An Acetobacter xylinum Insertion Sequence Element Associated with Inactivation of Cellulose Production

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Received 10 April 1991/Accepted 6 July 1991

An insertion sequence (IS) element, IS1031, caused insertions associated with spontaneous cellulose deficient (Cel') mutants of Acetobacter xylinum ATCC 23769. The element was discovered during hybridization analysis of DNAs from Cel' mutants of A. xylinum ATCC 23769 with pAXC145, an indigenous plasmid from a Cel' mutant of A. xylinum NRCC 17005. An IS element, IS1031/B, apparently identical to IS1031, was identified on pAXC145. IS1031 is about 950 bp. DNA sequencing showed that the two elements had identical termini with inverted repeats of 24 bp containing two mismatches and that they generated 3-bp target sequence duplications. The A. xylinum ATCC 23769 wild type carries seven copies of IS1031. Southern hybridization showed that 8 of 17 independently isolated spontaneous Cel' mutants of ATCC 23769 contained insertions of an element homologous to IS1031. Most insertions were in unique sites, indicating low insertion specificity. Significantly, two insertions were 0.5 kb upstream of a recently identified cellulose synthase gene. Attempts to isolate spontaneous cellulose-producing revertants of these two Cel' insertion mutants by selection in static cultures were unsuccessful. Instead, pseudorevertants that made waxlike films in the liquid-air interface were obtained. The two pseudorevertants carried new insertions of an IS1031-like element in nonidentical sites of the genome without excision of the previous insertions. Taken together, these results suggest that indigenous IS elements contribute to genetic instability in A. xylinum. The elements might also be useful as genetic tools in this organism and related species.

Transposable elements are discrete DNA segments capable of moving to new sites in the genome without requiring extensive sequence homology. Such elements seem to be present in all living organisms (for recent reviews, see references 6 and 20). In prokaroytes, insertion sequences (IS) are small, phenotypically cryptic transposable elements of 750 to 2,500 bp. IS elements are often present in the bacterial genome as repetitive sequences, and most contain terminal inverted repeats (IRs) and generate target sequence duplications upon insertion. Transposition of an IS may lead to gene inactivation or activation of nearby genes and may promote a variety of DNA rearrangements (for reviews, see references 15 and 19).

The genus Acetobacter contains several species which are being extensively studied in connection with acetic acid production and cellulose formation. There have been several reports of spontaneous mutations in Acetobacter strains affecting both morphological and physiological properties, including loss of the ability to produce cellulose (23, 29-33, 35). Bacteria of this genus are not genetically well characterized, and genetic investigations of the phenotypic instabilities have not been reported.

Gram-negative Acetobacter xylinum has been used in our laboratory as a model for genetic studies of cellulose biosynthesis (36-40). Most strains of A. xylinum examined contain a rather complex system of plasmids (11, 37-39). Alterations in the plasmid profile were found in both cellulose-negative (Cel') mutants and cellulose-producing (Cel') revertants of A. xylinum NRCC 17005 (formerly ATCC 10245). These plasmid rearrangements often generated either a 49-kb plasmid (pAXC245) of medium copy number or a 44-kb plasmid (pAXC145) of high copy number. A more detailed characterization of these two plasmids revealed that both shared extensive sequence homology with the host chromosome (or a nonisolatable replicon). pAXC145 was found to be almost identical to pAXC245, but the difference in size could not simply be explained by a deletion of 5 kb from the 49-kb plasmid (39). Recently, it was found that pAXC145 hybridized to several DNA fragments in digested total DNA from A. xylinum ATCC 23769, whereas no hybridization was observed with pAXC245 as the probe (11). These results indicated the presence of an additional DNA sequence in the 44-kb plasmid that was lacking in the 49-kb plasmid.

This paper reports the discovery of an IS, IS1031, whose transposition seems to cause spontaneous mutations interrupting cellulose formation in A. xylinum ATCC 23769. An apparently identical IS element (IS1031/B) was identified on pAXC145, and this element was absent on pAXC245. To my knowledge, this is the first identification of transposable elements in the genus Acetobacter.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. A. xylinum wild type and Cel' mutants were grown statically or with shaking as indicated at 30°C in a previously described medium (17). Escherichia coli cells were grown in Luria-Bertani medium (22) on a gyratory shaker at 37°C. Selective antibiotic concentrations for E. coli were as follows: 100 μg of ampicillin per ml and 15 μg of tetracycline per ml.

