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Cloning of an endoglucanase gene from *Pseudomonas fluorescens* var. *cellulosa* into *Escherichia coli* and *Pseudomonas fluorescens*

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SUMMARY

An endoglucanase chromosomal gene from the cellulolytic *Pseudomonas fluorescens* var. *cellulosa* (NCIB 10462) was cloned in *Escherichia coli.* Chromosomal DNA was partially digested with the restriction enzyme *Eco*RI and ligated into the broad host-range, mobilizable plasmid pSUP104 that had been linearized with the same enzyme. After transformation of *Escherichia coli,* an endoglucanase-positive clone was detected in situ by use of the Congo-red assay procedure. The endoglucanase gene on the recombinant plasmid pRUCL100 was expressed in the non-cellulolytic *Pseudomonas fluorescens* PF41. The DNA fragment carrying the gene was transferred to the plasmid pBR322, generating plasmids pRUCL150 and pRUCL151, and its restriction map was derived.

INTRODUCTION

The cellulolytic activities of bacteria have recently received more attention from both biotechnological [12] and ecological [24] perspectives. In this regard the cellulase system of Pseudomonads has been fairly well studied [28,39,40,43]. Endoglucanases are the major components of such enzyme complexes, with three being produced by *Pseudomonas fluorescens* var. *cellulosa* [43] and four or more by a *Pseudomonas* sp. [28]. Whether or not these multiple forms are synthesized from single or multiple genes is unknown. Similarly, the genetic relationships between cellbound and extracellular forms of these enzymes [42] have not been determined. The techniques for the cloning and expression of individual genes now facilitates the study of such fundamental questions. Indeed, considerable insight of bacterial cellulases has been gained by such approaches in *Bacillus* sp. N-4 [32], *Bacter-*

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Abbreviations: CMC, carboxymethylcellulose; EG, endoglucanase; kb, kilobase pairs; Mops, 4-morpholinepropanesulfonic acid; Ap^{r-s}, resistance-sensitivity to the antibiotic ampicillin; Cm^{r-s}, resistance-sensitivity to the antibiotic chloramphenicol; Tc^{r-s}, resistance-sensitivity to the antibiotic tetracycline; Sm^{r-s}, resistance-sensitivity to the antibiotic streptomycin; Tp^{r-s}, resistance-sensitivity to the antibiotic trimethoprim.

oides succinogenes [10], *Cellulomonas fimi* [15], *Clostridium thermocellum* [8], *Erwinia chrysanthemi* [2,21,41] and *Thermomonospora* YX [9]. With the prior biochemical/physiological understanding of Pseudomonad cellulases at hand, it is appropriate to extend these studies through molecular cloning of the individual genes. The initial results of the cloning of an endoglucanase gene from *P. fluorescens* var. *cellulosa* are presented.

MATERIALS AND METHODS

Bacterial strains and plasmids

Microorganisms used include the cellulolytic *Pseudomonas fluorescens* var. *cellulosa* (NCIB 10462), the non-cellulolytic *P. fluorescens* 6.2 strain PF41 (*ilv*32; [27]), *Escherichia coli HB101 (leu pro lac gal rpsL thi recA hsdS*; [36]), *E. coli* S17-1 (RP4-2-Tc::Mu-Km::Tn7, Tp^r, Sm^r *pro, hsdR*, mobilizing strain for conjugation [34]). The plasmids used included pSUP104 (Cm^r, Tc^r, broad host-range, mobilizable, 9.5 kb, [35]) and pBR322 [Ap^r, Tc^r, 4.362 kb, [4]).

Media and culture conditions

P. fluorescens var. *cellulosa* was grown at 28°C in M9 mineral medium [7] supplemented with 0.4% cellobiose and 0.01% $FeCl_3$. LB and the mineral medium of Schatz and Bovel [33] containing 0.5% sodium lactate and 40 μg/ml each of isoleucine-valine, were used for culture of *P. fluorescens* PF41 at 28°C. *E. coli* was cultivated at 37°C in LB. Agar (1.75%) and the antibiotics Tc (20 μg/ml), Cm (30 μg/ml) and Ap (50 μg/ml) were used as appropriate.

