Molecular Cloning, Expression, and Characterization of Endoglucanase Genes from *Fibrobacter succinogenes* AR1

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A cosmid gene library was constructed in *Escherichia coli* from genomic DNA isolated from the ruminal anaerobe *Fibrobacter succinogenes* AR1. Clones were screened on carboxymethyl cellulose, and 8 colonies that produced large clearing zones and 25 colonies that produced small clearing zones were identified. Southern blot hybridization revealed the existence of at least three separate genes encoding cellulase activity. prCO93, which is representative of cosmid clones that produce large clearing zones, was subcloned in pGem-1, and the resulting hybrid pRCEH directed synthesis of endoglucanase activity localized on a 2.1-kb EcoRI-HindIII insert. Activity was expressed from this fragment when it was cloned in both orientations in pGem-1 and pGem-2, indicating that *F. succinogenes* promoters functioned successfully in *E. coli*. A high level of endoglucanase activity was detected on acid-swollen cellulose, ball-milled cellulose, and carboxymethyl cellulose; and a moderate level was detected on filter paper, Avicel, lichenan, and xylan. Most activity (80%) was localized in the periplasm of *E. coli*, with low but significant levels (16%) being detected in the extracellular medium. The periplasmic endoglucanase had an estimated molecular weight of 46,500, had an optimum temperature of 39°C, and exhibited activity over a broad pH range, with a maximum at pH 5.0.

*Fibrobacter succinogenes* is one of the most prolific cellulolytic microorganisms in the rumen, possessing a highly active cellulase system (16, 46). However, difficulty in isolating *F. succinogenes* (43) has resulted in a large proportion of studies concentrating on strain S85, which was originally isolated by Bryant and Doetsch in 1954 (7). Extensive research has focused on the characterization of various enzymes involved in the saccharification of lignocellulosic compounds. Enzyme activities associated with the breakdown of cellulose and hemicellulose from S85 include endoglucanase (30, 31), cellobiosidase (22), esterase (29), and lichenanase (10).

Recently, *Bacteroides succinogenes* was reclassified to *Fibrobacter succinogenes* with two subspecies, *F. succinogenes* subsp. *succinogenes* and *F. succinogenes* subsp. longata (33). Strain AR1 has not been extensively characterized; however, it appears to belong to *F. succinogenes* subsp. longata (type strain HM2). Other strains belonging to *F. succinogenes* subsp. longata include MM4 and MB4 (1). Evidence that AR1 may belong to *F. succinogenes* subsp. longata is suggested by the fact that genomic DNA isolated from strains MM4, MB4, and HM2 (42a), like AR1 (7a), is not successfully digested with the restriction enzyme Sau3A1, whose recognition sequence is GATC. *BamHI* (whose core recognition sequence is GATC) also fails to digest AR1 DNA (7a). By comparison, genomic DNAs from strains BL2 and S85 are able to be restricted by *BamHI* (12). This property may be helpful in distinguishing the two subspecies.

The cloning of separate genes or operons would facilitate the analysis of individual cellulase enzymes, allowing a better understanding of their role in lignocellulose breakdown in the rumen. Elucidation of the mechanisms involved in gene expression would allow an informed attempt at genetic manipulation of these bacteria. Furthermore, it would provide important knowledge that is required before the release of genetically engineered microorganisms can be considered responsible. In this study, a gene library from strain AR1 was constructed to allow analysis of the genetic and enzymatic components responsible for the organism's ability to actively degrade lignocellulose. The isolation from *F. succinogenes* AR1 of three distinct endoglucanase genes, and in particular, the characterization of one clone with a high level of activity, is reported. The nucleotide sequence and novel aspects of gene control in the endoglucanase gene from AR1 with a high level of activity are presented in a separate report (7b).

**MATERIALS AND METHODS**

**Bacterial strains and cloning vectors.** *F. succinogenes* AR1 (formerly *B. succinogenes* [33]) was provided by Tom Bau-Chop. *Escherichia coli* HB101 and DH1 (17) were grown in 2× YT medium containing (per liter) 16 g of tryptone, 10 g of yeast extract, and 5 g of sodium chloride. Incubations were conducted at 37°C in flasks placed in an orbital shaker operating at 200 rpm (orbits per minute). Ampicillin was added at 100 μg/ml. For plate media, agar was added at 2%. Cosmid vector pHC79 (20) was used for cosmid cloning, and plasmids pGem-1, pGem-2, pGem7ZF*, and pGem7ZF− (Promega, Sydney, Australia) were used for subcloning and exonuclease deletions.

