Expression of the *exoY* gene, required for exopolysaccharide synthesis in *Agrobacterium*, is activated by the regulatory *ros* gene

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Some mutants of *Agrobacterium radiobacter*, defective in exopolysaccharide synthesis, were phenotypically complemented by two different regions of cloned chromosomal DNA. One of these had been shown to contain a gene termed *ros*, a novel class of transcriptional regulator. The other contains a gene termed *exoY* which encodes a glycosyltransferase that is involved in one of the early steps in exopolysaccharide synthesis. Mutations in *ros* reduced the expression of *exoY* and a model to account for the complementation of certain *exo* alleles by both *ros* and *exoY* is presented. *TnphoA* insertions into *exoY* which expressed alkaline phosphatase activity were isolated and mapped, confirming the membrane location of the *exoY* gene product. Some of these mutations were dominant, causing merodiploids to be non-mucoid. *exoY* is linked to two genes, one encoding an ω -aminotransferase and the other encoding an aldehyde dehydrogenase.

Keywords: *Agrobacterium*, dominant *exo* mutation, exopolysaccharide, gene regulation, *ros*

INTRODUCTION

The bacteria *Agrobacterium* and *Rhizobium* interact with higher plants, several of the former inducing tumours, and members of the latter forming nitrogen-fixing root nodules on legumes. Like many other Gram-negative bacteria, they make high-molecular-mass acidic exopolysaccharide (EPS) (reviewed by Leigh & Coplin, 1992, and Leigh & Walker, 1994). The rhizobial genes encoding these molecules have been studied in most detail in *Rhizobium meliloti* (now known as *Sinorhizobium meliloti*) the symbiont of alfalfa. By sequencing and genetic analyses, 22 *exo* genes, needed for the synthesis of the normal succinoglycan of *R. meliloti*, were identified and biochemical functions were ascribed to many of them (Reuber & Walker, 1993; Becker *et al.*, 1993a–c; Buendia *et al.*, 1991). In *R. meliloti*, nearly all the *exo* genes are contiguously arranged on a large indigenous plasmid (Finan *et al.*, 1986), and their transcription is controlled by two negatively acting transcriptional genes, *exoS* and

exoR, which are located on the *Rhizobium* chromosome (Doherty *et al.*, 1988; Reed *et al.*, 1991b).

Strains of *Agrobacterium* make an EPS that is chemically similar to that of *R. meliloti* (Sutherland, 1985) but the genetics of its production have been less well studied. Cangelosi *et al.* (1987) isolated several *exo* mutants of *A. tumefaciens* and cloned the corresponding DNA. Several of these were complemented by the corresponding *R. meliloti* genes, but some were not. Aird *et al.* (1991) also isolated non-mucoid *exo* mutants of a strain of *A. radiobacter* (which lacks any tumour-inducing plasmid). Analysis of these mutants revealed features that distinguished them from those of *R. meliloti*.

Firstly, the mutations were scattered in different regions of the genome; i.e. different mutants were complemented only by certain non-overlapping cosmids from an *A. radiobacter* gene bank – there were at least five such non-overlapping cosmid contigs. Secondly, despite the chemical similarity of the EPSs of the two genera, only mutations in *exoB* of *R. meliloti* were complemented by *A. radiobacter* *exo* genes. Some *exo* mutants were 'conditional', being EPS⁺ on medium containing glucose or glycerol but EPS[–] with dicarboxylic acids as sole C source. An unexplained observation was that certain *exo*

Abbreviations: AP, alkaline phosphatase; EPS, exopolysaccharide.

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mutants, which included some of the conditional ones, accumulated protoporphyrin IX, the immediate precursor of haem. Lastly, it was found that certain mutations, again including the conditional ones, were complemented by two non-overlapping cosmids, pBIO11 and pBIO21, which had no cloned DNA in common. To explain this last observation, Aird *et al.* (1991) suggested that such mutations were in a positively acting regulatory gene so that they could be complemented not only by cloning that gene itself but also by cloning, in a multi-copy vector, the 'target' structural gene that was activated by this proposed regulator. Thus, the target gene might become 'deregulated' and so no longer require activation of its transcription.