Isolation of DNA

A rapid alkaline extraction procedure was used for plasmid miniscreens [3]. Larger quantities of purified DNA were obtained by cleared lysis and cesium chloride centrifugation [25] or by a scale-up of the alkaline procedure, including a phenol extraction step (M.D. Yablonsky, personal communication). Chromosomal DNA was prepared essentially as described by Saito and Miura [31]. Several phenol, chloroform and ether extractions were performed and the DNA was recovered by ethanol precipitation. The preparation was further treated with pre-boiled RNase T1 (25 U/ml) and RNase A (50 μg/ml) and then re-extracted as described above.

Gel electrophoresis

DNA was prescreened using a horizontal minigel, and examined in greater detail by vertical agarose (0.8%) slab gel electrophoresis in TEA buffer. Small restriction fragments were analyzed using 6% polyacrylamide gels in TEB buffer. Phage λ cut with *Hin*dIII or with *Hin*dIII and *Eco*RI served as a size marker.

Restriction and ligation conditions

Restriction enzymes (Boehringer Mannheim Biochemica, Indianapolis, IN), and T4-DNA ligase (New England Biolabs, Beverly, MA) were used in the buffers recommended by the suppliers. Partial chromosome digests and ligations were performed as described in Refs. 25 and 30.

Transformation and conjugation

E. coli HB101 was transformed by the standard calcium chloride procedure [25] and *E. coli* S17-1 with the rubidium chloride-Mops method [22]. Bacterial conjugation was performed on plates [23].

Detection of enzymatic activity on plates

Endoglucanase [38]. Following growth of the colonies on plates, endoglucanase activity was detected by pouring (in the prsence or absence 0.1% sodium dodecyl sulfate) an overlay containing 0.1% CMC (type 7MF, Hercules, France) in Trypticase peptone agar (0.8%). After several hours of incubation, the plates were flooded with Congo red and saline. Endoglucanase activity around the active colonies was detected as pale yellow haloes on a red background.

β-Glucosidase [11]. Following growth of the colonies, they were covered by an agar overlay containing 0.1% methylumbelliferyl-β-D-glucose. The cultures were incubated at 50°C for 10 min. Positive colonies show a blue fluorescence under ultraviolet light.

Detection of endoglucanase activity by a rapid viscometric test

Bacteria expressing putative endoglucanase activity were grown overnight in complete medium and sonicated after resuspension of the cells in 0.05 M McIlvaine buffer (pH 7.0) [26]. The sonicate (100 μl) was incubated with 1.0 ml of a 1% solution of CMC in the same buffer at 30°C for various periods of time. The reaction was stopped by heating at 75°C for 10 min. The relative viscosity of the assay mixture was determined at 22°C by measuring the efflux time of the solution in a 0.2 ml pipette [8]. A decreased efflux time (i.e., a decrease in viscosity) compared to that of the control was used as an indicator of endoglucanase activity.

RESULTS

Cloning of the P. fluorescens var. cellulosa endoglucanase gene

The cloning approach used in this study is outlined in Fig. 1. It utilizes an in vitro cloning system with a broad host-range plasmid vector, combined with a further conjugation step into a non-cellulolytic *Pseudomonas* host. The purified chromosome of *P. fluorescens* var. *cellulosa* was partially digested with the restriction enzyme *Eco*RI to generate DNA fragments in the 10 kb range. These fragments were ligated with the *Eco*RI linearized plasmid vector pSUP104 at a concentration of 20 μg DNA/ml, using 1:1 and 1:2 insert to vector molar ratios. The ligation products were used to transform the plasmid mobilizing *E. coli* strain S17-1. Among 12 350 transformants detected by their resistance to Tc, 1004 (about 8% of the total) were Cm^s, thus harboring a recombinant plasmid containing a foreign DNA insert in the *Eco*RI site. This gene bank was then screened for in situ endoglucanase and β-glucosidase activities. One endoglucanase positive S17-1 was found (Fig. 2) and purified.

