Identification of the *exo* loci required for exopolysaccharide synthesis in *Agrobacterium radiobacter* NCIB 11883

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(Received 13 May 1991; revised 25 June 1991; accepted 8 July 1991)

We initiated a genetic analysis of *Agrobacterium radiobacter* NCIB 11883 with particular reference to the (*exo*) genes required for exopolysaccharide synthesis. Following mutagenesis with nitrosoguanidine, several *exo* mutant strains were isolated and several of the mutations were corrected by DNA cloned in a newly constructed cosmid library. Analysis of various complementing cosmids by genetic and physical criteria indicated that *exo* loci were quite widely dispersed in the bacterial genome. Certain *exo* mutations were corrected by different cosmids that shared no homologous DNA; possible explanations for this are presented. Using *phoA* fusions, it was shown that some *exo* genes were, or were closely linked to, genes that specified polypeptides associated with the bacterial cell surface. By introducing the cloned *exo* genes of *Rhizobium meliloti* it was found that only one out of thirty *exo* mutants of *A. radiobacter* was corrected by a defined *exo* locus of the former species; further analysis indicated that this particular *exo* gene corresponded to *exoB* of *R. meliloti*. Finally, it was found that several *A. radiobacter* *exo* mutants were non-mucoid on media with dicarboxylic acids as sole carbon source but appeared to be wild-type when sugars were the source of carbon.

Introduction

Members of the genera *Agrobacterium* and *Rhizobium* share several features. Both interact with higher plants; *Rhizobium* species form nitrogen-fixing root nodules in association with legumes, whereas *Agrobacterium* species induce crown gall tumours or hairy roots on a range of dicotyledonous plants.

Agrobacterium is closely related to *Rhizobium meliloti* as judged by sequence comparison of the 16S RNA. Both these bacteria make various extracellular polysaccharides that are important for their interactions with their host plants. Thus, both *R. meliloti* and *Agrobacterium tumefaciens* make an extracellular polysaccharide cyclic 1,2- β -D-glucan, that is required for normal nodule formation and the induction of crown galls respectively. In *R. meliloti*, two genes, *ndvA* and *ndvB* are required for the manufacture and export of this glucan (Geremia *et al.*, 1987) and in *A. tumefaciens* *chvA* and *chvB* are responsible for its synthesis and export, respectively. These two sets of genes (*ndv* and *chv*) are in both cases chromosomal and are homologous and functionally equivalent; the defects of *chv* mutants can be corrected

by cloned *ndv* genes and vice versa (Dylan *et al.*, 1986). In addition, the major high-molecular-mass acidic exopolysaccharide (EPS) produced by both genera is a succinoglycan, which consists of repeating units of β -linked glucose and galactose (7:1) with acidic side groups (Hisamatsu *et al.*, 1980; Sutherland, 1985). Mutants of *R. meliloti* which fail to make this polymer induce aberrant, non-fixing nodules which are devoid of bacteria (see for example Finan *et al.*, 1986; Leigh *et al.*, 1986).

There has been considerable interest in the *exo* genes of *R. meliloti*, which are needed for EPS synthesis. Several *exo* loci (*exoP*, *exoN*, *exoM*, *exoA*, *exoL*, *exoK*, *exoH*, *exoJ*, *exoG*, *exoF*, *exoQ* and *exoB*) have been located in a cluster on a large indigenous plasmid and two other loci, *exoC* and *exoD*, are on the chromosome (Long *et al.*, 1988b; Finan *et al.*, 1986). In addition there are two unlinked chromosomal loci, *exoR* and *exoS*, whose products negatively regulate the synthesis of EPS (Doherty *et al.*, 1988). The biochemical function of two of the *exo* genes has been established: *exoB* appears to encode a UDP-galactose-4-epimerase (Buendia *et al.*, 1991) and sequence analysis of *exoB* revealed that its product is similar (39% identity) to the epimerase encoded by the *galE* gene of *Escherichia coli*.

In *Agrobacterium*, studies on *exo* genes have been less detailed than in *Rhizobium*. Cangelosi *et al.* (1987)

Abbreviations: AMP, 2-amino-2-methyl-1-propanol; CAPSO, 3-(cyclohexylamino)-2-hydroxy-1-propanesulphonic acid; EPS, acidic exopolysaccharide; XP, 5-bromo-4-chloro-3-indolyl phosphate.

isolated several *exo* mutant strains of *A. tumefaciens* and isolated recombinant plasmids from a clone bank of genomic DNA which could correct them. Interestingly, more than 75% of these *exo* mutants could be corrected by cloned *exo* loci of *R. meliloti*. However, some mutants, in a locus termed *exoG*, failed to be corrected by any known *exo* gene of *R. meliloti* (Cangelosi *et al.*, 1987). Of the *exo* mutants of *A. tumefaciens*, only those in the *exoC* locus were defective in tumorigenesis. Significantly, the *exoC* mutants were pleiotropically defective; they were also unable to make cyclic 1,2- β -D-glucan and it was proposed that it was this that abolished tumour formation rather than the production of the acidic exopolysaccharide *per se*. Further, it has been shown that the inability of *exoC* mutants to synthesize 1,2- β -D-glucan and succinoglycan is due to a deficiency in the activity of the enzyme phosphoglucomutase which in wild-type bacteria converts glucose 6-phosphate to glucose 1-phosphate, an intermediate in the synthesis of UDP-glucose (Uttaro *et al.*, 1990).

The products of several *exo* genes appear to be associated with the bacterial membrane or periplasm (Long *et al.*, 1988b; Latchford *et al.*, 1991). This was deduced following the construction of fusions between *exo* genes and a defective form of *phoA*, the gene that specifies alkaline phosphatase (Manoil & Beckwith, 1985). This *phoA* lacks a promoter, ribosome binding site and the leader sequence that is responsible for the targeting of the enzyme to the periplasm. Since the enzyme is only active in the periplasm, this defective *phoA* can only express alkaline phosphatase if it is fused, in-frame, to a gene whose product is normally located in the periplasm or is inserted into the bacterial membrane such that at least part of the AP fusion is located in the periplasm.