Some support for this idea came from Brightwell *et al.* (1995) who showed that the gene in one of the cosmids (pBIO11) which complemented these alleles corresponded, both in function and in sequence, to a known transcriptional regulator, termed *ros*, (rough outer surface) in *A. tumefaciens* (Close *et al.*, 1985; Cooley *et al.*, 1991) which had the following properties. It represses transcription of itself and of two other loci, *virC* and *virD*, which are involved in tumour induction. It encodes a 14.5 kDa protein that binds to sequences (*ros* boxes) preceding genes that it regulates (D'Souza-Alt *et al.*, 1993). Brightwell *et al.* (1995) showed that this repressive activity was augmented by the presence of iron and glucose in the growth media. The *ros* gene of *A. radiobacter* and *A. tumefaciens* complemented both the EPS and the porphyrin defects of the relevant *A. radiobacter* mutants (Brightwell *et al.*, 1995). Mutations that deleted the 3' end of *ros* had a dominant mutant phenotype; when plasmids containing such cloned mutants were present in wild-type *A. radiobacter* or *Rhizobium*, the transconjugants were non-mucoid. If, as is normal with DNA-binding proteins, Ros is multimeric, maybe the presence of a truncated version of mutant *ros* results in the formation of an inactive protein complex (Brightwell *et al.*, 1995).

Keller *et al.* (1995) characterized a gene, *mucR*, of *R. meliloti* with a similar sequence to *ros*. The *mucR* mutants did not make the normal polysaccharide (EPS I) but made a different polymer, EPS II, which is not made by wild-type *R. meliloti* (Glazebrook & Walker, 1989). *mucR* repressed the *exp* genes that encode EPS II, but had no effect on transcription of the *exo* genes that encode EPS I, although *mucR* mutants made less EPS I than did the wild-type.

The second cosmid, with cloned *A. radiobacter* DNA that complemented those *exo* mutants that were also complemented by *ros*, was termed pBIO21. Aird *et al.* (1991) localized the *exo* DNA to a 4.5 kb region of pBIO21 and showed that some mutations in this DNA not only abolished its ability to complement the mutants, but were 'dominant', causing wild-type strains of *Agrobacterium* and also of *Rhizobium* to be non-mucoid when such mutant plasmids were present. Certain *exo-phoA* fusions in pBIO21 expressed alkaline phosphatase (AP), indicating that the product of the *exo* gene(s) is associated with the bacterial periplasm or membrane(s), something that has been established for several *exo* genes (Latchford

et al., 1990; Long *et al.*, 1988; Reuber *et al.*, 1991), and is consistent with the hydrophobicity pattern of ExoY and homologous proteins (e.g. Borthakur *et al.*, 1988). We therefore speculated (Aird *et al.*, 1991) that the *exo* gene(s) in pBIO21 included one or more of the targets for the regulatory gene that was later identified as *ros*. Here, we present a molecular analysis of this region and identify the relevant *exo* gene in pBIO21 and its relation to *ros* and other neighbouring genes.

METHODS

Strains and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1.

Media, general growth conditions and *in vivo* genetic manipulations. For *Agrobacterium*, these were as described by Aird *et al.* (1991). In triparental matings, pRK2013 (Figurski & Helinski, 1979) was used as the helper plasmid to mobilize recombinant plasmids based on pLAFR1 or pMP220 into *Rhizobium* or *Agrobacterium*. Transposon mutagenesis of pBIO60 was done as described for the mutagenesis of pBIO21 by Aird *et al.* (1991).

AP assay. Quantitative AP assays were performed as described by Brickman & Beckwith (1975), except that activities were expressed as a function of cellular protein concentration that was determined by the Bicinchoninic Acid Protein Assay Kit (Sigma) according to the manufacturers' instructions. Cells to be assayed were harvested at mid-exponential phase, thus minimizing the risk of spurious AP activity when the enzyme was present in the cytoplasm of cells that were in stationary phase (see Derman & Beckwith, 1995).

DNA manipulation. For routine analysis and isolation of DNA (transformation, restriction mapping, etc.), experiments were done using the protocols (or minor variants thereof) described by Sambrook *et al.* (1989). DNA was sonicated, fractionated into fragments approximately 400 bp in length, and the ends were filled in and ligated to appropriate vectors as described by Rossen *et al.* (1984). Fluorescently labelled oligonucleotides were synthesized by Richard James on a Bioresarch Cyclone oligonucleotide synthesizer (University of East Anglia, Norwich) according to the manufacturers' instructions, and the PCR products were prepared on an LEP Scientific cycler. DNA was sequenced using the dideoxy chain-termination method and gels were run on an ALF automated sequencer (Pharmacia) according to the manufacturers' instructions.