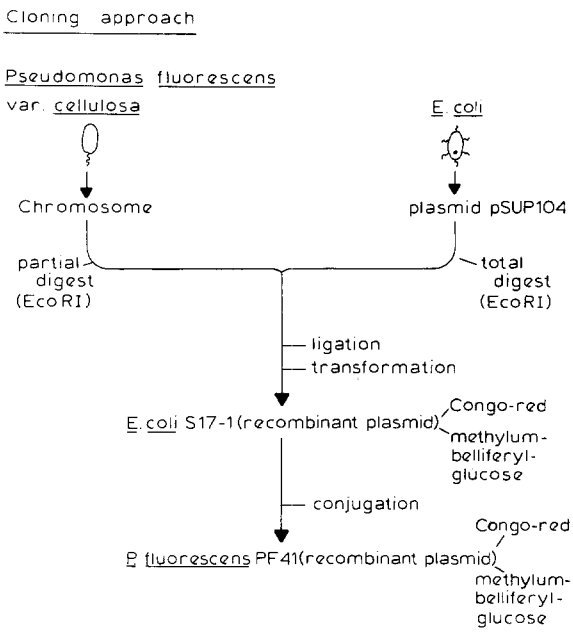

Fig. 1. Outline of the cloning protocol (see text for details).

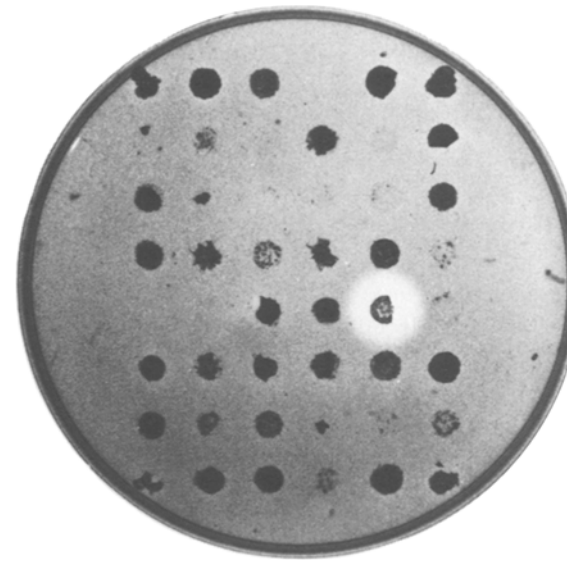

Fig. 2. Plate detection of endoglucanase activity. *E. coli* transformants containing *Pseudomonas* DNA inserts were overlaid with CMC and then with Congo red (see text). One colony shows the presence of a cloned endoglucanase gene by the clearing reaction. *E. coli* S17-1 (pSUP104) was used as a negative control (upper right colony).

The recombinant plasmid carried by this colony was named pRUCL100 (Fig. 3). It was extracted by the rapid alkaline method and used to transform *E. coli* S17-1 and HB101 to Tc resistance. All the transformants exhibited an endoglucanase positive phenotype on plates. *E. coli* S17-1(pRUCL100) was

conjugated with the non-cellulolytic *P. fluorescens* PF41. All *P. fluorescens* PF41 that acquired the Tc resistance concomitantly acquired endoglucanase activity, thus indicating that the gene was on the recombinant plasmid pRUCL100.

The presence of the endoglucanase gene was further demonstrated by a rapid qualitative viscometric test, as described in Materials and Methods. A sharp decrease in viscosity of CMC was observed after only a few minutes of incubation at 30°C with the sonicate fraction of *E. coli* HB101 (pRUCL100) and *P. fluorescens* PF41 (pRUCL100). Essentially no decrease in viscosity was noted with *P. fluorescens* PF41 or *E. coli* HB101 lacking the plasmid.

Physical characterization of the recombinant plasmids carrying the endoglucanase gene

The *P. fluorescens* var. *cellulosa* endoglucanase gene insert in plasmid pRUCL100 was subcloned into the *Eco*RI site of the plasmid pBR322. Both plasmids were totally digested with *Eco*RI, the fragments ligated at a DNA concentration of 10 μg/ml and an equimolar ratio, and the ligation products transformed in *E. coli* S17-1 (Fig. 3). Selection of the transformants was on Ap-containing plates (to counterselect for the transformant containing the pSUP104 plasmid) and further purification utilized Tc and Ap plates. Ten endoglucanase-positive colonies were selected for further study.