Isolation of cellulose-negative mutants of A. xylinum. Each spontaneous Cel' mutant was isolated as follows: one single wild-type colony from agar plates streaked from stocks of A. xylinum ATCC 23769 kept at -70°C was inoculated in liquid medium and incubated at 30°C with shaking. After a few days of incubation, a small aliquot of the dispersed phase of cells was transferred to fresh medium and incubated as
described above. A process of selective sampling leading to the accumulation of Cel− cells (40) was continued until Cel− colonies representing one spontaneous Cel− mutant were identified by visual inspection of the agar plates streaked from the last culture.

Mutagenesis of _A. xylinum_ cells with NG (N-methyl-N'-nitro-N-nitrosoguanidine) was essentially as described previously (40).

**Isolation and purification of plasmids and total DNA, restriction enzyme analysis, and agarose gel electrophoresis.** Total DNA from _A. xylinum_ was isolated and purified as described previously (39). Large-scale isolation and purification of _E. coli_ plasmids and minipreparation of recombinant plasmids were performed by the methods of Davis et al. (12). Purified DNA was digested with restriction endonucleases purchased from Amersham, Bethesda Research Laboratories (BRL), and New England BioLabs according to the manufacturers’ instructions. Restriction digest of total DNA and plasmid DNA were separated by horizontal agarose gel electrophoresis (39).

**Recovery of DNA from agarose gels.** DNA fragments generated by restriction enzyme digestion and separated by agarose gel electrophoresis were recovered from gel slices by electroelution in dialysis bags (34) or in a Biotrap BT 1000 device (Schleicher & Schuell) as described by the manufacturer or by electroelution from agarose gels onto DE81 filters (Whatman) (13). pAXC145 was purified from low-melting-point agarose gels by a combination of agarose melting at 65°C and phenol extraction (41).

**Molecular cloning of DNA.** The plasmids pBR322, pUC18, pUC19, pGEM3, and pGEM4 were used as cloning vectors (Table 1). Cloning was performed essentially as described elsewhere (22). Ligated DNA was transformed into HB101 or DH1 cells made competent by the CaCl2 method (22) or into “Library Efficiency,” DH5 or DH5α competent cells (BRL) according to the manufacturer’s instructions, selecting for resistance to ampicillin (100 µg/ml).

**Southern blot, slot blot, and colony blot hybridizations.** DNA was transferred from agarose gels to Hybond-N membranes (Amersham) by electroblotting (38) or modified Southern blotting (25). Recombinant plasmids were depurinated (0.25 N HCl; 15 min), denatured by the addition of NaOH to 0.5 N, and then transferred to nylon membranes with a Bio-Dot SF microfiltration apparatus (Bio-Rad Laboratories) according to the manufacturer’s instructions. Colonies containing pBR322 with inserts were transferred to and grown on nitrocellulose filters (BA85; Schleicher & Schuell) (22). Lysis of bacteria on filters and prewashing were performed as described elsewhere (43). DNA probes were labeled with [α-32P]dCTP (3,000 Ci/mmol; Amersham) by using nick translation kits (Amersham) according to instructions provided by the manufacturer. DNA hybridizations were performed either at 37°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–0.1% sodium dodecyl sulfate (SDS)–1 mM Na₂EDTA–75 µg of calf thymus DNA (sonicated and denatured) per ml–1× Denhardt’s solution followed by washes of blots at 65°C in SSC–0.1% SDS to a final concentration of 0.2× SSC, essentially as described previously (1) or at 65°C in 0.5 M NaP, (pH 7.2)–7% SDS–1 mM Na₂EDTA, followed by washes at 65°C first in 40 mM NaP,1% SDS and finally in 20 mM NaP,1% SDS (9, 16).
DNA sequence determination. The sequenced DNAs are shown in Fig. 2B and C and in Fig. 4B. pDCG9ΔB10 and pDCG9ΔB29 were constructed by unidirectional exonucleolyse III-generated deletions of the insert in pDCG9 (Fig. 2B) with the Erase-a-Base System (Promega) according to the instructions of the manufacturer. All plasmids were propagated in DH5 or DH5α. Double-stranded plasmid was isolated by the boiling miniprep method (1), and DNA templates were denatured by alkali and prepared for sequencing essentially as previously described (8). DNA sequence determinations were performed by the chain-terminating method (27) employing the Sequenase kit (United States Biochemical Corp.) and end-labeled ([γ-32p]ATP; >5,000Ci/ mmol; Amersham) M13 Universal primer (U.S. Biochemical) for inserts in pUC19 in addition to SP6 and T7 promoter primers (Promega) for inserts in pGEM3 and pGEM4.