**Gene library and screening for endoglucanase activity.** Genomic DNA was isolated essentially by the procedure of Anderson and McKay (2). Genomic DNA was partially digested with *HindIII*, and following separation on a 0.7% agarose gel, appropriately sized fractions were pooled and ligated into the *HindIII* site of pHC79. Recombinant cosmids were packaged and infected into *E. coli* DH1 by using the Promega Packagene kit. Cells were plated onto ampicillin (100 μg/ml)-containing plates, and 2,000 colonies were screened onto plates containing (per liter) 2 g of carboxym-
methyl cellulose (CMC; low viscosity; Sigma Chemical Co., St. Louis, Mo.), 5 g of yeast extract, the minimal salt of Gilardi (13), 100 mg of ampicillin, and 50 mg of cycloheximide. Plates were incubated for 3 days at 37°C and then flooded with 1 mg of Congo red per ml for 15 min. Plates were drained and then washed with 1 M NaCl. Endoglucanase-positive clones were identified by the clearing zones surrounding the colonies (45). Thirty-three clones were identified as endoglucanase positive, with 8 large clearing zones (LCZs; 3 cm in diameter after 2 days of growth) and 25 small clearing zones (SCZs; 1 cm in diameter after 2 days of growth). Recombinants pRC093 and pDA84 were chosen as representatives of cosmid clones, producing large and small clearing zones, respectively (see Table 1).

**Subcloning and exonuclease deletions.** Plasmid DNA was isolated by the alkaline lysis procedure (5). Plasmid pRCE was constructed by cloning an approximately 3.3-kb EcoRI fragment from pRC093 into pGem-1 (Table 1). A 2.075-bp EcoRI-HindIII fragment encoding FSendA (7b) was subcloned from pRCE into pGem-1, pGem-2, pGem7Zf, and pGem7Zf , generating pRCEH, pRCE2H, pRCZ , and pRCEZ , respectively. The 1.55-kb HindIII-Apal, 1.35-kb EcoRI-SphI, 1.38-kb EcoRI-Sau3AI, 1.62-kb EcoRI-XbaI, and 1.86-kb EcoRI-BglII restriction enzyme fragments were cloned from pRCE into pGem7Zf . Exonuclease deletions of pRCZ and pRCEZ were generated by using the Erase-a-base kit (Promega). Deletions from the HindIII end of pRCZ were obtained by first digesting it with SaeI, to produce a 3’ overhang (to inhibit exonuclease III digestion), followed by digestion with HindIII to generate a 5’ overhang. Nested sets of deletions were obtained by exonuclease III digestion for set time periods. Deletions from the EcoRI end were performed by first linearizing pRCZ with XhoI (5’ overhang), end filling with α-phosphorothioate deoxyoligonucleotide triphosphates (39), restricting with EcoRI, and sequentially digesting with exonuclease III.

**Southern hybridization.** Cosmid clones were prepared from *E. coli* DH1 by using Qiagen plasmid preparation columns (Qiagen Inc., Studio City, Calif.). A small amount of *E. coli* genomic DNA was also extracted on the Qiagen columns because of the size (>20 kb) of the cosmid recombinants. DNA hybridization was performed essentially as described by Southern (42). Cosmids and *F. succinogenes* AR1 genomic DNA were digested to completion with HindIII. These digests, together with an EcoRI-HindIII fragment from pRCEH and an approximately 6-kb HindIII fragment encoding cellulase activity from pDA84 (8), were loaded onto 1% agarose gels and electrophoresed for 2 h at 50 V. Lambda DNA HindIII and EcoRI-HindIII digests were included as molecular weight markers. Gels were capillary blotted onto N+nylon filters (Amersham, Sydney, Australia) according to the directions of the manufacturer and hybridized by using the insert from pRCEH which was labeled with [32P]dATP by using a random priming kit (Bresa, Adelaide, Australia). High- and low-stringency hybridization and washing procedures were performed as outlined in the Amersham blotting and hybridization protocols for Hybond membranes.