Their plasmids were isolated by the rapid alkaline procedure and cleaved with the restriction enzyme *Sal*I. Preliminary work with the plasmid pRUCL100 indicated that the insert carrying the endoglucanase gene contained a unique decentered restriction site for *Sal*I (see Fig. 3). Determination of the size of the DNA fragments by agarose electrophoresis indicated that two types of plasmids in *E. coli*, differing only in the orientation of the insert in the *Eco*RI site of pBR322, both conferred the endoglucanase positive phenotype. They were termed pRUCL150 and pRUCL151 (Fig. 3). Large amounts of plasmid pRUCL150 were extracted using the scale-up modification of the miniscreen procedure and this DNA was used for restriction analysis. Several restriction enzymes were used, both singly and in combination. Fig. 4 shows the

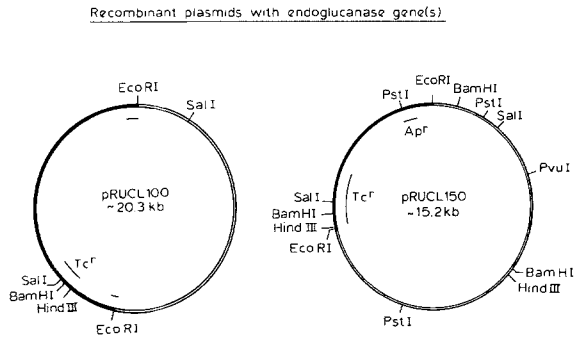

Fig. 3. The plasmids pRUCL100 and pRUCL 150 (pRUCL151) (not drawn to scale). The heavy line represents the pSUP104 for pRUCL100 and pBR322 for pRUCL150 (pRUCL151), and the thin lines represent the insert carrying the endoglucanase gene. The insert is in opposite orientations in pRUCL150 and pRUCL151, as indicated by the location of the *Sal*I restriction site. In addition to the restriction sites indicated, the insert contains unique unlocalized restriction sites for the following enzymes: *Bcl*I, *Bgl*II and *Kpn*I. It also contains 5 *Ava*I sites and 6 *Pvu*II sites, but it lacks an internal *Eco*RI site.

band patterns of the plasmid fragments obtained after agarose or polyacrylamide gel electrophoresis.

These experiments indicated that the endoglucanase gene is on an insert of approximately 10.8 kb. Thus, plasmid pRUCL100 is approximately 20.3 kb and plasmids pRUCL150 and pRUCL151 are 15.2 kb. The restriction map of pRUCL150 derived from these experiments is presented in Fig. 3.

DISCUSSION

This study adds to the known examples of cloned cellulase genes. Several points are pertinent. We used a broad host-range, mobilizable cloning vehicle. Several such broad host-range plasmids are available for cloning (see Refs. 1, 14, 16 and 35). They are larger than the narrow host-range, *E. coli* cloning vectors, and thus their transformation efficiency is relatively low. However, this impediment can be circumvented by the use of broad host-range cosmids. A technical noteworthy point was that the transformation of *E. coli* S17-1 was performed using the Mops-RbCl procedure rather than the $CaCl_2$ procedure, as we found the mortality of the

