm ^r <i>celZ</i> ⁺ broad-host-range replicon <i>mob</i> site	This paper

* Abbreviations: Sm, streptomycin; Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; *celZ*, endoglucanase gene from *E. chrysanthemi* strain 3665.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are summarized in Table 1. *Z. mobilis* strains were grown at 30 °C without agitation in a complex medium (Skotnicki *et al.*, 1981) containing 20 g glucose l⁻¹ unless otherwise stated. *Escherichia coli* strains were grown aerobically at 37 °C in Luria broth (Luria & Delbruck, 1943). Solid complex medium containing 10 g carboxymethylcellulose and 10 g fructose l⁻¹ was used to screen colonies of *Z. mobilis* for carboxymethylcellulase (CMCase) activity. Solid minimal medium M9 (Miller, 1972) supplemented with 10 g carboxymethylcellulose, 2 g glycerol and 0.2 g yeast extract l⁻¹ was used to screen *E. coli* colonies. Antibiotics were added at the following concentrations: for *E. coli*, ampicillin (50 µg ml⁻¹), tetracycline (25 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹), kanamycin (25 µg ml⁻¹); and for *Z. mobilis*, rifampicin (50 µg ml⁻¹) and chloramphenicol (100 µg ml⁻¹).

Preparation of plasmid DNA and recombinant DNA techniques. Plasmid DNA was prepared from *E. coli* and *Z. mobilis* strains by the alkaline lysis procedure of Birnboim & Doly (1979) and purified, when necessary, by centrifugation on caesium chloride/ethidium bromide gradients (Maniatis *et al.*, 1982). Restriction endonucleases and T4 DNA ligase were used according to the manufacturer's instructions (Bethesda Research Laboratories). Restriction fragments were analysed by agarose gel electrophoresis (Maniatis *et al.*, 1982).

Conjugation and transformation. *E. coli* was transformed using the calcium chloride procedure described by Maniatis *et al.* (1982). Plasmid pNB20 was transferred from *E. coli* into *Z. mobilis* ZM1-3 by conjugation using the filter mating technique as previously described (Brestic-Goachet *et al.*, 1987). Plasmid RP4 was used as mobilizing plasmid (Datta *et al.*, 1971). *Z. mobilis* transconjugants were isolated on complex media containing chloramphenicol to select the strains carrying the recombinant plasmid and rifampicin to inhibit the growth of *E. coli*.

Enzyme assays. CMCase activity on plates was detected using the Congo red technique: the dye forms a deep pink complex with the β-glucan substrate (carboxymethylcellulose) leaving clear haloes around active colonies (Teather & Wood, 1982). Endoglucanase activity was measured as described by Boyer *et al.* (1948b) using carboxymethylcellulose (C-4888 from Sigma) as substrate. Reducing sugars were estimated by the ferricyanide method (Park & Johnson, 1949). One unit of enzyme activity (IU) corresponds to 1 µmol glucose equivalent liberated min⁻¹ from carboxymethylcellulose at 37 °C. Alcohol dehydrogenase activity was determined by measuring the ethanol-dependent reduction of NAD⁺. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 µmol NAD⁺ min⁻¹ at 25 °C. Cell protein concentration was measured by the Lowry method with bovine serum albumin as standard. For measurements of intracellular enzyme activities, the cells were disrupted by sonication using a Branson sonifier (three cycles at 40 W for 30 s each). The extracellular enzyme activities were assayed in the culture medium which had been dialysed against 0.01 M-potassium phosphate buffer pH 7.0. For alcohol dehydrogenase activity, the culture medium was concentrated 50-fold by ultrafiltration using an Amicon cell with a PM 10 membrane.

Localization of endoglucanase activity. The release of periplasmic proteins was achieved by two procedures. In method A, spheroplasts were formed by the action of glycine as described by Yanase *et al.* (1985) except that sorbitol was used instead of sucrose. The spheroplasts were harvested by centrifugation (5000 g, 15 min, 4 °C), suspended in 0.01 M-potassium phosphate buffer pH 7.0 and disrupted by sonication (as described above). Enzyme activities were assayed in the culture medium (extracellular or periplasmic components) and in the

spheroplast pellet after sonication (soluble cytoplasmic or membrane components). In method B, shock fluids were obtained by the sucrose/EDTA method of Willis *et al.* (1974). Enzyme activities were assayed in the soluble fraction of the shocked cells (periplasm), in the cell pellet (after sonication) and in the extracellular medium.