In this study we report the isolation of mutants of *A. radiobacter* strain NCIB 11883 which are defective in the production of EPS and the molecular cloning and characterization of some of the *exo* genes in this strain.

Methods

Bacterial strains, plasmids, media and growth conditions. The strains used are presented in Table 1, together with the plasmids that were employed and constructed in this study. *Escherichia coli* strains were cultured on LB medium (Maniatis *et al.*, 1982) at 37 °C. *Rhizobium leguminosarum* strain 8401 was grown on TY medium (Beringer, 1974) at 28 °C. *A. radiobacter* was grown at 28 °C on either LB or TY medium or minimal medium MOD-D2, pH 7.0, containing (g l⁻¹): KH₂PO₄, 3.0; Na₂HPO₄, 3.0; (NH₄)₂SO₄, 0.3; MgSO₄·7H₂O, 0.2; CaCl₂·2H₂O, 0.015; FeCl₃, 0.067; plus trace elements; 1 ml from a stock solution containing (g l⁻¹): CaCl₂·6H₂O, 0.66; ZnSO₄, 0.18; CuSO₄, 0.16; MnSO₄, 0.15; CoCl₂, 0.18; H₃BO₃, 0.10; Na₂MoO₄·2H₂O, 0.30. MOD-D2 was supplemented with D-glucose (5 g l⁻¹), glycerol (5 g l⁻¹) or sodium succinate (5 g l⁻¹) as sole C source as indicated.

Genetic manipulations. pLAFR1-based plasmids were transferred into *A. radiobacter* strain T1305 (a derivative of NCIB 11883) in triparental crosses in which *E. coli* containing the plasmid pRK2013 was used as the helper strain (Figurski & Helinski 1979). Selection for transfer of the recombinant plasmids was done by plating on medium containing streptomycin at 200 µg ml⁻¹ (to which T1305 is resistant) plus tetracycline at 2–5 µg ml⁻¹ (against which pLAFR1 confers resistance).

Cells of *A. radiobacter* were mutagenized with nitrosoguanidine (NTG) as follows. Approximately 10⁸ cells were spread on a Petri dish of TY medium and, following incubation at 28 °C for 8 h, some solid NTG was deposited in the centre of the plate. After 2 d incubation there was a zone of clearing around the NTG; with a sterile loop, bacteria were harvested from the edge of this zone of clearing, suspended in 20% (v/v) glycerol and then plated on various media following appropriate dilutions.

Transposon mutagenesis. (a) Tn5lac (Simon *et al.*, 1989). *E. coli* strain A118 contains a chromosomally located copy of Tn5lac (A. Economou, unpublished). Plasmids pBIO11 and pBIO21 (see Results) were introduced into strain A118 by transformation and the derivatives were used as donors in triparental crosses with the *polA* Nal^r *E. coli* strain C2110 as recipient (the mobilizing plasmid pRK2013 does not survive in this strain). Selection was made on media containing nalidixic acid, kanamycin and tetracycline (50, 20 and 5 µg ml⁻¹ respectively). Thus derivatives of these two plasmids containing random insertions of the transposon were isolated. Initially, a collection of Tn5lac insertions into each plasmid was mobilized *en masse* from *E. coli* C2110 to one or more of the *A. radiobacter* *exo* mutants that was corrected by pBIO11 and/or pBIO21. Several non-mucoid transconjugants were obtained, suggesting that they contained mutant plasmids with the transposon in the appropriate *exo* gene. However, despite repeated attempts, using different methods, it proved impossible to transform *E. coli* using preparations from these non-mucoid bacteria. Therefore, colonies of C2110(pBIO11::Tn5lac) or C2110(pBIO21::Tn5lac) were gridded out and the various mutant plasmids were mobilized into appropriate *exo* mutant strains and into wild-type *A. radiobacter*. In the latter case, attempts were made to introduce the insertions into the genome by marker exchange (Ruvkun & Ausubel 1981).

(b) TnphoA (Manoil & Beckwith, 1985). Plasmids pBIO11 or pBIO21 were introduced into *E. coli* strain A143, which contains a chromosomal copy of TnphoA (A. Economou, unpublished). *R. leguminosarum* strain 8401 was used as a recipient in triparental crosses with these *E. coli* strains, selection being for Kan^r (TnphoA), Tet^r (pLAFR1), Str^r (8401) transconjugants. The reasons for this choice of recipient were (1) that we thought TnphoA insertions might be rare and (2) the difficulty – see above – of getting recombinant plasmids out of strain T1305. We felt that the behaviour of any fusions would be similar in *Rhizobium* and in the closely related *A. radiobacter*, although of course this could not be guaranteed. Thus derivatives of pBIO11 and pBIO21 containing TnphoA were isolated. Colonies were replicated to medium containing 5-bromo-4-chloro-3-indolyl phosphate (XP; 40 µg ml⁻¹). From those colonies that were blue, plasmid DNA was isolated (Hombrecher *et al.*, 1984) and the plasmids were introduced into *E. coli* by transformation.

DNA manipulations. Transformation of *E. coli*, digestion of DNA with restriction endonucleases and ligations were done essentially as described by Maniatis *et al.* (1982). Plasmid DNA was isolated from *E. coli* as described by Holmes & Quigley (1981) or according to the protocol recommended by Hybaid with their Qiagen kit.

Plasmid DNA was isolated from *Rhizobium* and *A. radiobacter* as described by Grosveld *et al.* (1981) for *E. coli* and modified for these bacteria as described by Hombrecher *et al.* (1984).