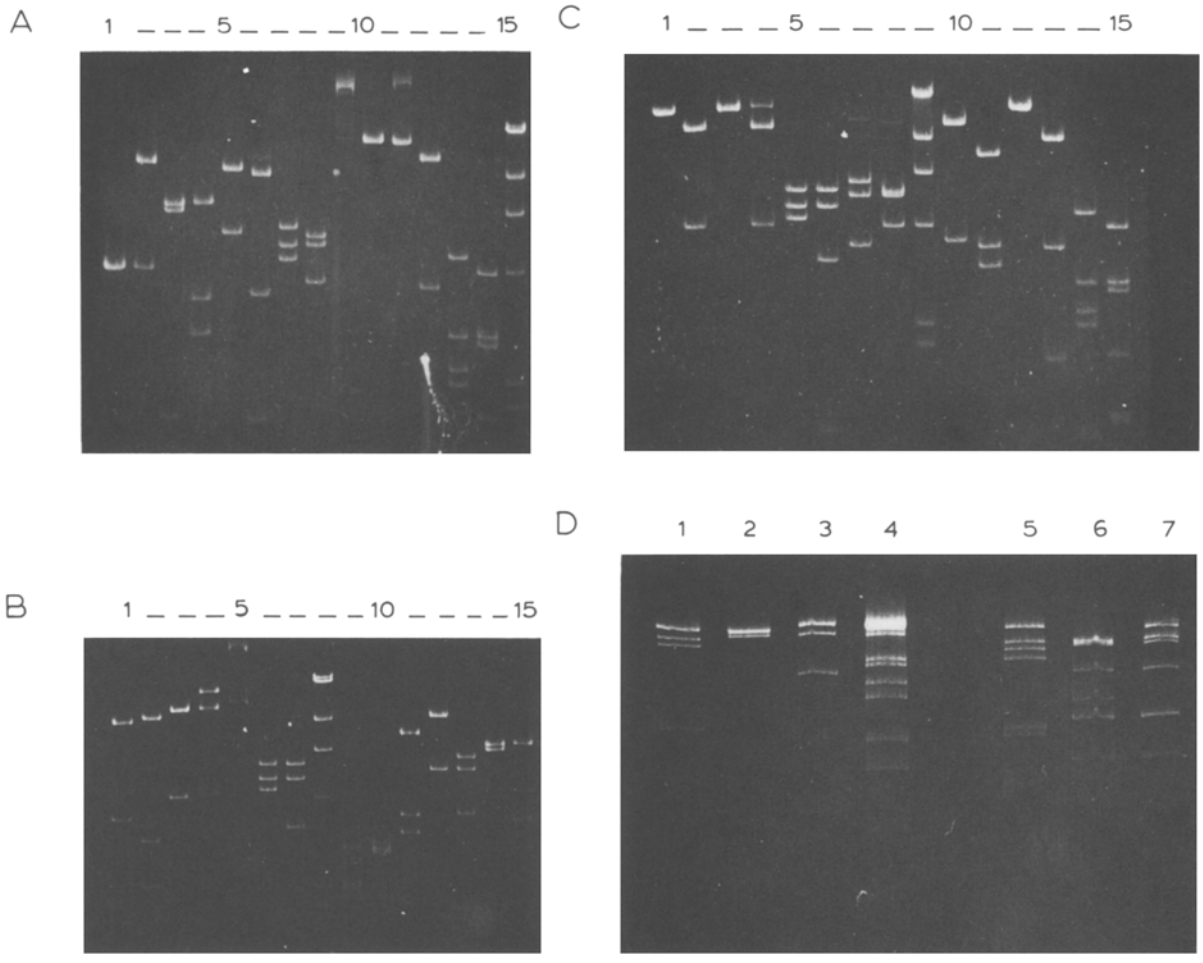

Fig. 4. Agarose gel electrophoresis (A–C) and polyacrylamide gel electrophoresis (D) analysis of DNA from the recombinant plasmid pRUCL150 cleaved with various restriction enzymes. Lanes are numbered, left to right.
(A) Lane 2,*Eco*RI; 3,*Pst*I; 4,*Eco*RI and *Pst*I; 5,*Sal*I; 6,*Eco*RI and *Sal*I; 7,*Bam*HI; 8,*Eco*RI and *Bam*HI; 9,*Bcl*I; 10,*Bgl*II; 11,*Sca*I; 12,*Pvu*I; 13,*Pvu*II; 14,*Ava*I; 1,*Eco*R I-linearized pBR322; 15,λ phage cut with *Hin*dIII.
(B) Lane 1,*Eco*RI and *Sal*I; 2,*Sca*I and *Sal*I; 3,*Eco*RI and *Bcl*I; 4,*Eco*RI and *Bgl*II; 5,*Kpn*I; 6,*Bam*HI; 7,*Sca*I and *Bam*HI; 9,*Eco*RI and *Pvu*II; 10,*Eco*RI and *Ava*I; 11,*Eco*RI and *Pvu*I; 12,*Hin*dIII; 13,*Eco*RI and *Hin*dIII; 14,*Pst*I; 15,*Eco*RI and *Pst*I; 8,phage λ cut with *Hin*dIII.
(C) Lane 1,*Sca*I and *Bcl*I; 2,*Eco*RI and *Bcl*I; 3,*Sca*I and *Bgl*II; 4, *Eco*RI and *Bgl*II; 5,*Bam*HI; 6,*Sca*I and *Bam*HI; 7,*Sca*I and *Hin*dIII; 8,*Eco*RI and *Hin*dIII; 10,*Pvu*I; 11,*Eco*RI and *Pvu*I; 12,*Sca*I and *Sal*I; 13, *Eco*RI and *Sal*I; 14,*Pvu*II; 15,*Ava*I; 9, phage λ cut with *Hin*dIII.
(D) Lane 1,*Eco*RI and *Pst*I; 2,*Eco*RI and *Bam*HI; 3,*Eco*RI and *Sal*I; 5,*Pvu*II; 6,*Eco*RI and *Ava*I; 7,*Ava*I; 4,phage λ cut with *Eco*RI and *Hin*dIII.

cells when stored overnight at 4°C in $CaCl_2$ to be very high.