RESULTS

Discovery of a putative transposable element in cellulose-negative mutants of A. xylinum. The observation that a DNA sequence on pAXC145, absent on pAXC245 (see Introduction), hybridized to identical and/or similar DNA sequences reiterated in the genome of A. xylinum ATCC 23769 suggested the existence of putative mobile DNA elements (11).

The biosynthesis of cellulose by several A. xylinum strains has been shown to be a rather unstable trait, and isolation of spontaneous Cel− mutants is easily accomplished. Therefore, it was interesting to examine whether the formation of spontaneous Cel− mutants of A. xylinum ATCC 23769 was associated with the movement of a DNA sequence homologous to that in pAXC145, thereby indicating transposition.

Southern blots of HindIII-digested total DNA from the wild type and five independently isolated spontaneous and two NG-induced Cel− mutants of A. xylinum ATCC 23769 were hybridized with purified 32P-labeled pAXC145. The result showed that two of the spontaneous Cel− mutants contained an extra, but presumably identical, hybridization signal that was lacking in the wild type (Fig. 1). This extra hybridization signal indicated insertion of a mobile DNA element. No additional hybridization signals were observed in the three other spontaneous Cel− mutants or the two NG-induced Cel− mutants (data not shown).

Molecular cloning and characterization of a DNA fragment showing similarity to that in pAXC145. Total DNA from pAXC145 was digested with HindIII, and DNA fragments were separated by preparative electrophoresis on an agarose gel (1.0%). DNA fragments in a gel slice containing the 3.1-kb DNA fragment (Fig. 1) were electroeluted and cloned into the HindIII site of pBR322. Recombinant plasmids containing DNA fragments homologous to pAXC145 were identified by colony blot, slot blot, and Southern blot hybridizations. Of 200 recombinant plasmids, only three hybridized to pAXC145. Restriction enzyme analysis revealed that all three plasmids contained an identical DNA fragment of 3.1 kb cloned in the same orientation. A restriction map of the 3.1-kb fragment in pDCB188 is shown in Fig. 2A. Hyridization analysis of Southern blots of pDCB188, singly or doubly digested with several restriction endonucleases, showed that pAXC145 hybridized to a DNA sequence of about 950 bp, the location of which is shown in Fig. 2A.

The 3.1-kb HindIII fragment in pDCB188 was purified by electroleution from an agarose gel slice. Southern hybridization of restriction enzyme-treated total DNA from Cel1 and Cel2 with 32P-labeled 3.1-kb fragment and pAXC145 plasmid, respectively, showed that the sites inserted by the IS element were within 100 bp (data not shown). Thus, the insertion sites may be identical in these two independent mutants.

Evidence of transposition of IS1031, an indigenous IS of A. xylinum. pDCB188 was digested with BamHI and religated, generating the recombinant plasmid pDCB188ΔIS, containing the flanking sequence of the putative IS element (Fig. 2A). Hybridization of digested total DNA from A. xylinum ATCC 23769 wild type and Cel1 with pDCB188ΔIS as a probe is shown in Fig. 3. The results demonstrated that the flanking sequence hybridized to a fragment about 950 bp larger in PstI- and BglII-digested total DNA from Cel1 compared with the wild type. Since PstI and BglII cut outside the element, these results indicate that the insertion in Cel1 occurred by transposition of a DNA element, hereafter called IS1031 (registered by the Plasmid Reference Center [21]), of approximately 950 bp.

Hybridization analysis of HindIII-digested total DNA from the wild type and Cel1 revealed that HindIII cut inside the IS element (Fig. 3). However, DNA sequencing showed that HindIII cuts only 24 bp from the right end (see below and Fig. 5A).