Sequences were assembled using the DNAStar and Gene Jockey programs, and sequence comparisons were done using BLASTX and FASTN searches of data bases.

RESULTS

Sequence of the *exoY* region of *A. radiobacter*

The relevant *exo* region of cosmid pBIO21 region had been localized to a 4.5 kb *EcoRI*-*Bam*HI fragment that, when cloned in the wide-host-range plasmid pMP220 to form pBIO60, corrected all the mutants that were complemented by the original cosmid. Further, all the transposon insertions that abolished the ability of pBIO21 to complement the *exo* mutants were located in the same fragment (Aird *et al.*, 1991).

This cloned DNA was sonicated and the resulting small fragments were cloned into pUC18 for sequencing of both

Table 1. Bacterial strains and plasmids

Strain/plasmid	Characteristics	Reference/source
<i>E. coli</i>		
803	Met ⁻ , used as a recipient in routine transformations	Wood (1966)
JM101	F ⁻ <i>traD36 proA⁺ proB⁺ lac^a lacZAM15 supE thi Δ(lac-proAB)</i>	Messing <i>et al.</i> (1983)
<i>A. radiobacter</i>		
T1305	Wild-type; Str ^R	Aird <i>et al.</i> (1991)
Exo 51.1	Exo ⁻ , complemented by pBIO11 and pBIO21	Aird <i>et al.</i> (1991)
Exo 53.1	Exo ⁻ , complemented by pBIO11 and pBIO21	Aird <i>et al.</i> (1991)
Exo 63	Exo ⁻ , complemented by pBIO11 and pBIO21	Aird <i>et al.</i> (1991)
Exo 66.1	Exo ⁻ , complemented by pBIO11 and pBIO21	Aird <i>et al.</i> (1991)
Exo 70.1	Exo ⁻ , complemented by pBIO21 only	Aird <i>et al.</i> (1991)
Exo 73.1	Exo ⁻ , complemented by pBIO21 only	Aird <i>et al.</i> (1991)
Exo 81	Exo ⁻ , complemented by pBIO11 and pBIO21	Aird <i>et al.</i> (1991)
Plasmids		
pUC18	Amp ^R	Vieira & Messing (1984)
pRK2013	Kan ^R , used for mobilizing P1-group plasmids	Figurski & Helinski (1979)
pLAFR1	Tet ^R , wide-host-range P-group cloning vector	Friedman <i>et al.</i> (1982)
pMP220	Tet ^R , <i>lacZ</i> , wide-host-range P-group cloning vector	Spaink <i>et al.</i> (1987)
pBIO21	pLAFR1-based cosmid, contains <i>A. radiobacter</i> <i>exoY</i>	Aird <i>et al.</i> (1991)
pBIO60	4.5 kb <i>EcoRI</i> - <i>Bam</i> HI fragment in pMP220, contains <i>exoY</i>	Aird <i>et al.</i> (1991)
pBIO903	3.1 kb <i>Sma</i> I-deletant of pBIO60	This work
pBIO912	1.6 kb <i>Pst</i> I- <i>Bam</i> HI fragment in pMP220, contains <i>exoY</i>	This work
pBIO917	1.2 kb <i>Sma</i> I fragment in pMP220, <i>exoY</i> not intact, contains 700 bp upstream of <i>exoY</i>	This work
pBIO918	1.1 kb <i>Nde</i> I- <i>Bam</i> HI fragment in pMP220, contains intact <i>exoY</i> and 300 bp upstream of <i>exoY</i>	This work
pBIO92	<i>TnphoA</i> pBIO21 mutant containing the dominant <i>exoY1::TnphoA</i> allele	This work
pBIO69	<i>TnphoA</i> pBIO21 mutant containing the <i>exoY2::TnphoA</i> allele	This work
pBIO72	<i>TnphoA</i> pBIO21 mutant containing the <i>exoY3::TnphoA</i> allele	This work

Fig. 1. Diagrammatic representation of the *exoY* region of *A. radiobacter*. Dimensions and orientations of the four genes referred to in the text are shown as hatched boxes. The sizes of the fragments cloned into pMP220 and the names of the resultant plasmids are presented; cloning sites used were *Bam*HI (B), *Sma*I (Sm), *Nde*I (Nd), *Pst*I (P) and *Eco*RI (RI). The ability of the plasmids to complement the EPS-defect of mutant Exo 81 is indicated.