A cosmid clone bank of *A. radiobacter* was constructed by isolating genomic DNA then, following partial digestion with *EcoRI* and size-fractionation on a NaCl gradient, ligating fragments 25–30 kb in size

Table 1. *Bacterial strains and plamids*

Strain or plasmid	Relevant properties	References
<i>A. radiobacter</i>		
NCIB 11883	Field isolate, produces large amounts of succinoglycan EPS	Linton <i>et al.</i> (1987)
T1305	Derivative of NCIB 11883; Str ^r	J. D. Linton (unpublished)
Exo 7	EPS ⁻ derivatives of T1305	This study
Exo 51.1		
Exo 53.1		
Exo 53.2		
Exo 61.1		
Exo 63		
Exo 64		
Exo 65.2		
Exo 66.1		
Exo 66.3		
Exo 70.1	EPS ⁻ derivative of T1305 (EPS ⁻ on succinate, EPS ⁺ on glucose)	
Exo 73.1	EPS ⁻ derivative of T1305	
Exo 73.2	EPS ⁻ derivative of T1305 (EPS ⁻ on succinate, EPS ⁺ on glucose)	
Exo 74	EPS ⁻ derivative of T1305 (EPS ⁻ on succinate, EPS ⁺ on glucose)	
Exo 81	EPS ⁻ derivatives of T1305	
Exo 82		
Exo 84.1		
Exo 84.2		
Exo 85		
Exo 87.1	T1305::Tn5, EPS ⁻ derivative of T1305	
Exo 87.2		
SGN-1	J. D. Linton (unpublished)	
<i>R. leguminosarum</i>		
8401	A derivative of strain 8002 cured of the symbiolytic plasmid pRP2JI, Str ^r	Lamb <i>et al.</i> (1982)
<i>E. coli</i>		
803	Met ⁻ ; used as a recipient in routine transformations	Wood (1966)
C2110	Nal ^r PolA ⁻	Stachel <i>et al.</i> (1985)
CC118	Lac ⁻ ; used for routine transformations	Manoil & Beckwith (1985)
A118	CC118::Tn5lac; Kan ^r	A. Economou (unpublished)
A143	C2110::Tnp ϕ A; Nal ^r , Kan ^r	
Plasmids		
pRK2013	Used for mobilizing P1-group plasmids, Kan ^r	Figurski & Helinski (1979)
pPH1J1	P-group plasmid used for marker exchange; Gent ^r , Spc ^r , Str ^r	Beringer <i>et al.</i> (1978)
pLAFR1	Wide-host-range P-group cloning vector; Tet ^r	Friedman <i>et al.</i> (1982)
pMP220	Wide-host-range P-group cloning vector; Tet ^r , lacZ	Spaink <i>et al.</i> (1987)
Recombinant plasmids		
pD34	<i>R. meliloti</i> DNA containing <i>exoP</i> , <i>N</i> , <i>M</i> , <i>A</i> , <i>L</i> , <i>K</i> , and <i>H</i> cloned in pLAFR1; Tet ^r	Long <i>et al.</i> (1988b)
pD56	<i>R. meliloti</i> DNA containing <i>exoJ</i> , <i>G</i> , <i>F</i> , <i>Q</i> , and <i>B</i> cloned in pLAFR1; Tet ^r	
pD15	<i>R. meliloti</i> DNA containing <i>exoC</i> cloned in pLAFR1; Tet ^r	Leigh <i>et al.</i> (1986)
pEX312	<i>R. meliloti</i> DNA containing <i>exoP</i> , <i>N</i> , <i>M</i> , <i>A</i> , <i>L</i> , <i>K</i> , <i>H</i> , <i>J</i> , <i>G</i> , <i>F</i> , <i>Q</i> cloned in pLAFR1	Long <i>et al.</i> (1988b)

to *Eco*RI-cut pLAFR1 DNA (Downie *et al.*, 1983). Following packaging into λ phage packaging mix (Stratagene), approximately 10000 Tet^r transfectants were obtained.

For hybridization experiments, genomic or plasmid DNA was transferred to a Hybond-N filter (Maniatis *et al.*, 1982) and was probed with digoxigenin-labelled DNA probes according to the manufacturer's (Boehringer) instructions.

Results

Isolation of Exo⁻ mutants of *A. radiobacter*

Following mutagenesis of *A. radiobacter* strain T1305 with NTG, approximately 2000 survivors were plated on

MOD-D2 medium containing one of L-glycerol, D-glucose or sodium succinate as sole C source. After 3 d incubation at 28 °C the colonies were inspected and any that were less mucoid than the wild type were purified and retained for further study.

Completely non-mucoid colonies occurred at a frequency of approximately 1% on each of the media. In addition to the completely non-mucoid mutants, other colonies were seen that were semi-mucoid or which had an altered 'wrinkled' appearance, indicating that they too were in some way affected in EPS production. However, these 'leaky' mutants were not studied further.

Some Exo mutants are EPS⁻ on media containing succinate but are EPS⁺ on medium containing glucose or glycerol

Following purification of the EPS⁻ mutants from the three different media they were each examined on media containing glycerol, glucose or succinate. All those that were EPS⁻ on the glucose or glycerol media were also non-mucoid on all three media. However, five of the mutants (Exo 63, 73.1, 73.2, 66.1 and 74) that were EPS⁻ on medium containing succinate as sole C source were fully mucoid and had an apparently wild-type phenotype on media containing glycerol or glucose as carbon source. These five strains were then plated on MOD-D2 supplemented with one of several carbon sources. All of these mutants were fully mucoid on media containing glucose, galactose, ribose, fructose, maltose, citrate or pyruvate, but on media in which the dicarboxylic acids fumarate or succinate were the sole C source, the colonies appeared to be completely EPS⁻.