Localization and analysis of an IS element apparently identical to IS1031 in pAXC145. Hybridization of pAXC145 to IS elements in total DNA from A. xylinum ATCC 23769 suggested that this 44-kb plasmid contained a putative IS element. To localize the IS element in pAXC145 and more directly test whether this element was absent from pAXC245, the 3.1-kb HindIII fragment containing IS1031 (Fig. 2A) was used to probe a Southern blot of BamHI-digested pAXC145 and pAXC245. Figure 4A shows hybridization to a 18.9-kb BamHI fragment from pAXC145 (lanes 1 and 3), whereas no hybridization is observed to any of the BamHI fragments (strong bands in lane 2) generated from

FIG. 1. Southern blot hybridization of HindIII-digested total DNA from the A. xylinum ATCC 23769 wild type (Wt) and a spontaneous Cel− mutant (Cel1) with 32P-labeled pAXC145. The DNA fragment responsible for the extra hybridization signal at 3.1 kb was electroeluted from a gel slice and cloned into pBR322. Size markers are HindIII-digested λ DNA.

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pAXC245 (lane 4). Since the plasmid preparation of pAXC245 also contained plasmids other than pAXC245 present in A. xylulm NRCC 17005 Cell1R (39), the weak bands in lane 2 are due to DNA fragments generated by BamHI digestions of these plasmids. The hybridization to weak bands (Fig. 4A, lane 4) showed that, although pAXC245 lacked IS1031-like elements, other plasmids residing in Cell1R contained sequences homologous to IS1031. That the hybridization signals in Fig. 4A, lane 4, were due to homology with IS1031 and not the flanking sequence in the 3.1-kb fragment was confirmed by using an internal fragment of IS1031 as the probe (data not shown).

Probing Southern blots of restriction enzyme-digested pAXC145 with the 3.1-kb HindIII fragment localized the IS element to a 1.8-kb Clal fragment (Fig. 4B). This Clal fragment was cut out of pSVK2, electroeluted from the agarose gel, and cloned into the AccI site of pUC19 (in DH5α). The recombinant plasmid pDCU41 was mapped, and the putative IS element was localized by hybridization analysis with 32P-labeled 3.1-kb HindIII fragment. The results depicted in Fig. 4B showed that EcoRV, HindII and HindIII cut inside the IS element from pAXC145 at the same sites as found within IS1031 (Fig. 2A). The size of the two elements was also equal. Therefore, IS1031 and the IS element in pAXC145, hereafter called IS1031B, may be identical.

FIG. 2. Restriction endonuclease maps of DNA fragments containing the putative IS element cloned from total DNA of A. xylulm ATCC 23769 Cell (A and B) and of the DNA fragment generated by the IS insertion in Cell1 cloned from total DNA of ATCC 23769 wild type (C). For a description of the restriction fragments cloned, see Table 1. Localization of the IS element is shown ( ) (only the HinfI site inside the element is shown). ( ), site of the IS insertion in Cell1; ——, direction and extent of nucleotide sequencing; , pBR322; , pGEM3 and pGEM4; , pUC18.

FIG. 3. Evidence of transposition of IS1031. Total DNAs from A. xylulm ATCC 23769 wild type (Wt) and Cell1 digested with PstI, BgIII, and HindIII were Southern blot hybridized with 32P-labeled pDCB188AIS (Fig. 2A) containing a flanking sequence of IS1031. A DNA fragment with IS1031 insertion (Cell1); 0, same fragment without IS insertion (Wt).

Nucleotide sequence analysis of the borders of IS1031 and IS1031B and of the IS1031 target site in the wild type. Since HindIII cut inside IS1031, a small part of the IS element was absent in pDCB188 (Fig. 2A). However, Southern hybridization of total DNA from the wild type and Cell1 with 32P-labeled pDCB188AIS and the 3.1-kb HindIII fragment from pDCB188 (Fig. 2A), respectively, showed that the complete IS1031 element was located within a 2.5-kb BamHI-PstI fragment (data not shown). This BamHI-PstI fragment was electroeluted from a gel slice, cloned into the BamHI-PstI site of pGEM3 (in DH5), and identified by Southern blot and slot blot hybridizations with the 3.1-kb HindIII fragment as the probe. Figure 2B shows the restriction map of the 2.5-kb BamHI-PstI fragment (pDCG9) with the location of IS1031 and the maps of pDCG9A10 and pDCG9A29 that were used to sequence the borders of IS1031. The nucleotide sequence around the ends of IS1031/B was determined with the subclones pDCU41 and pDCU41A1ES as depicted in Fig. 4B.