Electrophoresis. Electrophoresis in the presence of sodium dodecyl sulphate (SDS) was carried out in 8% (w/v) acrylamide gels according to the method of Laemmli (1970). Proteins were visualized by Coomassie blue staining.

Immunological techniques. A rabbit antiserum raised against the purified endoglucanase Z from *Er. chrysanthemi* 3665 was kindly provided by Dr J. Cattaneo. Immunodiffusion tests were performed according to Ouchterlony (1958). The immunoblot analysis after SDS-PAGE was carried out as described by Towbin *et al.* (1979). The second antibody was a goat anti-rabbit immunoglobulin G coupled to peroxidase (Biosys, Compiègne, France) and was revealed by the H₂O₂-diaminobenzidine technique (Diano *et al.*, 1987).

RESULTS

Subcloning of the celZ gene

The major extracellular CMCase of *Er. chrysanthemi* strain 3665 has been characterized as an endo- β -1,4-glucanase and is called endoglucanase Z (Boyer *et al.*, 1984 *a, b*). Its molecular mass is 45 kDa. This type of enzyme is thought to perform the initial attack on amorphous cellulose and appears to be the predominant type of cellulolytic enzyme in bacteria. The structural gene coding for endoglucanase Z has been cloned and expressed in *E. coli* RR1 Δ M15 (Boyer *et al.*, 1987). A 14.5 kb fragment was integrated into the pUC18 vector, generating the plasmid pMH13. Subsequent deletion of a 6 kb *Sph*I fragment did not abolish CMCase activity and in the resulting plasmid pMH14 (Fig. 1), the *celZ* gene was expressed from its own promoter (Boyer *et al.*, 1987).

Plasmid pGSS33 is a broad-host-range, mobilizable plasmid containing the replicon of plasmid RSF1010. We have previously shown that pGSS33 can be easily transferred from an *E. coli* donor strain into *Z. mobilis* after mobilization by the helper plasmid RP4 (Brestic-Goachet *et al.*, 1987). Plasmid pGSS33 was stably maintained in *Z. mobilis* as assayed by resistance to chloramphenicol and tetracycline.

The 6.6 kb *Bgl*II-*Hind*III fragment from pMH14, carrying the *celZ* gene, was ligated to pGSS33 opened at unique *Bam*HI and *Hind*III sites (Fig. 1). The recombinant plasmid pNB20 was isolated from chloramphenicol- and ampicillin-resistant, tetracycline-sensitive *E. coli* HB101 transformants which showed CMCase activity when screened using the Congo red dye staining method. Restriction endonuclease analysis (not shown) of pNB20 by *Pvu*II revealed four fragments of 6.0, 5.0, 4.3 and 4.1 kb.

Transfer of the celZ gene into Z. mobilis

Plasmid pNB20 was transferred from *E. coli* HB101(pNB20) into *Z. mobilis* ZM1-3 by conjugation using the filter mating technique and plasmid RP4 as helper (Brestic-Goachet *et al.*, 1987). Transconjugants were screened for their resistance to chloramphenicol since *Z. mobilis* exhibits natural resistance to streptomycin and ampicillin. Transconjugants were obtained at a frequency of 10⁻⁴ per recipient cell. In our conditions we were not able to obtain transconjugants using *Z. mobilis* ZM4-1 (a spontaneous rifampicin-resistant mutant of strain ZM4). This strain (ATCC 31821) is more efficient than strain ZM1 for ethanol production and is widely used for fermentation studies (Rogers *et al.*, 1982). One hundred *Z. mobilis* ZM1-3 transconjugants were randomly selected and screened for CMCase activity using the Congo red dye staining method. All of them showed a clear halo around colonies, indicating the presence of endoglucanase activity. The integrity and the function of plasmid pNB20 obtained from a *Z. mobilis* transconjugant were determined by restriction analysis and further confirmed by back-transfer to *E. coli* HB101. All chloramphenicol-resistant *E. coli* transformants tested (more than 100) were CMCase positive. The restriction analysis patterns of the plasmid from the *E. coli* transformants were identical to that of the original pNB20 plasmid.