**Cellular fractionation.** Cells in the late logarithmic phase were harvested at 11,000 × g for 10 min, and the supernatant was taken as the extracellular phase. Periplasmic enzymes were prepared by the osmotic shock procedure (19), with modifications. Cells were washed in 10 mM Tris hydrochloride (pH 8), resuspended in 25% sucrose–10 mM Tris hydrochloride (pH 8), and shaken gently at 22°C for 10 min. Cells were pelleted (11,000 × g for 10 min) and resuspended in ice water with further gentle shaking at 4°C for 10 min. Cells were pelleted, and the supernatant that was recovered was used as the periplasmic fraction. Cytoplasmic fractionation was performed by sonicating the remaining cell pellet (resuspended in 50 mM sodium citrate buffer [pH 5.7]) with a Branson sonifier fitted with a B-30 microtip at 70% maximum power and 50% duty cycle. The cytoplasmic fraction was the supernatant after centrifugation at 16,000 × g for 1 h at 4°C. β-Galactosidase and alkaline phosphatase were assayed to determine the activities of these respective cytoplasmic (36) and periplasmic (19) marker enzymes. Activity was determined by a spectrophotometric method (34) by using paranitrophenyl (pNP) derivatives.

**Endoglucanase activity and molecular weight determination.** Endoglucanase activity was assayed by measuring the release of reducing sugars (35) on 0.5% CMC, phosphoric acid-swollen cellulose (Avicel PH101 prepared by the method of Wood (48)), oatspelt xylan (batch no. X-0376; Sigma), lichenan (batch no. 124F-0106; Sigma), laminarin (batch no. 78F-3798; Sigma), ball-milled cellulose (Whatman no. 1; Whatman, Maidstone, England), and 5% Avicel PH101 (38-μm average particle size; Honeywell and Stein, Poole, United Kingdom), filter paper (Whatman No. 1), and sliva cotton buffered in 50 mM citrate phosphate buffer (pH 6). Activity was measured as (i) pNP liberated from pNP-glucoside, pNP-xylloside, pNP-lactoside, and pNP-cellobiose (34) and (ii) glucose liberated from maltose, cellobiose, lactose, sucrose, melibiose, and melezitose with a glucose oxidase-peroxidase kit (Sigma). For evaluating the effect of pH, HCl-KCl (100 mM) was used for pH 1.0 to 2.2, citrate-phosphate buffer (various concentrations of citrate and phosphate were used, depending on the pH) was used for pH 2.6 to 7.0, 50 mM Tris hydrochloride was used for pH 7.2 to 9.0, and 50 mM glycine–NaOH was used for pH 9.0 to 12.5 (15). The pH of the final endoglucanase assay mixture (including added enzyme fractions) was measured directly, as the buffering capacities of final solutions were found to vary markedly. One unit of activity was defined as that which released 1 μmol of glucose equivalent as reducing sugar or 1 μmol of pNP per min at 39°C. Protein concentration was determined by the Lowry procedure (28). Thermal inactivation of endoglucanase activity was determined by measuring reducing sugar from CMC at 39°C, after preincubation in 50 mM citrate phosphate (pH 6) at 4, 39, and 50°C. The mode of cellulase action was determined in an Ubbelohde viscometer by quantitating the decrease in viscosity with time caused by enzyme action in a solution of 1% CMC–50 mM citrate phosphate (pH 6). The molecular weight of the endoglucanase was determined from periplasmic extracts by gel filtration (3) by using Sephadex G100 (Pharmacia, Sydney, Australia). The column was calibrated with bovine serum albumin, ovalbumin, pepsin, and lysozyme, and the void volume was calculated with blue dextran (Sigma). The column was eluted with 50 mM citrate buffer (pH 6), and fractions were assayed for endoglucanase activity by measuring the release of reducing sugar by the para-hydroxybenzoic acid hydrazide method (26), with CMC used as the substrate.