The 300 nucleotides of IS1031 and IS1031B sequenced to date were identical. Both elements carry terminal, imperfect IRs of 24 bp (with two mismatches) and are flanked by 3-bp direct repeats as shown in Fig. 5A (IS1031) and B (IS1031B). However, a duplication of 5 bp and IRs of 22 bp also fit with the DNA sequence of the borders of IS1031. The DNA sequence of the insertion site in the wild-type DNA could not discriminate between these two possible sizes of the target sequence (Fig. 5C; see below). On the other hand, the DNA sequence of the junctions of IS1031/B revealed an unambiguous 3-bp (TTA) duplication of target DNA (Fig. 5B). Hence, it is reasonable to conclude that a 3-bp sequence (TGA) is also the target site for IS1031 (Fig. 5A). The structural features of the borders of the IS1031 elements together with their sizes classified them as IS elements.

To confirm that IS1031 had inserted into the new site in Cell1 by a transposition event, I electroeluted the 2.46-kb PstI fragment from the wild-type DNA (Fig. 3), cloned it into pUC18 (Fig. 2C), and sequenced the target site region in the subcloned 437-bp BamHI-EcoRI fragment (pTDG211), as
shown in Fig. 2C. Comparison of the DNA sequence of the target region (Fig. 5C) with the sequences flanking IS1031 in Cell (Fig. 5A) provided evidence of a transposition of IS1031 into the target site with a concomitant duplication of the target sequence. IS1031 has inserted upstream of the cellulose synthase catalytic subunit gene in Cell. Recently, Wong et al. (42) reported the characterization and nucleotide sequence of a cellulose synthase operon from A. xylinum 1306-3, while Saxena et al. (28) described the cloning and sequencing of a cellulose synthase catalytic subunit gene of A. xylinum ATCC 53582.

I have sequenced a 700-bp region in the A. xylinum ATCC 23769 wild-type genome containing the target site inserted by IS1031 in Cell. This sequence (data not shown) extending from the BamHI site to 263 bp downstream of the EcoRI site in pTDU21 (Fig. 2C) was compared with the sequences from A. xylinum 1306-3 (42) and ATCC 53582 (28) deposited in the EMBL data base. The alignment showed that the last 510 bp of the sequenced part of the target region was almost identical (98% identity) with the first 502 nucleotides of the sequence from A. xylinum ATCC 53582, while no similar homology to the DNA sequence from A. xylinum 1306-3 was found. Furthermore, the restriction enzyme sites mapped downstream of the IS insertion site in pTDU21 (Fig. 2C) were localized at the same positions in the sequence from ATCC 53582 (28). Thus, the 9.9-kb HindIII fragment from the A. xylinum ATCC 23769 wild type (Fig. 3) seems to be identical to the HindIII fragment which Saxena et al. (28) found to contain the cellulose synthase catalytic subunit gene. Interestingly, the insertion site of IS1031 in ATCC 23769 Cell1 was localized 518 bp upstream of the translation start codon (position 636) of the gene for the catalytic subunit of cellulose synthase from ATCC 53582 (see Discussion).