Stability of plasmid pNB20 in Z. mobilis

One of the transconjugant ZM1-3(pNB20) strains was cultured for 55 generations in a medium without antibiotic (chloramphenicol) in conditions previously described (Brestic-

Fig. 1. Construction of plasmid pNB20. The stippled lines represent *Er. chrysanthemi* DNA; the arrows indicate the direction of transcription of the *celZ* gene. Sites for restriction endonucleases are: *AccI* (Ac), *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hind*III (H), *Pst*I (P), *Pvu*I (PvI), *Pvu*II (PvII).

Goachet *et al.*, 1987). One hundred randomly selected colonies were screened for chloramphenicol resistance and CMCase activity. Eighty-three clones were CMCase-positive and all of these were chloramphenicol-resistant. Therefore, plasmid pNB20 was reasonably stable in *Z. mobilis* in the absence of selective pressure.

Expression of the celZ gene in Z. mobilis

In the parent strain of *Er. chrysanthemi*, the endoglucanase Z activity is found in the culture medium. The biosynthesis of the enzyme seems to be constitutive and subject to catabolite repression (Boyer *et al.*, 1984a). In cultures of this strain in both mineral and rich media the activity of the enzyme was highest at the end of the exponential phase, reaching 1.6 IU ml⁻¹ [specific activity 3–4 IU (mg cellular protein)⁻¹]. The kinetics of growth and endoglucanase production was studied in the recombinant strain *Z. mobilis* ZM1-3 (pNB20) in a rich medium containing a low fructose concentration (10 g l⁻¹) to minimize catabolite repression by the carbon source. On this medium, growth of the recombinant strain was similar to that of the wild-type ZM1-3, with a specific growth rate of 0.22 h⁻¹ (Fig. 2). Endoglucanase Z was produced during the exponential growth phase up to a maximum of 1 IU ml⁻¹. This maximal value was stably maintained for at least 14 h. The activity was stable in the culture medium of *Z. mobilis*

Fig. 2. Growth and kinetics of endoglucanase production by *Z. mobilis* ZM1-3(pNB20). Cells were grown at 30 °C on a complex medium containing 10 g fructose l⁻¹ as carbon source and 100 µg chloramphenicol ml⁻¹. (a) Total specific activity expressed as units (mg cellular protein)⁻¹ (▲). (b) Growth (OD₆₀₀) (○), and total activity (□), cellular activity (◆) and extracellular activity (●) of endoglucanase. The kinetics were done three times and mean values are given for each parameter.

while it rapidly decreased in the case of *Er. chrysanthemi* (Boyer *et al.*, 1984a). In *Z. mobilis* ZM1-3(pNB20), the specific activity of endoglucanase Z was constant during the whole growth phase (Fig. 2a), which strongly suggests a lack of catabolite repression in these conditions. This result was further confirmed by an experiment run at a fructose concentration of 20 g l⁻¹. A similar specific activity was observed at this concentration (3.9 compared to 4.2 IU mg⁻¹). On glucose or sucrose media the enzyme level was in the same range, with specific activities of 3.8 and 3.9 IU mg⁻¹ respectively. It was consequently concluded that in ZM1-3(pNB20) the biosynthesis of endoglucanase Z was not regulated by catabolite repression of the carbon source in the conditions tested. The biosynthesis of the enzyme was not affected in cells grown on media containing different carbon sources.

The *celZ* gene in *Z. mobilis* was expressed efficiently, as the specific activity of the enzyme was fivefold higher than in *E. coli* HB101(pNB20) (0.8 IU mg⁻¹) and in the same range as that of *Er. chrysanthemi* (3–4 IU mg⁻¹). Thus the recombinant *Z. mobilis* strain showed the same potential for cellulose degradation as *Er. chrysanthemi*. However, the cellular localization of the endoglucanase was different in the two organisms: all the activity was found in the culture medium in *Er. chrysanthemi* (Boyer *et al.*, 1984a), whereas in *Z. mobilis* ZM1-3(pNB20) the activity was cell-associated during the exponential growth phase and some activity was seen in the culture medium during the stationary phase. After 28 h of culture about 40% of the total activity was recovered from the culture medium. The amount of extracellular enzyme increased from 0.12 IU ml⁻¹ (13 h of culture) to 0.40 IU ml⁻¹ (28 h of culture) with a parallel decrease in the intracellular activity (from 0.83 to 0.60 IU ml⁻¹) (Table 2). During this period (13–28 h) the total activity remained constant (Fig. 2), consistent with the protein being released from the cell into the culture medium. This process was not due to cell lysis, at least up to 20 h of growth, as demonstrated by the assay of alcohol dehydrogenase activity in the medium. For instance only 0.2% of the activity of this cytoplasmic protein was extracellular for a 20 h old culture while the value was 32% for the endoglucanase Z (Table 2).