Prior studies on the cloning of *Pseudomonas* genes into *E. coli* have indicated that the expression of these genes is often weak in this new background [5,13,18,20,29], although some reports note good expression levels [18,23]. With the possibility of weak expression, we adopted the cloning approach illustrated in Fig. 1. Thus, in the general screening protocol, should no expression of the genes of interest have been found in *E. coli*, we planned to conjugate the recombinant plasmids 'en masse' to the non-cellulolytic *P. fluorescens* PF41 in order to gain a better chance of expression of the genes. This

was not necessary for after transformation of *E. coli* S17-1 and screening of the colonies containing recombinant plasmids with the very sensitive Congo red assay technique, one endoglucanase gene-positive clone was evident by direct endoglucanase expression.

Studies currently in progress show that the level of expression of the endoglucanase gene in *E. coli* HB101 is lower than in *P. fluorescens* PF41 and that the enzyme is partially excreted by the latter organism. The subcloning of the endoglucanase gene into the small, multicopy and amplifiable pBR322 plasmid permitted preparation of the endoglucanase insert in good yield. Furthermore, as pBR322 has been sequenced [37], and the exact position of the cleavage sites for many restriction endonucleases is known, its use will facilitate the restriction analysis of the cloned endoglucanase gene containing insert, and will enable us to subclone smaller fragments and locate the gene on the insert.

The two plasmids pRUCL150 and pRUCL151 differ only in the orientation of the insert in pBR322. The endoglucanase gene is expressed by both plasmids in *E. coli,* and their level of expression is equal (unpublished results). Thus, the EG gene has been cloned with, and is transcribed from, its own promoter.

Although *P. fluorescens* var. *cellulosa* contains two distinct β-glucosidases [19], no such activity could be detected in our gene bank. Several reasons can be considered to explain this result; the β-glucosidase genes may have been cut by the restriction enzyme *Eco*RI used in the cloning experiment, even though partial digests of chromosomal DNA were used. Their promoters may not be recognized in *E. coli.* Alternatively, if one assumes that there is but one β-glucosidase gene and that the genome of *P. fluorescens* is 7600 kb in length [17], then, using the formula of Clarke and Carbon (1976) [6], the probability to find one positive β-glucosidase clone among one thousand recombinant ones is only 0.73. However, the size of the genome of *P. fluorescens* is not known with certainty and estimates vary widely [17]. The probability rises to 0.94 if one assumes the chromosome to be 3600 kb in length and over 0.99 if one assumes, based on the observations of Hwang and Suzuki [19] that two β-glucosidase genes are present on the chromosome. Similar comments apply to the cloning of the endoglucanase gene.

This initial study opens the way for further investigations. *P. fluorescens* var. *cellulosa* is known to produce several different endoglucanase genes [42,43]. We now have a methodology to investigate promoter(s) function, how many endoglucanase genes exist, in what manners the expression of the gene(s) are regulated, and to compare the endoglucanases produced from the cloned gene(s) and the native ones. A further important area of investigation will be to gain expression of the endoglucanase gene(s) in *Zymomonas mobilis.* This bacterium is able to ferment glucose, fructose and sucrose into ethanol and one of the objectives of our study is to extend the range of substrates that it can use.

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REFERENCES

1 Bagdasarian, M. and K.N. Timmis. 1982. Host: vector systems for gene cloning in *Pseudomonas.* In: Current Topics in Microbiology and Immunology, Vol. 96. Gene cloning in organisms other than *Escherichia coli,* (Hofschneider, P.H. and W. Goebel, eds), pp. 47–67, Springer-Verlag, Berlin, Heidelberg and New York.

2 Barras, F., M.-H. Boyer, J.-P. Chambost and M. Chippaux. 1984. Construction of a genomic library of *Erwinia chrysanthemi* and molecular cloning of the cellulase gene. Mol. Gen. Genet. 197: 513–514.