When *A. radiobacter* strains were grown on minimal medium (pH 7.0) containing glucose and a universal indicator (BDH) the pH was found to drop to around 6.5 after 4–5 d growth. However, if succinate was used as the C source the pH rose from 7 to 9 during the same period of time. The wild-type strain of *A. radiobacter* is slightly less mucoid on medium containing succinate as a C source than when glucose is added. To establish whether the 'Suc⁻ Glu⁺' strains are EPS⁻ as a result of the high pH on medium containing succinate, Exo 73.1 and the wild-type strain T1305 were grown on MOD-D2 medium supplemented with succinate and 50 or 100 mM-HEPES (pH 7) for 2–3 d. The presence of 50 or 100 mM-HEPES maintained a pH of around 7 in cultures supplemented with succinate but EPS production was not improved and growth was unaffected. Conversely, maintaining a pH of 9 in media buffered with 10 mM-CAPS or AMP and supplemented with glucose as C source did not reduce EPS production in the wild-type or strain Exo 73.1. Therefore we conclude that the conditional phenotype of this mutant is not due to differences in pH.

Identification of recombinant plasmids that correct exo mutants of A. radiobacter.

Fragments of *EcoRI*-digested *A. radiobacter* DNA approximately 30 kb in size were ligated to pLAFR1 and packaged into bacteriophage λ . Approximately 10000 primary transfectants were obtained. Given the size of the bacterial genome and the size of the cloned inserts, this number is certainly sufficient to represent the entire genome in the clone bank.

To identify recombinant plasmids which corrected *exo* mutants, the recombinant plasmids were mobilized *en*

masse in triparental matings into representative *exo* mutants of *A. radiobacter*. Approximately 1000 transconjugants for each recipient were plated on MOD-D2 medium containing the C source on which the mutants were initially identified. These colonies were inspected for any that were mucoid, indicating that the *exo* mutation had been corrected by a recombinant plasmid. For approximately half of the recipient strains at least one colony was mucoid; for the remaining *exo* mutants which failed to be corrected it is likely that this may be due simply to statistical sampling since approximately 1000 recombinant plasmids is only just sufficient to give a 90% probability of isolating a given gene.

Plasmid DNA was isolated from the mucoid colonies and was introduced into *E. coli* by transformation, selecting for tetracycline resistance. The recombinant plasmids were then re-introduced by conjugation from *E. coli* into the *A. radiobacter* strain in which their effect on EPS production had initially been detected. In most cases, as expected, the appropriate *exo* mutant recipient was uniformly corrected by the reintroduced plasmid; where this was not the case, it is possible that the original mucoid colonies were due to reversion of the *exo* mutation, or, perhaps the recombinant plasmid had undergone some rearrangement during the course of its isolation or transformation. In any event, these plasmids were not examined further.

exo genes are located in at least five different regions of the genome

To determine if any of the plasmids that contained *exo* genes shared the same cloned DNA, representative plasmids were labelled with digoxigenin and used as probes to DNA from each of the other plasmids which had been digested with *EcoRI*, run on an agarose gel and transferred to a nylon membrane (Southern, 1975). As judged by the sizes of restriction fragments in different plasmids, together with the patterns of hybridizing fragments, it was clear that the eleven plasmids that were studied fell into five groups in which the cloned DNA of one group did not hybridize with that of any of the others. Three of the groups contained more than one, non-identical plasmids which had two or more *EcoRI* fragments in common.

As shown in Fig. 1, the plasmids in group 1 (pBIO15, pBIO16 and pBIO17) shared a total of 18 kb and those in group 3 (pBIO12 and pBIO24) have 10 kb (two fragments) in common. The five plasmids (pBIO2, pBIO10, pBIO13, pBIO14 and pBIO21) in group 2 all shared two fragments comprising 12.1 kb. Note that in this group, pBIO2 also contained an *EcoRI* fragment that did not 'fit' – it seems likely that this was due to the addition of a piece of non-contiguous DNA during the cloning steps.

Fig. 1. Restriction maps of recombinant plasmids containing cloned *exo* genes. Plasmids are arranged in groups; those in the same group share cloned DNA as judged by hybridization studies. RI, *Eco*RI sites. An asterisk indicates contiguous fragments that have not been ordered. The dotted line in pBIO2 refers to a fragment that probably came from elsewhere in the genome. The numbers refer to the sizes of fragments (kb).

Table 2. *exo* alleles corrected by *A. radiobacter* recombinant plasmids

Plasmids	<i>exo</i> alleles corrected
Group 1 pBIO17	87.1, 87.2, 7
pBIO15	87.1, 87.2, 7
pBIO16	87.1, 87.2, 7
Group 2 pBIO13	81, 82, 66.1*, 70.1, 51.1, 73.2*, 66.3, 53.1, 61.1
pBIO21	81, 82, 66.1*, 70.1, 51.1, 73.2*, 66.3, 53.1, 61.1, 65.2, 73.1*, 53.2, 63*, 74*
pBIO10	81, 82, 66.1*, 70.1, 51.1, 73.2*, 66.3, 53.1, 61.1, 65.2, 73.1*, 53.2, 63*, 74*
pBIO2	81, 82, 66.1*, 70.1, 51.1, 73.2*, 66.3, 53.1, 61.1, 65.2, 73.1*, 53.2, 63*, 74*
pBIO14	81, 82, 66.1*, 70.1, 51.1, 73.2*, 66.3, 53.1, 61.1, 65.2, 73.1*, 53.2, 63*, 74*
Group 3 pBIO12	84.1, 84.2
pBIO24	84.1, 84.2, 85
Group 4 pBIO25	SGN-1
Group 5 pBIO11	81, 82, 66.1*, 51.1, 53.1, 65.2, 53.2, 63*

* indicates *exo* strains which were EPS⁻ on medium containing succinate but EPS⁺ on glucose.

Two other plasmids, pBIO11 and pBIO25 each contained approximately 30 kb of cloned DNA which, as judged by the hybridization experiments, did not overlap with each other nor with any of the plasmids named above.

The incomplete *Eco*RI restriction maps of these 5 'contigs' are shown in Fig. 1 and the *exo* alleles that they correct are listed in Table 2.