Table 2. Activity of exocellular endoglucanase Z of *Z. mobilis* ZM1-3(pNB20)

The values are means of three separate experiments.

Time of growth (h)	Endoglucanase activity		Alcohol dehydrogenase activity	
	Total* (IU ml ⁻¹)	Extracellular (%)	Total* (IU ml ⁻¹)	Extracellular (%)
10	0.57	10	0.04	<0.2
13	0.95	13	0.14	0.4
16.5	0.98	22	0.28	0.4
20	0.99	32	0.82	0.2
27.5	1.00	40	1.02	4

* Cell-bound and extracellular activity.

Characterization of the endoglucanase produced by recombinant *Z. mobilis* ZM1-3(pNB20)

The endoglucanase Z isolated from the parent *Er. chrysanthemi* strain 3665 or from *E. coli* RR1ΔM15(pMH14) showed identical physico-chemical and immunological properties (Boyer *et al.*, 1987). We compared the enzyme produced by the recombinant *Z. mobilis* ZM1-3(pNB20) with that of *E. coli* RR1ΔM15(pMH14) by immunological techniques using a rabbit antiserum raised against the purified enzyme from *Er. chrysanthemi*. For this purpose, cell extracts and concentrated culture media were prepared from cultures of *Z. mobilis* ZM1-3(pNB20) and ZM1-3 (as a negative control).

The Ouchterlony double immunodiffusion test showed complete homology between the enzymes from *E. coli* and *Z. mobilis* (Fig. 3). In addition, the intra- and extracellular proteins of *Z. mobilis* ZM1-3(pNB20) were identical. To confirm this immunological identity, the *Z. mobilis* enzymes were characterized further by immunoblotting. The crude protein fractions were separated by SDS-PAGE, transferred to nitrocellulose paper and treated with the antiserum against the purified endoglucanase. *E. coli* RR1ΔM15(pMH14) and *Z. mobilis* ZM1-3(pNB20) both showed a single band of molecular mass 45 kDa at the same level as that of the purified enzyme from *Er. chrysanthemi* (Fig. 4). In addition, the intra- and extracellular enzymes of *Z. mobilis* ZM1-3(pNB20) showed identical electrophoretic migration.

Localization of the endoglucanase Z produced by *Z. mobilis*

The cellular location of endoglucanase Z was studied with exponentially growing cells (OD₆₀₀ = 1.0) of the recombinant strain ZM1-3(pNB20). Spheroplasts were obtained using glycine (see Methods) and harvested by centrifugation. Most of the enzyme activity (89%) was recovered in the supernatant and only 11% was associated with spheroplasts (Table 3). In the same conditions, 93% of alcohol dehydrogenase activity, a cytoplasmic marker, was associated with the spheroplast fraction indicating that spheroplast integrity was maintained. From this experiment a periplasmic location was deduced for endoglucanase Z.

This location was confirmed by an osmotic shock treatment of growing cells (OD₆₀₀ = 1.0). At this growth stage 10% of the enzyme was already present in the culture medium (Fig. 2, Table 3). Cells were separated by centrifugation and subjected to osmotic shock in the conditions described by Willis *et al.* (1974) for *E. coli*. As much as 80% of the cell-associated activity was recovered from the supernatant after centrifugation of the shocked cells. In the same conditions only 2% of the alcohol dehydrogenase activity was released into the supernatant. Therefore, it is concluded that the endoglucanase activity was released by the osmotic shock in *Z. mobilis* as observed for periplasmic enzymes of *E. coli* (Neu & Heppel, 1965; Nossal & Heppel, 1966). Unfortunately, no periplasmic marker is described in *Z. mobilis* to further confirm the periplasmic location of endoglucanase Z.