3 Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acid. Res. 7: 1513–1523.

4 Bolivar, F., R.L. Rodriguez, P.G. Green, M.C. Betlach, H.L. Heinecker, H.W. Boyer, J.H. Crosa and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2: 95–113.

5 Buckel, P. and E. Zehelein. 1981. Expression of *Pseudomonas fluorescens* D-galactose dehydrogenase in *E. coli*. Gene 16: 149–159.

6 Clarke, L. and J. Carbon. 1976. A colony bank containing synthetic *Col*El hybrid plasmids representative of the entire *E. coli* genome. Cell 9: 91–99.

7 Clowes, R.C. and W. Hayes. 1968. Experiments in Microbial Genetics. 244 pp. Blackwell Scientific Publications, Oxford.

8 Cornet, P., D. Tronik, J. Millet and J.-P. Aubert. 1983. Cloning and expression in *Escherichia coli* of *Clostridium thermocellum* genes coding for amino acids synthesis and cellulose hydrolysis. FEMS Microbiol. Lett. 16: 137–141.

9 Collmer, A. and D.B. Wilson. 1983. Cloning and expression of a *Thermomonospora* YX endocellulase gene in *E. coli*. Bio/Technology 1: 594–601.

10 Crosby, B., B. Collier, D.Y. Thomas, R.M. Teather and J.D. Erfle. 1984. Cloning and expression in *Escherichia coli* of cellulase genes from *Bacteroides succinogenes*. Bio/Joule 7: (Feb/March) 14.

11 Cuskey, S.M., B.S. Montenecourt and D.E. Eveleigh. 1981. Overproduction of cellulase – screening and selection. In: Overproduction of Microbial Metabolites. (Krumphanzl, V., B. Sikyta and Z. Vanek, eds.), pp. 405–416, Pub. Czechoslovak Academy of Sciences and Academic Press, New York, NY.

12 Doelle, H.W. 1984. Microbial cultures in the utilization of cellulosic materials. Biotech. Adv. 2: 1–19.

13 Durham, D.R., R.A. Roehl, J.L. Flickinger, J.E. Gifford and R.E. Wattam. 1984. Genetic engineering in *Pseudomonas*: cloning and expression of catabolic genes. Dev. Indust. Microbiol. 25: 163–174.

14 Eaton, R.W. and K.N. Timmis. 1984. Genetics of xenobiotic degradation. In: Current Perspectives in Microbial Ecology (Klug, M.J. and Reddy, C.A., eds.), pp. 694–703, American Society for Microbiology, Washington D.C.

15 Gilkes, N.R., D.G. Kilburn, M.L. Langsford, R.C. Miller, Jr., W.W. Wakarchuk, R.A.J. Warren, D.J. Whittle and W.K.R. Wong. 1984. Isolation and characterization of *Escherichia coli* clones expressing cellulase genes from *Cellulomonas fimi*. J. Gen. Microbiol. 130: 1377–1384.

16 Haas, D. 1983. Genetic aspects of biodegradation by Pseudomonads. Experientia 39: 1199–1213.

17 Holloway, B.W. (1975) Genetic organization of *Pseudomonas*. In: Genetics and Biochemistry of *Pseudomonas*, (Clarke, P.H., and M.H. Richmond, eds.), pp. 133–161. John Wiley and Sons, London.

18 Holloway, B.W. 1984. Pseudomonads. In: Genetics of Industrial Microorganisms. (C. Ball, ed.), pp. 63–92. CRC Press, Boca Raton, FL.

19 Hwang, J.-T. and H. Suzuki. 1976. Intracellular distribution and some properties of β-glucosidases of a cellulolytic Pseudomonad. Agric. Biol. Chem. 40: 2169–2175.

20 Jacoby, G.A., J.E. Rogers, A.E. Jacob and R.W. Hedges. 1978. Transposition of *Pseudomonas* toluene degrading genes and expression in *Escherichia coli*. Nature 274: 179–180.

21 Kotoujansky, A., A. Diolez, M. Boccara, Y. Bertheau, T. Andro and A. Coleno. 1985. Molecular Cloning of *Erwinia chrysanthemi* pectinase and cellulase structural genes. EMBO J. 4: 781–786.