Genetic characterization of the plasmids containing *exo* genes

Each of the plasmids identified above, in addition to being transferred into the *exo* mutant which they had initially been found to correct, was also mobilized separately into all of the *exo* mutants that had been isolated. To a large extent, the results were consistent with the physical classification of the plasmids in that plasmids in the same group tended to correct the same sets of *exo* alleles. Thus pBIO15, pBIO16 and pBIO17 (group 1) all corrected *exo*7, *exo*87.1 and 87.2, pBIO12

and pBIO24 corrected *exo84.1* and *exo84.2* and pBIO24 also corrected *exo85*. pBIO25, which did not overlap with any of the other cosmids, only corrected the EPS⁻ strain SGN-1, the strain which had initially been used to detect it.

For the group 2 plasmids the patterns were more complex (Fig. 1, Table 2). Three alleles, *exo73.2*, *exo81* and *exo82*, were fully corrected by all five plasmids and six others, *exo51.1*, *exo53.1*, *exo70.1*, *exo61.1*, *exo66.3* and *exo66.1* were partially corrected by all the plasmids; in those that were partially corrected the transconjugants had an appearance intermediate between the wild-type and the original mutant. A further five alleles, *exo53.2*, *exo65.2*, *exo63*, *exo73.1* and *exo74*, were corrected by four of the plasmids (pBIO21, pBIO10, pBIO2 and pBIO14) suggesting that the above four alleles are in or near the DNA common to these plasmids but absent from pBIO13 which failed to correct these alleles. As indicated in Fig. 1 and Table 2, the correction pattern of a few alleles was not readily reconcilable with the maps of the overlapping plasmids. One possibility is that those alleles may in fact not be single point mutations; in this connection, it is relevant to note that the mutagen used, NTG, is known to induce multiple mutations at the region of the DNA replication fork.

As indicated in Table 2, several of the mutants that were EPS⁻ on succinate media but EPS⁺ on glucose media were corrected by members of the group 2 plasmids. Thus these C-source-dependent *exo* mutations are closely linked to several 'unconditional' *exo* alleles.

Correction of some exo alleles by different DNA sequences

The mutant containing the *exo82* allele was the one that had been initially used to isolate pBIO11. It was surprising therefore to find that this mutation was also corrected by all the plasmids in group 2; as described above, these plasmids have completely different restriction patterns from pBIO11 and, as judged by the hybridizations, contain no homologous DNA. Further, pBIO11 was found to correct several of the *exo* mutants that had originally been used to obtain members of the group 2 plasmids (Table 2). Some, but not all, of the C-source-dependent mutants were corrected by pBIO11 but there was no clear pattern relating those alleles that were corrected by pBIO11 to those that were corrected only by the other group 2 plasmids.

The group 2 region (represented by pBIO21) and pBIO11 were studied in more detail (a) because of the apparently paradoxical ability of different regions of DNA to correct the same alleles and (b) because each could complement the novel C-source-dependent *exo* mutants.

Subcloning of exo DNA from pBIO11 and pBIO21

(i) *pBIO11*. By a combination of single and double digests a restriction map of pBIO11 was established (Fig 2a). One feature of the cloned DNA was that it appeared to be under-represented in restriction sites. For example the only *EcoRI* sites were at the site of ligation to the vector, the insert DNA being a single 30 kb *EcoRI* fragment. Also the cloned DNA contained no *BamHI* or *SacI* sites. The significance of this paucity of certain restriction sites is not clear.

Following digestion of pBIO11, six *SphI* fragments were obtained. Each was individually cloned into pMP220 and the resultant plasmids were individually mobilized into each of the *exo* mutants that were corrected by pBIO11. One of these plasmids, pBIO343 (Fig 2a), containing an 11.8 kb *SphI* fragment, corrected all the mutants. A 4 kb *HindIII* fragment from pBIO343 was further subcloned into pMP220 to form pBIO344. Again, the resultant plasmid corrected all the *exo* mutants that were complemented by pBIO11. (Note that, as shown in Fig. 2a, the 'right hand' *HindIII* site was derived from the linker in pBIO344.)

(ii) *pBIO21*. A preliminary restriction map of pBIO21 is shown in Fig. 1 and a more extensive one is in Fig. 2(b). By subcloning various fragments of the insert DNA from pBIO21, it was found that a 4 kb *EcoRI*-*BamHI* fragment, cloned into pMP220 to form pBIO60, was sufficient to restore EPS synthesis to all of the *exo* mutants that were corrected by pBIO21 itself.

Thus the *exo* regions in pBIO11 and pBIO21 were located in two fairly small segments of DNA in the two plasmids.

Mutagenesis of pBIO11 and pBIO21 with Tn5lac

Approximately 400 derivatives of *E. coli* C2110 containing pBIO11 or pBIO21 with *Tn5lac* insertions were used as donors in crosses with wild-type *A. radiobacter* or appropriate *exo* mutants as recipients. Following transfer into the wild-type, attempts were made to introduce the *Tn5lac* into the corresponding position in the genome by introducing the PI group plasmid pPH1JI and screening for derivatives that were Kan^r (*Tn5lac*) Gent^r (pPH1JI) and Tet^s (pLAFR1). However, no non-mucoid mutants were found, indicating either that, by chance, none of the insertions were into cloned *exo* genes, or that there is a problem in marker exchanging in this system. Subsequent observations indicated that the latter was the case.

When mutant derivatives of pBIO11 and pBIO21 were introduced into appropriate *exo* mutants, the following results were obtained.

Fig. 2. Localization of *exo* genes in pBIO11 and pBIO21. (a) Map of pBIO11 showing *Eco*RI (RI), *Sal*GI (Sa) and *Sph*I (Sp) sites. Dimensions of the subcloned fragments in pBIO343 and pBIO344 are shown. The box denotes the region in which the 30 'active' *phoA* insertions were located. For pBIO343, only relevant *Hind*III sites (H) are indicated; H† in pBIO344 represents a *Hind*III site from the pMP220 polylinker. (b) Map of pBIO21 showing *Eco*RI (RI) and relevant *Bam*HI (B) and *Hind*III (H) sites. The 4.0 kb *Eco*RI-*Bam*HI fragment which corrects the *exo* mutants and in which all the *exo*::*TnphoA* insertions were located is indicated as fragment A. Other *phoA* insertions which expressed alkaline phosphatase activity but which did not affect EPS production were located in fragments B and C.