When osmotic shock was applied to growing cells of *E. coli* HB101(pNB20) (OD₆₀₀ = 1.0), more than 94% of the endoglucanase activity was released from the cells. No activity was present in the culture medium. It was concluded that, as in *Z. mobilis* ZM1-3(pNB20), in *E. coli* HB101(pNB20) the endoglucanase Z was located in the periplasm.

Fig. 3

Fig. 4

Fig. 3. Double immunodiffusion test of the endoglucanase produced by *E. coli* RR1ΔM15(pMH14) and *Z. mobilis* ZM1-3(pNB20). The centre well contained 10 μl of antiserum against purified endoglucanase Z from *Er. chrysanthemi* 3665. The sample wells contained 10 μl of concentrated cell extract from RR1ΔM15(pMH14) (A), ZM1-3(pNB20) (B) and ZM1-3 control (F), or 10 μl of concentrated supernatant from ZM1-3(pNB20) (C) and ZM1-3 control (E).

Fig. 4. Immunoblot analysis of endoglucanase in culture supernatants or cell extracts of *Z. mobilis* ZM1-3(pNB20) and *E. coli* RR1ΔM15(pMH14). Samples (50 μg) of concentrated culture supernatants or cell extracts were subjected to SDS-PAGE (8%) analysis and transferred to nitrocellulose. Antibodies against purified endoglucanase Z from *Er. chrysanthemi* 3665 were used. Lanes: 1, RR1ΔM15 cell extract; 2, RR1ΔM15(pMH14) cell extract; 4, ZM1-3(pNB20) cell extract; 5, ZM1-3 cell extract; 6, culture supernatant from ZM1-3(pNB20). Molecular mass markers stained with Ponceau red are shown in lane 3: phosphorylase *b* (94 kDa), bovine serum albumin (63 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa). The arrow indicates the position of the protein (45 kDa) reacting with anti-endoglucanase Z serum.

Table 3. Distribution of endoglucanase activity in exponentially growing cells of *Z. mobilis* ZM1-3(pNB20)

Preparation procedure*	Fraction	Percentage of total enzyme activity in fraction†	
		Endoglucanase	Alcohol dehydrogenase
A	Spheroplasts	10.9	92.5
	Culture medium	89.1	7.5
B	Culture medium	10.2	<0.2
	Supernatant of shocked cells	80.4	2.5
	Pellet of shocked cells	9.4	97.5

* In method A (spheroplast formation), the enzyme activities were measured in the sonicated spheroplasts and in the culture medium; in method B (osmotic shock), the enzyme activities were measured in the culture medium and in the supernatant and pellet (after sonication) of shocked cells. See Methods for details.

† The experiment was done twice and mean values are given.

DISCUSSION

In previous work from our laboratory (Brestic-Goachet *et al.*, 1987), derivatives of the broad-host-range plasmid RSF1010 were used for transfer of heterologous genes into *Z. mobilis*. In this study, plasmid pGSS33 was used to transfer an endoglucanase gene of a Gram-negative bacterium, *Er. chrysanthemi*, into *Z. mobilis*. In separate work, plasmid pKT210 allowed the transfer of an α -amylase gene from a Gram-positive bacterium (unpublished results). These two successful gene transfers clearly demonstrate the usefulness of mobilizable plasmids derived from RSF1010 for cloning in *Z. mobilis*. In both cases, plasmid RP4 was used as helper.

The transfer frequency was 10^{-4} per recipient cell, a value 20-fold higher than that reported (Brestic-Goachet *et al.*, 1987) for pGSS33 in *Z. mobilis* ZM4 (ATCC 31821). Thus the efficiency of transfer by conjugation seems to be strain-dependent, *Z. mobilis* ZM1 (ATCC 10988) being the best recipient. Our results are in agreement with those of Afendra & Drainas (1987), who reported the same transfer frequency (10^{-4} per recipient cell) for a shuttle vector carrying an origin of replication of a *Z. mobilis* plasmid. Conway *et al.* (1987a) also reported a transfer frequency of 10^{-4} for a plasmid, pLOI197, derived from RSF1010, into *Z. mobilis* CP4. The same authors succeeded in increasing the transfer frequency to 10^{-2} by including a *mob* site from RP4 in their vector, in addition to the original RSF1010 *mob* site.