**RESULTS**

**Isolation of endoglucanase genes from *F. succinogenes* AR1 genomic DNA.** Two thousand cosmid clones were screened in *E. coli* DH1 for endoglucanase activity. Eight clones exhibited LCZs on CMC plates, and 25 clones exhibited SCZs. Plasmid pRC093 (Table 1), which was representative...
TABLE 1. Recombinant plasmids and cosmids used in this study

<table>
<thead>
<tr>
<th>Plasmid or cosm</th>
<th>Vector and construction</th>
<th>Endoglucanase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRC093</td>
<td>pHCG79 cosmide bank</td>
<td>LCZ</td>
</tr>
<tr>
<td>Cosmids 2 to 8</td>
<td>pHCG79 cosmide bank</td>
<td>LCZ</td>
</tr>
<tr>
<td>pDA44</td>
<td>pHCG79 cosmide bank</td>
<td>SCZ</td>
</tr>
<tr>
<td>pRCE</td>
<td>pGEM-1, approx. 3.3-kb HindIII insert from pRC093</td>
<td>LCZ</td>
</tr>
<tr>
<td>pRCEH</td>
<td>pGEM-1, 2.1-kb EcoRI-HindIII insert from pRC093</td>
<td>LCZ</td>
</tr>
<tr>
<td>pRCEH2</td>
<td>pGEM-2, 2.1-kb EcoRI-HindIII insert from pRC093</td>
<td>LCZ</td>
</tr>
<tr>
<td>pRCZ+</td>
<td>pGEM7Zf', 2.1-kb EcoRI-HindIII insert from pRC093</td>
<td>LCZ</td>
</tr>
<tr>
<td>pRCZ-</td>
<td>pGEM7Zf', 1.97-kb HindIII-ecori insert from pRC093</td>
<td>—</td>
</tr>
<tr>
<td>pRCZEM+</td>
<td>pGEM7Zf', 1.35-kb EcoRI-SphI insert from pRC093</td>
<td>—</td>
</tr>
<tr>
<td>pRCZEM*</td>
<td>pGEM7Zf', 1.38-kb EcoRI-Sau3AI insert from pRC093</td>
<td>—</td>
</tr>
<tr>
<td>pRCZEX+</td>
<td>pGEM7Zf', 1.62-kb EcoRI-XbaI insert from pRC093</td>
<td>—</td>
</tr>
<tr>
<td>pRCZEB+</td>
<td>pGEM7Zf', 1.86-kb EcoRI-BglII insert from pRC093</td>
<td>LCZ</td>
</tr>
</tbody>
</table>

Exonuclease deletions

<table>
<thead>
<tr>
<th>Plasmid or cosm</th>
<th>Restriction map analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRCZ-1</td>
<td>pRCZ+, deletion from HindIII terminus, 1.97-kb insert</td>
</tr>
<tr>
<td>pRCZ-2</td>
<td>pRCZ-, deletion from HindIII terminus, 1.74-kb insert</td>
</tr>
<tr>
<td>pRCZ-1</td>
<td>pRCZ+, deletion from EcoRI terminus, 1.93-kb insert</td>
</tr>
<tr>
<td>pRCZ-2</td>
<td>pRCZ-, deletion from EcoRI terminus, 1.58-kb insert</td>
</tr>
</tbody>
</table>

* Restriction enzyme sites and exonuclease deletion sites have been mapped by DNA sequence analysis (7b).

—. No clearing zone and no detectable activity by reducing sugar assay.