Mutagenesis in A. xylinum by indigenous IS elements. As shown above, two of five spontaneous Cel' mutants of A. xylinum ATCC 23769 were apparently due to the transposition of IS1031. In spite of the lack of a selectable marker in IS1031, this rather high frequency of IS-induced Cel' mutants of ATCC 23769 would facilitate the analysis of cellulose biosynthesis in this strain. However, the identical insertions of IS1031 in the two independently isolated Cel' mutants (Cell1 and Cell2) could indicate a preferred insertion site. To clarify whether indigenous IS elements could be used in transposon mutagenesis and examine the distribution of insertion sites, I isolated 12 new independent, spontaneous Cel' mutants of A. xylinum ATCC 23769. Southern blots of HindIII- and BglII-digested total DNAs from these mutants were probed with 32P-labeled 3.1-kb HindIII fragment containing IS1031 (Fig. 2A). The results showed that six of the mutants had alterations in their IS1031 profiles compared with that of the wild type, as demonstrated by the HindIII digestions in Fig. 6. Comparison of the autoradiograms from the HindIII (Fig. 6) and BglII (data not shown) analyses indicated that four of the six mutants had single insertions of an element homologous with IS1031. Cel9, on the other hand, had two insertions, while Cel17 exhibited a more complex alteration of its IS profile, indicating a DNA rearrangement. Interestingly, at least four of the insertions are in different sites in the genome, showing that the IS element has no pronounced target site specificity. It is also interest-
ing to observe that the flanking DNA sequence of IS1031 in the 3.1-kb HindIII fragment (Fig. 2A) hybridized to the wild-type HindIII fragment of 9.9 kb (Fig. 3) in apparently all the new transposon mutants, showing that the six IS insertions are outside this 9.9-kb fragment (Fig. 6).

It has been reported previously (40) that cellulose production can be induced phenotypically in some Cel− mutants of A. xylinum when protein synthesis is blocked. In preliminary experiments, the 17 spontaneous Cel− mutants were tested for cellulose induction by arresting the protein biosynthesis in cells of early-log-phase cultures with tetracycline, as described by Valla and Kjosbakken (40). By visual inspection of aggregate formation, I found that one insertion mutant (Cel9) and five of the nine noninsertion mutants showed induction of cellulose biosynthesis. The lack of cellulose induction in the seven Cel− mutants containing new IS insertions indicates that these insertions are in genes encoding proteins involved in the formation of cellulose.

Reversion of IS-mediated Cel− mutants. Cel− cells of A. xylinum outgrow Cel− cells in static cultures by enabling the Cel+ cells to reach the air-liquid interface, where the supply of oxygen is abundant (30, 40). The Cel1 and Cel2 mutants were grown in static cultures to select Cel+ revertants and thus investigate whether the cellulose-producing ability could be restored in them and whether reversion was due to excision of the additional IS insertions. However, in several attempts, no normal cellulose Pellicle appeared on the surface of the medium. Rather, in every reversion experiment, waxlike films were formed at the air-liquid interface. The films were very fragile and easily broken into small pieces by light shaking. Cells in these waxlike films formed by the Cel1 and Cel2 mutants were pseudorevertants (designated Cel1RW and Cel2RW); when streaked on agar plates, they formed colonies that were morphologically distinguishable from both the wild type and the parental Cel− mutants. Interestingly, Schramm and Hestrin (30) also observed formation of zoogale films on the surface of liquid medium by Cel− mutants grown in static cultures. The films were fragile and dissolved in hot 4% sodium hydroxide, indicating that the cementing substance was not cellulose.

In order to examine the profile of IS elements in the two pseudorevertants, Cel1RW and Cel2RW, total DNA isolated from cells in the waxlike films was digested with HindIII and was Southern blot hybridized with 32P-labeled 3.1-kb HindIII fragment containing IS1031 (Fig. 2A). Figure 7 shows that both pseudorevertants have new IS1031-like insertions in nonidentical sites without losing the insertions at 3.1 kb. It has been shown that transposition usually occurs at a higher frequency than the precise excision of an IS element from an inactivated gene that is required to restore gene activity (5, 19). Consequently, an interpretation of the reversion experiments described here could be that the new insertions of the IS element induced the production of a waxlike substance, making the cells float to the surface, where the oxygen supply is sufficient. This would release the selective pressure necessary for the selection of cellulose revertants formed by a precise excision of IS1031.

One attempt to revert the other six insertion Cel− mutants and the nine Cel− mutants without detectable IS insertions was made. Only the insertion mutant Cel9, which showed cellulose induction, and six noninsertion mutants reverted to cellulose-producing cells in this single experiment. The rest of the insertion mutants (five) and noninsertion mutants (three) made thin films more or less similar to the waxlike
clear whether IS elements were involved in the generation of these two plasmids.