(a) *pBIO11*. The pBIO11::Tn5*lac* mutant plasmids were mobilized from derivatives of *E. coli* C2110 (see Methods) containing the mutant plasmids into strains carrying the *exo81*, *exo82*, *exo63* and *exo53.1* alleles and the transconjugants were plated on MOD-D2 supplemented with glycerol or succinate as appropriate. For 10 of these mutant plasmids, the transconjugants of all four recipients were Exo⁻ (for the recipient with the *exo63* allele, the transconjugants with each of these 10 mutant plasmids were non-mucoid on succinate medium but Exo⁺ on glucose medium, just like the mutant strain itself). This observation indicated that the four alleles were in the same complementation group even though they differed in their phenotype.

Plasmid DNA was isolated from the corresponding ten C2110(pBIO11::Tn5*lac*) donor strains and used to transform *E. coli* 803 (it is difficult to isolate sufficient amounts of plasmid DNA from C2110 itself for restriction analysis: unpublished observation). The mutant plasmid DNA was digested with various restriction enzymes; consistent with the sub-cloning experiments (see above) all 10 mutant plasmids contained Tn5*lac* in the 4.0 kb *Hind*III fragment (cloned in pBIO344) that had been shown to correct all four *exo* mutants. The precise locations of the Tn5*lac* insertions within this fragment were not determined.

Having isolated insertional mutations which were clearly in *exo* genes corresponding to the original *exo* mutations, these pBIO11 *exo*::Tn5*lac* plasmids were transferred to wild type strain T1305 and attempts were made to introduce the Tn5*lac* into the genome by marker exchange. Following introduction of pPH1J1 into these derivatives of T1305, several hundreds of Gent^r Kan^r colonies were examined; all were mucoid, indicating that, for some unknown reason, this strain was refractory to the procedure of marker exchange which has been so widely used in genetic studies of other Gram-negative bacteria.

(b) *pBIO21*. Using the same method as described in the latter part of the above section, 400 pBIO21::Tn5*lac* mutant plasmids were transferred from C2110 into each of the *exo* mutants that are complemented by pBIO21 itself. None of the transconjugants of any of the mutants was EPS⁻. It is not clear why such a low proportion of transposon insertions was into *exo* genes – restriction digests of a random selection of the pBIO21::Tn5*lac* plasmids showed that they did contain insertions of Tn5*lac* and that the insertions were in different positions (either in the vector or the cloned DNA).

Isolation of 'dominant' exo mutations in cloned A. radiobacter DNA following mutagenesis with Tn5lac

Following the mutagenesis of pBIO11 and pBIO21, mutant derivatives containing Tn5lac insertions were transferred into the wild-type strain. With pBIO11, it was found in 10 cases that the transconjugants were non-mucoid. Plasmid DNA was isolated from the corresponding *E. coli* donor strains and restriction mapping revealed that the insertions were close to the previously isolated *exo::Tn5lac* mutations; i.e. within the 4 kb fragment that had been cloned in pBIO344.

This observation was reminiscent of those of Gray *et al.* (1990) and Reed *et al.* (1991), who identified two genes, *exoX* and *exoY*, in *Rhizobium* sp. NGR 234 and *R. meliloti*, respectively. When these two pairs of genes were both cloned in a recombinant plasmid, such constructs had no obvious effects on EPS production. However, if there was an 'imbalance' in the ratio of *exoX* to *exoY* such that the former was in excess, then the strain would be non-mucoid. Such an 'imbalance' was obtained by cloning *exoX* separately from *exoY* or, more relevant to the observation reported here, by mutagenesis of the plasmid containing both *exoX* and *exoY* and isolation of insertions into the latter.

It is possible that in pBIO11 there may also be two genes, perhaps similar to *exoX* and *exoY* and that mutations in the latter cause a relative excess of *exoX* gene product. In order to locate the proposed *exoX*-like gene in pBIO11, derivatives of pBIO11 that contained the 'dominant' *exo* alleles were re-mutagenized with a derivative of Tn3 (Tn3 HoHo1) (Stachel *et al.*, 1985) and these secondarily mutated plasmids were re-introduced into wild-type strain T1305. Some mucoid colonies were identified, consistent with Tn3 HoHo1 having inserted into the *exoX*-like gene. Restriction mapping showed that these Tn3 HoHo1 insertions were closely linked to the original Tn5lac-induced dominant *exo* mutations; again they were located in the 4.0 kb *Hind*III fragment mentioned above.

It should be noted that when the pBIO11 *exo::Tn5lac* mutant plasmids which were identified on the basis of their inability to correct the NTG-induced *exo* mutants were introduced into wild-type strain T1305, the transconjugants were still mucoid showing that these mutations were not dominant.

Following a similar procedure involving the mutagenesis of pBIO21 with Tn5lac no dominant mutations were identified out of 200 tested.

TnphoA mutagenesis of pBIO11 and pBIO21

As described in the Introduction, the use of translational fusions between a 'target' gene and a defective *phoA* gene, lacking the signal sequence, has been of value for

deducing if the product of the target gene is located in the bacterial membranes or the periplasm. Indeed, Long *et al.* (1988a) thus showed that products of several *exo* genes had such a subcellular location. To test if any of the products of the genes (and, in particular, *exo* genes) in pBIO11 or pBIO21 were also at or near the cell surface, they were each mutagenized with TnphoA (see Methods). For each mutagenesis, approximately 5000 transconjugants were plated on complete medium supplemented with XP, a chromogenic indicator for alkaline phosphatase. Approximately 1% of the colonies were bluer than the background colour. Plasmid DNA was isolated from these and introduced into *E. coli* by transformation and these mutant plasmids were then re-introduced into both wild-type and appropriate *exo* mutant strains of *A. radiobacter*.