22 Kushner, S.R. 1978. An improved method for transformation of *Escherichia coli* with *Col*El – derived plasmids. In: Genetic Engineering (H.B. Boyer and S. Nicosia, eds.), p. 17, Elsevier/North Holland, Amsterdam.

23 Lejeune, P., M. Mergeay, F. Van Gijsegem, M. Faelen, J. Gerits and A. Toussaint. 1983. Chromosome transfer and R-prime plasmid formation mediated by plasmid pULB113 (RP4::Mini-Mu) In: *Alcaligenes eutrophus* CH34 and *Pseudomonas fluorescens* 6.2. J. Bacteriol. 155: 1015–1026.

24 Ljundahl, L.J. and K.-E. Ericksson. 1985. Ecology of Microbial Cellulose Degradation. Adv. Microbial Ecol. 8: 237–299.

25 Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular cloning, a laboratory manual. 545pp., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., U.S.A.

26 McIlvaine, T.C. 1921. A buffer solution for colorimetric comparison. J. Biol. Chem. 49: 183–186.

27 Mergeay, M. and J. Gerits. 1978. F′-plasmid transfer from *Escherichia coli* to *Pseudomonas fluorescens*. J. Bacteriol. 135: 18–28.

28 Ramasamy, K. and H. Verachtert. 1980. Localization of cellulose components in *Pseudomonas* sp. isolated from activated sludge. J. Gen. Microbiol. 117: 181–191.

29 Ribbons, D.W., G.J. Wigmore and A.M. Chakrabarty. 1979. Expression of TOL genes in *Pseudomonas putida* and *Eschericia coli*. Soc. Gen. Microbiol. Quart. 6: 14–25.

30 Rodriguez, R.L. and R.C. Tait. 1983. Recombinant DNA techniques, an introduction. 236 pp. Addison-Wesley Publishing Company, Reading, MA, U.S.A.

31 Saito, H. and K.-I. Miura. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta 72: 619–629.

32 Sashihara, N., T. Kudo and K. Horikoshi. 1984. Molecular cloning and expression of cellulase genes of alkalophilic *Bacillus* sp. strain N-4 in *Escherichia coli*. J. Bacteriol. 158: 503–506.

33 Shatz, A. and C. Bovell, Jr. 1952. Growth and hydrogenase activity in a new bacterium. *Hydrogenomonas facilis*. J. Bacteriol. 63: 87–98.

34 Simon, R., U. Priefer and A. Pühler. 1983a. A broad host range mobilizing system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology 1: 784–790.

35 Simon, R., U. Priefer and A. Pühler. 1983b. Vector plasmids for in vivo and in vitro manipulations of gram negative bacteria. In: Molecular genetics of the bacteria-plant interaction (A. Pühler, ed.), pp. 98–106, Springer-Verlag, Berlin.

36 So, M., H.W. Boyer, M. Betlach and S. Falkow. 1976. Molecular cloning of an *Escherichia coli* plasmid determinant that encodes for the production of heat-stable enterotoxin. J. Bacteriol. 128: 463–472.

37 Sutcliffe, J.G. 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. 43: 77.

38 Teather, R.M. and P.J. Wood. 1982. Use of Congo-red-polysaccharide interaction in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. 43: 777–780.

39 Tewari, H.K. and D.S. Chahal. 1977. Production, purification and properties of extracellular cellulase from *Pseudomonas* Sp.-1. Ind. J. Microbiol. 17: 88–92.

40 Thayer, D.W. 1978. Cellulolytic and physiological activities of bacteria during production of single-cell protein from wood. Am. Inst. Chem. Eng. Symp. Ser. 74: 126–135.

41 Van Gijsegem, F., A. Toussaint and E. Schoonejans. 1985. In vivo cloning of the pectate lyase and cellulase genes of *Erwinia chrysanthemi*. EMBO J. 4: 787–792.

42 Yamane, K., H. Suzuki and K. Nisizawa. 1970. Purification and properties of extracellular and cell-bound cellulase components of *Pseudomonas fluorescens* var. *cellulosa* J. Biochem. 67: 19–35.

43 Yoshikawa, T., H. Suzuki and F. Nisizawa. 1974. Biogenesis of multiple cellulase components of *Pseudomonas fluorescens* var. *cellulosa*. 1. Effects of culture conditions on the multiplicity of cellulase. J. Biochem. 75: 531–540.