of LCZ, was isolated and completely digested with HindIII, EcoRI, or BamHI. Digests were ligated into appropriately restricted pGem-1, transformed into E. coli HB101, and screened for endoglucanase activity on CMC plates. A number of transformants derived from each digest gave CMC-positive clones, indicating that all three restriction enzyme sites were absent from the functional endoglucanase gene. The EcoRI-derived clone pRCE harbored an insert of approximately 3.3 kb. HindIII digestion of this insert resulted in a 2.1-kb EcoRI-HindIII fragment which was cloned in pGem-1, generating pRCEH. Restriction map analysis of the pRCEH insert (Fig. 1) indicated an absence of sites for AatII, NsiI, PstI, SacI, SalI, Smal, and XhoI. Fragments from digestion with Sau3AI, which cut three times, and Apal, BglII, SphI, and XbaI, which cut only once each, were ligated into pGEM7Zf+, transformed into E. coli HB101, and screened onto CMC plates. Only clone pRCE+ (EcoRI-BglII insert) exhibited cellulase activity. However, activity was not stably maintained in cells harboring this recombinant, and the 2.1-kb EcoRI-HindIII fragment was considered to be the minimum-sized restriction enzyme fragment that would encode stable endoglucanase activity. It was noted that cloning of DNA derived from F. succinogenes AR1 in pGem-1, pGEM7Zf+, pGEM7Zf*, pHCG79, and pKK232-8 (6) was generally successful, whereas cloning of recombinants constructed in pTZ18U and pTZ19U (Biorad, Sydney, Australia) and PBS* (Stratagene, Sydney, Australia) were frequently unstable. pGem7Zf* and pGem7Zf+ were most frequently used for subcloning and exonuclease deletion analysis because of the large number of suitable restriction enzyme sites in the multiple cloning site. Endoglucanase activity from the 2.1-kb fragment in pRCEH and pRHE2 was independent of orientation, indicating that the gene was expressed from its own promoter in E. coli. Deletion analysis (Table 1) indicated that the functional gene was encoded within a fragment of approximately 1.7-kb delimited at the EcoRI end by pRCZ+1 and at the HindIII end by pRCZ−1.

Southern blot analysis. Seven of the recombinant cosmids producing LCZ, pRC093, and cosmids 2 to 7 (see Table 1 for description) showed equivalent bands of approximately 3.4 kb when they were hybridized with 32P-labeled probe synthesized from the pRCEH insert (Fig. 2). By contrast, cosmids 8 (LCZ) and pDA44 (SCZ) revealed an absence of hybridization under both high- and low-stringency conditions of hybridization and washing. Moreover, when the filter was stripped and then reprobed with a 6-kb HindIII fragment (cellulase encoding) derived from pDA44, only the corresponding 6-kb band in lanes B and N (Fig. 2a) indicated hybridization (data not shown). These observations indicated that three distinct genes are encoded by (i) pRC093 and cosmids 2 to 7, which produce LCZs, (ii) cosmids 8 (LCZ), and (iii) pDA44 (SCZ). Southern blot analysis of the EcoRI-HindIII fragment from pRCEH hybridized to F. succinogenes AR1 genomic DNA digested with HindIII showed a single band of hybridization under high- and low-stringency conditions. This indicated that F. succinogenes AR1 probably encodes only a single copy of the gene and that there is an absence of other genes showing a high degree of similarity to it. It was noted that the genomic band (Fig. 2a, lane L) appeared to be slightly smaller than the corresponding cosmids bands (Fig. 2a, lanes D through J). This may have been due to a gel artifact, perhaps caused by overloading.

Cellular localization of endoglucanase in E. coli. Determination of the cellular distribution of endoglucanase activity, as estimated by reducing sugar analysis by using the dinitrosalicylic acid method (32) or the procedure of Nelson (35)
Physiochemical properties of endoglucanase activity. It was noteworthy that expression of endoglucanase activity of E. coli clones was observed under aerobic or anaerobic growth conditions. Cellobiose or glucose (100 mM) had no effect on the expression of endoglucanase activity, as determined by clearing zone size on plates containing CMC and glucose or cellobiose. Endoglucanase pH profiles were constructed, and activity was high (>50% of maximum activity) between pH 4.5 and 8.0, peaking at about pH 5.0 for E. coli HB101 harboring pRCEH (Fig. 3). Endoglucanase activity showed a sharp decrease below pH 4.5 and a more gradual decrease at alkaline pH values. Endoglucanase activity was maximum at

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TABLE 2. Cellular distribution of endoglucanase in E. coli HB101 harboring pRCEH and E. coli DH1 harboring pRCO93

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (mU/ml) of original culture</th>
<th>Endoglucanase&lt;sup&gt;a&lt;/sup&gt;</th>
<th>pNP-galactosidase</th>
<th>pNP-alkaline phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pRCO93</td>
<td>pRCEH</td>
<td></td>
</tr>
<tr>
<td>Extracellular</td>
<td></td>
<td>6 (16)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7 (17)</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Periplasmic</td>
<td></td>
<td>30 (78)</td>
<td>32 (78)</td>
<td>ND</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td></td>
<td>2 (6)</td>
<td>2 (5)</td>
<td>0.4 (100)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>38 (100)</td>
<td>41 (100)</td>
<td>0.4 (100)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Endoglucanase activity was determined by the method of Nelson (35), and cellular distribution was confirmed by a viscometric assay.