With pBIO11, 30 'active' insertions of TnphoA expressed alkaline phosphatase activity in *Rhizobium*. Each of these mutant plasmids also conferred enzymic activity when transferred to *A. radiobacter*. When mobilized into each of the *exo* mutants that are corrected by pBIO11, all of the 'active' TnphoA insertions restored them to an EPS⁺ phenotype, showing that the mutations were not in the *exo* loci defined by *exo81*, *exo82*, *exo63* and *exo51.1*. These TnphoA insertions were mapped and were found to be located in a cluster in the cloned DNA in pBIO11 approximately 12 kb from the *exo* loci that had been identified earlier (Fig. 2a). It remains to be determined if these *phoA* fusions are in other *exo* genes, which are in a different complementation group.

Following TnphoA mutagenesis of pBIO21, 22 alkaline-phosphatase-positive derivatives of *R. leguminosarum* were obtained. Each of these mutant plasmids was shuttled through *E. coli* and then into wild-type *A. radiobacter*; in all cases the *A. radiobacter* transconjugants were blue on media containing XP. Initially the mutant plasmids were introduced into the Exo⁻ strains containing the unusual (because they were also corrected by pBIO11) alleles *exo81* and *exo82*. It was found that 12 of the 'active' mutant plasmids no longer corrected the Exo⁻ defect of these strains. All 12 such insertions were in the same 4 kb *Eco*RI-*Bam*HI fragment that had been shown to correct all the *exo* alleles that were corrected by pBIO21 itself (region A in Fig. 2b). In all the other mutant plasmids which contained alkaline-phosphatase-positive insertions, the transposon was located close to, but not in the 4.0 kb *Eco*RI-*Bam*HI fragment (regions B and C in Fig. 2b). Complementation tests between the *exo* mutant AP⁺ plasmids and the other, original *exo* mutants were also attempted but the results were not readily interpretable (not shown). The arrangement of genes and complementation groups within the *exo* region will be precisely determined when the sequence of this region of pBIO21 and the exact location of the TN5*phoA* insertions are established.

Correction of one *exo* mutant of *A. radiobacter* by cloned *exoB* from *R. meliloti*

The succinoglycan of *R. meliloti* strain 1021 is similar if not identical in structure to that of *A. radiobacter* strain NCIB 11883. To test if the *exo* genes of the former could complement any of the *exo* mutants that we had isolated, four different plasmids containing defined *exo* loci of *R. meliloti* were introduced into each of the *exo* mutants of *A. radiobacter*.

Plasmids pD34 (containing *exoP*, *N*, *M*, *A*, *L*, *K*, *H*) and pD15 (*exoC*) corrected none of the *A. radiobacter* mutants but pD56 restored EPS production to one mutant, SGN-1, which had been induced by Tn5 mutagenesis (A. Cornish, personal communication). Plasmid pD56 contains the cloned *exoJ*, *G*, *F*, *Q* and *B* loci. To determine which of these corresponded to the locus mutated in strain SGN-1, different derivatives of pD56 with Tn5 in each of these loci were transferred to SGN-1. It was found that the mutant derivative with Tn5 in *exoB* was completely non-mucoid. Further, plasmid pEX312, which contains *exoP*, *N*, *M*, *A*, *L*, *K*, *H*, *J*, *G*, *F* and *Q*, but not *exoB*, also failed to correct the EPS-defect of SGN-1. It has recently been shown that the *exoB* locus encodes a UDP-galactose 4-epimerase (Buen-dia *et al.*, 1991).

Given the similarity in the EPSs of the two genera, we were surprised that so few of the *exo* mutants of *A. radiobacter* were corrected by *exo* loci of *R. meliloti*. In contrast, in similar experiments, Cangelosi *et al.* (1988) found that the majority of the *exo* mutants in a strain of *A. tumefaciens* were restored by one of the *exo* loci of *R. meliloti*.

In addition to the cloned *exo* genes of *R. meliloti*, plasmids pIJ3040 (Borthakur *et al.*, 1986) and pI10, which contain undefined *exo* genes required for the production of the xanthan polysaccharide of the phytopathogen *Xanthomonas campestris*, were mobilized into the *exo* mutants of *A. radiobacter*. None of the mutants was complemented. Further, plasmid pIJ1427 (Borthakur *et al.*, 1986) and seven cosmids containing cloned *exo* genes (Diebold & Noel, 1989) of *R. leguminosarum* were transferred to the *Agrobacterium* mutants; again none was restored to an EPS⁺ phenotype.

Discussion

In this paper we have described the isolation of mutants of a strain of *A. radiobacter* which are defective in the synthesis of the succinoglycan EPS and have initiated the characterization of the corresponding cloned DNA. A number of features have emerged from this work.

Firstly, it is clear that the *exo* genes are quite widely dispersed in this strain. We have identified five non-

overlapping groups of plasmids or 'contigs,' and we have isolated several other *exo* mutants which are corrected by none of the plasmids in these five groups. This dispersal of *exo* genes is in contrast to the situation in *R. meliloti*, where, apart from *exoC* and *exoD*, the known *exo* 'structural' genes are clustered on a large plasmid (Long *et al.*, 1988b; Finan *et al.*, 1986). Although not stated explicitly, examination of the results of Cangelosi *et al.* (1987) suggests that in *A. tumefaciens* the *exo* genes are also more scattered than in *R. meliloti*.

Several phenotypically distinct classes of *exo* mutants were isolated. These ranged from those that appeared to be completely non-mucoid to those which made reduced amounts of the polymer to some in which the colonies had a rough appearance. Of particular interest were those which had a wild-type morphology on media containing glucose or glycerol as sole C source but which were EPS⁻ on succinate- or fumarate-containing media. This difference was not due to the effects of pH on EPS production and at present its biochemical basis is not known. It is obvious that these mutants are not grossly defective in succinate uptake and/or utilization since they appear to grow perfectly well with succinate as the sole C source. Interestingly, these C-source-conditional alleles are closely linked to mutations that block EPS production irrespective of the C-source, since some of the 'group 2' plasmids and pBIO11 correct representatives of both classes of mutant.