Although pAXC245 lacks IS1031B, other plasmids residing in *A. xylinum* NRCC 17005 Cel1R exhibit homology with IS1031 (Fig. 4A, lanes 2 and 4). Hence, IS elements are probably located in several of the plasmids detected in various *A. xylinum* strains. These elements may play an important role in the plasmid profile alterations observed in this bacterium (11, 39).

Half of the spontaneous Cel" mutants of *A. xylinum* ATCC 23769 contained insertions of IS1031-like elements. Similar frequencies of spontaneous mutations caused by IS insertions in *E. coli* (15) and *Halobacterium halobium* (24) have also been seen. Since the majority of the insertions in the mutants of *A. xylinum* ATCC 23769 are in different sites and the target site is only 3 bp, no insertion hot spot seems to be involved. However, some kind of regional insertion specificity cannot be excluded. It is worth mentioning that recent reports have suggested the involvement of IS elements in the control of extracellular polysaccharide formation in *Pseudomonas atlantica* (3, 4) and in the variation of xanthan formation in *Xanthomonas campestris* pv. *campesiris* (18). In *Zoogloea ramigera*, instability in extracellular polysaccharide production seemed to be associated with DNA rearrangements (14).

Genes encoding proteins directly involved in the biosynthesis of cellulose have recently been isolated and cloned (28, 42). Interestingly, the cellulose deficiency in the spontaneous Cel1 (and Cel2) mutant of *A. xylinum* ATCC 23769 seems to be caused by the insertion of IS1031 upstream of the cellulose synthase catalytic subunit gene (28). Furthermore, the preliminary sequence (700 bp) of the target site region in the ATCC 23769 wild type contains a putative open reading frame preceding the *BamHI* site (Fig. 2C) and with a translation stop codon at position 606 (position 408 in the sequence from *A. xylinum* ATCC 53582 (28)). The last 117 bp of this open reading frame showed 66% identity with a continuous stretch (nucleotides 29 to 145) in the sequence from *A. xylinum* 1306-3 (42).

It should be noted, however, that in two previous reports (28, 42) different polypeptides have been assigned to be the catalytic subunit of cellulose synthase. The deduced amino acid sequence of the cellulose synthase catalytic subunit described by Saxena et al. (28) is equivalent to the sequence of the putative protein encoded by the *bcsA* gene and not the *bcsB* gene which Wong et al. (42) found to encode the catalytic subunit of cellulose synthase in *A. xylinum* 1306-3. However, the protein encoded by the *bcsB* gene was found by computer sequence analysis to be equivalent to a putative protein encoded by an open reading frame downstream of the catalytic subunit gene in ATCC 53582 (28). Thus, the cellulose synthase operon seems to have a similar organization in both of these *A. xylinum* strains and accordingly in ATCC 23769. This suggests that the insertion of IS1031 in Cel1 (and Cel2) is upstream of the transcription start of the operon. If it is assumed that the cellulose synthase operon in *A. xylinum* ATCC 23769 is of similar size as that in strain 1306-3 (42), a few kilobases of the downstream part of the operon are lacking in the 9.9-kb *HindIII* fragment. Six spontaneous Cel" mutants contained IS insertions outside this 9.9-kb fragment. The cellulose deficiency in these mutants may be due to insertions (i) in the region of the operon outside this 9.9-kb fragment, (ii) in genes representing known functions in the production of cellulose but which are not part of the cellulose synthase operon (26), or (iii) in genes whose role in this formation has not yet been identified.
However, direct evidence for the association between these insertions and the formation of Cel+ mutants remains to be found.

In summary, the rather frequent transposition of IS1031 observed as insertions in Cel+ mutants and pseudorevertants of *A. xylinum* suggests that indigenous IS elements might be important contributors to genetic instability. These IS elements might facilitate the elucidation of cellulose biosynthesis in *A. xylinum* and provide new tools for genetic studies both in this organism and in other related species of the genus *Acetobacter*.

ACKNOWLEDGMENTS

I thank Rune Standal for computer sequence analysis.

This study was supported by grants from the Norwegian Research Council for Science and Humanities and the Royal Norwegian Council for Scientific and Industrial Research and by a fellowship from The Norwegian Institute of Technology, the University of Trondheim.

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