<sup>b</sup> Values in parentheses are percentages of total activity.

<sup>c</sup> ND, Not detectable (0.1 mU/ml or less).
37 to 39°C, decreasing markedly at elevated temperatures. This was reflected in thermal stability at 39°C compared with that at 50°C (Fig. 4). At 39°C, the activities of the enzyme fractions, which were buffered in 50 mM citrate phosphate (pH 6), remained high (75 to 100%) for 8 h, whereas endoglucanase was completely lost after 3 h when it was stored at 50°C. By comparison, endoglucanase activity was retained for several months at 0 to 4°C. The molecular weight of the endoglucanase was estimated by gel filtration to be approximately 46,500.

Mode of action and substrate specificity. As measured with an Ubbelohde viscometer, a rapid increase in CMC fluidity was observed with periplasmic enzymes from E. coli harboring pRCEH, and this was indicative of an endo-acting enzyme. Activity on natural substrate (cellulbiose) or substituted substrate (PNP-glucoside) was not detectable. These substrates are diagnostic for β-glucosidase activity. Low activity on pNP-cellulobiose, 0.72 nmol/min/mg of protein, indicated that exoglucanase activity was also minimal. Similarly, activity was not detected on other natural or substituted short-chain glycans (maltose, lactose, sucrose, melibiose, melezitose, pNP-xylolse, pNP-lactoside, and pNP-glucoside). It was noted that trehalose was actively hydrolyzed by periplasmic enzymes from E. coli HB101 and DH1 (7a).

Activity on recalcitrant substrates such as filter paper, cotton, and Avicel was significant (Table 3). Less crystalline celluloses were more extensively hydrolyzed, with the highest activity detected on acid-swollen cellulose. It was noted that hydrolysis of CMC resulted in the rapid production of reducing sugar, with >70% maximum production observed after 1 h and essentially 100% observed by 4 h. Specific activity on CMC was about 70 nmol/min/mg of protein (30-min assay). Activity on lichenan (1,3-1,4-β-D-glucan), but not on laminarin (1,3-β-D-glucan), indicated that it was probably the 1,4-β-D-glucan component of lichenan that was being hydrolyzed. It was noteworthy that the endoglucanase was also active on xylan.

### TABLE 3. Hydrolysis of glycan substrates after 24 h of incubation with periplasmic enzymes from E. coli HB101 harboring pRCEH

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reducing sugar (μg of glucose)</th>
<th>% Activity relative to CMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC</td>
<td>26.8b</td>
<td>100</td>
</tr>
<tr>
<td>Avicel PH101</td>
<td>10.9</td>
<td>41</td>
</tr>
<tr>
<td>Filter paper</td>
<td>8.5</td>
<td>32</td>
</tr>
<tr>
<td>Cotton</td>
<td>2.4</td>
<td>9</td>
</tr>
<tr>
<td>Ball-milled cellulose</td>
<td>46.2</td>
<td>173</td>
</tr>
<tr>
<td>Acid-swollen cellulose</td>
<td>165.4</td>
<td>618</td>
</tr>
<tr>
<td>Laminarin</td>
<td>NDc</td>
<td>ND</td>
</tr>
<tr>
<td>Lichenan</td>
<td>13.4</td>
<td>50</td>
</tr>
<tr>
<td>Xylan</td>
<td>15.8</td>
<td>59</td>
</tr>
</tbody>
</table>

* Reducing sugars were determined by the method of Nelson (35) after incubation of periplasmic enzymes in 50 mM sodium citrate buffer (pH 6) for 24 h. Each substrate was 5 mg/ml, except for Avicel, filter paper, and cotton, which were 50 mg/ml.