Plasmid pNB20 was stably maintained in *Z. mobilis*, as was the original vector. Thus the introduction of the *celZ* gene in plasmid pGSS33 had no great influence on plasmid stability.

The specific activity of endoglucanase from *Z. mobilis* ZM1-3(pNB20) was about the same as that observed in *Er. chrysanthemi* and much higher than in *E. coli* HB101(pNB20). Although the gene copy number was higher in *Z. mobilis* ZM1-3(pNB20) (pGSS33 is a multicopy plasmid) than in *Er. chrysanthemi* (*celZ* is a chromosomal gene) the observed high expression was the result of efficient transcription and translation processes in *Z. mobilis*. From the genetic construction of pNB20 (Fig. 1) the *celZ* gene is probably expressed from its own promoter. If this is the case, it would mean that the *Z. mobilis* expression system can recognize a promoter sequence from *Er. chrysanthemi*. This hypothesis is supported by the results of Conway *et al.* (1987c), which showed that the sequences of some *Z. mobilis* promoters are similar to those of other prokaryotes (Hawley & McClure, 1983). However, a different conclusion was drawn for the promoter sequence of *Z. mobilis* pyruvate decarboxylase, which does not contain sequences resembling those of the *E. coli* consensus sequence (Conway *et al.*, 1987b).

In contrast to our results, expression of other endoglucanases recently transferred into *Zymomonas* species has been low. Expression of endoglucanase gene from *Bacillus subtilis* in '*Z. anaerobia*' (= *Z. mobilis*) was very poor: 0.025 IU mg^{-1} , a value more than 100-fold lower than in *E. coli* (Yoon *et al.*, 1988). Higher levels were obtained for the *Pseudomonas fluorescens* var. *cellulosa* endoglucanase (Lejeune *et al.*, 1988) transferred to *Z. mobilis* CP4 (0.35 IU mg^{-1}) but this was still 10 times lower than our results. This low expression of heterologous genes seems general in *Z. mobilis*, since neither the *B. licheniformis* α -amylase (unpublished observations), β -galactosidase nor lactose permease (Carey *et al.*, 1983) were well expressed. High β -galactosidase activity was only obtained with truncated *E. coli lacZ* gene fusions with *Z. mobilis* promoters (Byun *et al.*, 1986; Yanase *et al.*, 1986). Thus, the high expression of heterologous genes in *Z. mobilis* does not seem to be a general rule even when the genes are expressed from their own promoters. In this respect the *celZ* gene of *E. chrysanthemi* appears to be an exception. Confirmation of this hypothesis will lead to the conclusion that fusion with a promoter of *Z. mobilis* is the only way of getting a high expression in this bacterium.

An interesting property of the endoglucanase Z in *Z. mobilis* is its excretion. Our experimental results favour the hypothesis of a periplasmic location of the enzyme. Consequently, it is assumed that the excretion signals from *E. chrysanthemi* were correctly recognized in *Z. mobilis*. A secretion mechanism has recently been demonstrated for the levansucrase of this bacterium (L. F. Preziosi and others, unpublished); it might be able to process the precursor of endoglucanase Z to allow the passage of the protein through the cytoplasmic membrane. A similar situation was observed with the *B. licheniformis* α -amylase (N. Brestic-Goachet and others, unpublished), which was also exported through the inner membrane of *Z. mobilis*. Thus the export mechanism of *Z. mobilis* seems to accept export signals from other bacteria and

correctly process the proteins to the cell periplasm. It is less clear whether the release of endoglucanase Z into the culture medium is due to an active secretion mechanism or to an increased leakiness of the outer membrane at the end of the growth phase. The outer membrane might be leaky by the effect of ethanol produced during growth. Further confirmation of this will require the study of a periplasmic marker protein in *Z. mobilis*.

The endoglucanase Z protein produced by *Z. mobilis* appeared to be identical to that synthesized by *E. coli* and *Er. chrysanthemi* on the basis of molecular mass and immunological properties. In *Z. mobilis*, both extra- and intracellular forms were identical.

From this work it is concluded that a cellulase gene is stably maintained and expressed in an efficient ethanol producer. More genes need to be cloned, for instance β -glucosidase and exoglucanase, to obtain a *Z. mobilis* strain that can degrade cellulose. In addition, these enzymes will need to be secreted into the culture medium in order to degrade this high molecular mass substrate.

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