It was striking that several *exo* mutants could be corrected by different cloned fragments of DNA that shared no detectable homology. We have no clear answer to this apparent paradox, but two testable models are presented here. One is that, like *R. meliloti* (Glazebrook & Walker, 1989), *A. radiobacter* has the potential to make two types of EPS molecule but that normally the synthesis of one of these is suppressed. However, by cloning the 'silent' *exo* genes, their expression may become constitutive (due, for example, to the activity of a vector promoter) and the 'second' EPS would be made. Thus, correction of these mutants by the group 2 plasmids would be by the restoration of the genuinely corresponding wild-type DNA but correction by pBIO11 would be due to the deregulated synthesis of the 'new' EPS (or vice versa). A second hypothetical model invokes a regulatory system. If the mutants that were corrected by both classes of plasmid were in a gene whose function were to activate expression of other 'structural' *exo* genes, then such mutants might be corrected either by introducing the corresponding DNA or by cloning the 'structural' *exo* genes such that they were expressed from a vector promoter and so are independent of the normal regulatory control.

Another unexplained phenomenon concerns the difficulty encountered in marker exchanging transposons from

a recombinant plasmid to the corresponding location in the genome. It may simply be that this strain of *A. radiobacter* has a much lower efficiency of recombination than (for example) *Rhizobium*. Whatever the reason, this deficiency, unless overcome, will hamper future genetic analysis in this system.

Sequence analysis initially indicated that at least some *exo* genes encoded proteins that were located in the bacterial membrane, since some had extremely hydrophobic regions (Borthakur *et al.*, 1988; Gray & Rolfe, 1990). This was confirmed by the use of *phoA* fusions into *exo* genes, several of which expressed alkaline phosphatase activity in *R. meliloti* and *R. leguminosarum* (Long *et al.*, 1988a; Latchford *et al.*, 1991).

Following mutagenesis of pBIO21 with Tn*phoA*, several 'active' insertions were identified which were found to be in *exo* genes since they abolished the ability of pBIO21 to complement one or more *exo* mutants. In contrast, the insertions of Tn*phoA* into pBIO11 which expressed alkaline phosphatase were not in the previously identified *exo* DNA but were 12 kb from it. These mutations were not marker-exchanged so it is not possible to say if they are in another, linked, *exo* locus on pBIO11.

The first demonstration that EPS production could be inhibited by the presence of multiple copies of a gene came from the work of Borthakur *et al.* (1985) and Borthakur & Johnston (1987). In *R. leguminosarum* biovar *phaseoli*, there is a gene termed *psi*, on the Sym plasmid, close to other nodulation and nitrogen-fixation genes. When *psi* is cloned, it inhibits EPS synthesis but this can be counteracted by the introduction of a cloned *pssA* (*exo*) gene which is required for EPS production (Borthakur & Johnston 1987). Thus the inhibitory effects of the excess of the *psi* product can be overcome by a corresponding increase in the levels of the *pss* product. Strikingly, as judged by sequence analysis and the use of *phoA* fusions, the products of both *psi* and *pssA* are located in the membrane, suggesting that there may be some post-translational interaction between them (Borthakur & Johnston, 1987; Latchford *et al.*, 1991).

A similar situation appears to exist in other rhizobial strains. Two genes, *exoX* and *exoY*, have been identified in *Rhizobium* sp. NGR234 and in *R. meliloti* which share some homology in their sequences and have similarities in their function relative to *psi* and *pssA*, respectively (Gray *et al.*, 1990; Zhan & Leigh, 1990). However, in these two strains *exoX* and *exoY* are adjacent to each other whereas *psi* and *pssA* are not only unlinked, but are on different replicons. When *exoX* and *exoY* are cloned on the same recombinant plasmid, mutations in the latter cause a relative excess of *exoX* gene product and hence result in a reduction of EPS production (Reed *et al.*, 1991; Gray *et al.*, 1990). A similar case seems to apply to

some *exo* genes of *A. radiobacter* cloned in pBIO11 since certain insertions, close to mutations that abolish EPS synthesis, result in a non-mucoid phenotype when the mutant plasmids are transferred to the wild-type strain of *A. radiobacter*. It will be of interest to see if pBIO11 contains genes equivalent to *exoX* and *exoY* or whether we have identified a different 'two-component' system.

Whereas Cangelosi *et al.* (1987) found that most of the *exo* mutants of *A. tumefaciens* could be corrected by the introduction of one of the cloned *exo* genes of *R. meliloti*, we found that only one out of 44 of the *exo* mutants that we had isolated in *A. radiobacter* was restored by an *R. meliloti* *exo* gene. This mutant, SGN-1, was corrected by the *R. meliloti* *exoB* gene, which has recently been shown to encode a UDP-galactose 4-epimerase (Buendia *et al.*, 1991). In view of the similarities of the succinoglycan EPS molecules of *R. meliloti* and of *A. radiobacter* it seems surprising that such a low proportion of the *exo* mutants in the latter have functional equivalents in the former. Given the relatively close taxonomic relatedness of *Agrobacterium* and *R. meliloti*, it is unlikely that this failure of intergeneric complementation is due to an inability of the *R. meliloti* genes to be expressed in *Agrobacterium*; in fact, using *exo::phoA* fusions, it was found that intergeneric expression of these fusions occurred (unpublished observations). Again, it seems inherently unlikely that a very similar EPS polymer would be made by different biochemical routes.

This work was funded by Shell Research Ltd. We are grateful to Allan Downie for useful discussions, to Graham Walker, Mike Daniels and Dale Noel for generously supplying strains and plasmids, and to Alan Cavill for technical assistance.

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