* Production of reducing sugar on CMC at 1 h was 72% of the level at 24 h and was essentially 100% by 4 h.

* ND. Not detectable (1 μg or less in 24 h).

### DISCUSSION

Genomic DNA from F. succinogenes AR1 was successfully cloned into E. coli by using cosmid pH79, and a number of recombinants expressed endoglucanase activity. Distinct LCZs and SCZs on CMC plates were features of endoglucanase expression in E. coli. A similar phenomenon has been reported by other authors during the construction of gene libraries of Cellulomonas (14), Clostridium thermocellum (40), Ruminococcus albus (21), and F. succinogenes (8, 9, 24). In all cases a correlation between clearing zone diameter and endoglucanase activity was observed. Southern blot analysis revealed the isolation of three separate genes encoding endoglucanase (Fig. 2). It is possible that other unique endoglucanase genes are represented in the family of 25 clones that produced SCZs. The fact that two separate genes were found for the eight clones that produced LCZs indicates that this possibility is likely. Multiple endoglucanase genes have been identified for other cellulolytic bacteria, and in C. thermocellum, for example, 15 distinct endoglucanase genes have been isolated (18). In F. succinogenes S85, a number of endoglucanases have been identified, and three have been characterized in particular (30, 31). It will thus be useful to isolate and characterize all the genes encoding endoglucanases from our gene bank, because this will lead to a comprehensive understanding of the endoglucanase requirements for this microorganism.

In view of the recent reclassification of Fibrobacter as a genus distinct from the genus Bacteroides and, furthermore, to the suggestion that it may form a new family distinct from the family Bacteroidaceae (33), it was significant that the endoglucanase genes in pRCEH and pRCEH2 were expressed in E. coli, indicating that promoters from F. succinogenes may be recognized by E. coli RNA polymerase. Data from DNA sequencing and primer extension analysis to map transcription start sites would be useful to confirm the presence of functional promoters within the cloned insert.

Delimitation of the boundaries of the functional gene in pRCEH was undertaken by subcloning restriction fragments in pGem7Zf+ and constructing nested deletions from pRCZ+ and pRCZ−. As a result, a 1.7-kb fragment was identified as being important for the expression of endoglucanase. This indicated that the possible molecular weight of the encoded protein could be up to 62,000. However, preliminary data
indicated that the molecular weight of the periplasmic endoglucanase was 46,500, which suggested that regions in addition to the coding sequence are required for activity.

In _F. succinogenus_ AR1, 80% of endoglucanase activity was found in the extracellular medium (7a), and a similar level was found in strain S85 (16). In _E. coli_ harboring pRCEH and pRC093, most activity (approximately 80%) was localized in the periplasm (Table 2). _E. coli_ is not noted for its ability to secrete cloned products into the extracellular medium; however, secretion to the periplasm is normally efficient (38). Most cloned cellulases are secreted to the periplasm, and DNA sequence analysis has revealed that all cellulases to date have signal peptides predicted for their amino acid sequences (4).

Maximum endoglucanase activity was at pH 5.0, which is around the range (pH 5.5 to 7.0) reported for cloned endoglucanases from _F. succinogenes_ (44), _R. albus_ (25, 37, 47), and _Bacteroides ruminocloea_ (48). Maximum activity of extracellular endoglucanase from _F. succinogenes_ AR1 is observed at pH 5.5 (7a), which is similar to that for the single gene product cloned in _E. coli_.

_F. succinogenes_ exhibits a diverse range of enzyme activities with the ability to solubilize lignocellulosic materials (23). Some of these properties were reflected in the broad range of substrates hydrolyzed by the gene product of pRCEH (Table 3). Most endoglucanases that have been cloned from ruminal bacteria show broad-spectrum activity on celluloseous compounds. Kinetic studies were not undertaken; however, it was noteworthy that activities on acid-swollen cellulose and ball-milled cellulose were higher than that on CMC. Initial production of reducing sugars on CMC was, in fact, high, but within 4 h, hydrolytic action essentially ceased. This can be explained by an endoglucanase attack on the portions of CMC that have at least three or more consecutive unsubstituted glucose units, resulting in only about 2% of CMC that can be readily hydrolyzed (11, 27).

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