r	1	MKSATQSAEQ	TLSSSEDFDV	RVPIGGIAKR	SFDMTSAALA	LLIFSPIFLL
		*****	*	***	***	***
m	1	MKSATRSASS	PFFIPEETGA	VRPIGGMAKR	SFDVLAASVA	LLLFSPLFLL
		3	▼	2	▼	1
r	51	IAVLVKMSDP	GPIFYGHRV	GHNGRYFHCL	KFRTNMGND	EILROYLAAN
		* ** *	* ** *	***	***	*
m	51	IMALVKFSDG	GSVYGHRR	GHNGQSPKCL	KFRINMEQGD	EVLEEFPRIN
r	101	PEGAEWRAT	RKLNDPRVT	AVGARVRKLS	LDELPLQINI	LRGEMSVVGP
		*****	*****	*****	*****	*****
m	101	PDAYEWRAT	RKLNDPRVT	VVGALVRKLS	LDELPLQINI	LRGEMSVVGP
r	151	RPVVDEELSY	YESAAAYLS	TRPGLTGLWQ	ISGRNDVSYK	TRVAFDTQYV
		***	***	*****	*****	*****
m	151	RPVVEDELEL	YDSAAVFLR	SRPGLTGLWQ	ISGRNDVSYA	TRVAFDTQYV
r	201	QNWSMRQDNF	IIVKTIPAVC	LSRGSY		
		***	*****	*****		
m	201	QNWSLFADLV	IIVKTIPAVC	LSRGSY		

Fig. 2. Sequence of the deduced ExoY product of *A. radiobacter* (r), compared with the corresponding gene in *R. meliloti* (m). Identical amino acid residues are asterisked. The sites of the three *exoY::TnphoA* insertions are shown as triangles; the *exoY* alleles in each mutant are indicated.

forward and reverse strands. Once about 80% of the sequence had been obtained, the gaps were filled in either by primer walking or by making specific subclones designed from restriction sites that had been revealed from the sequence. The 4.5 kb sequence was searched for the presence of ORFs and one obviously relevant gene was apparent (Fig. 1).

Identification of *exoY*

The deduced protein product of an ORF that extended from position 737 to 61 (Fig. 2) had a striking resemblance in sequence, size and hydrophobicity (Fig. 3) to that of

several other genes that had been shown to be involved in EPS synthesis in various bacteria (Borthakur *et al.*, 1988; Reuber & Walker, 1993; Vanderslice *et al.*, 1989; Jiang *et al.*, 1991). These included the products of the *exoY* gene of *R. meliloti* (82% amino acid identity), *pssA* of *R. leguminosarum* (81%), *rfbP* of *Salmonella* (70%), *gumD* of *Xanthomonas* (71%) and *cpsD* (Rubens *et al.*, 1993) of *Streptococcus* (81%). The succinoglycan of *Rhizobium* and, presumably, *Agrobacterium* is synthesized from UDP on membrane-bound lipid carriers, as is the case for other Gram-negative bacteria. The *exoY*, *rfbP* and *gumD* genes are believed to encode a glycosyltransferase that adds the first sugar to these isoprenoid carriers (see Reuber & Walker, 1993). Despite the sequence similarities, neither the cloned *pssA* gene of *R. leguminosarum*, nor the related *exoY* of *R. meliloti*, complemented the EPS⁻ defect of any of the *A. radiobacter* mutants that were restored by pBIO60, nor did pBIO60 complement *pssA* mutants of *R. leguminosarum* (Aird *et al.*, 1991). It has been noted that the ExoY (PssA) proteins of *Rhizobium* are both located in the bacterial membrane (see Reuber & Walker, 1993; Latchford *et al.*, 1990).

To determine if *exoY* of *A. radiobacter* was sufficient to complement the mutants that were complemented by the intact pBIO60, various subclones of pBIO60 were made, the cloned DNA being inserted into the wide-host-range vector pMP220. The resulting plasmids were mobilized into the *exo* mutants listed in Table 1 and the trans-conjugants were examined for their ability to restore EPS synthesis on media with glucose or succinate as sole C source. All plasmids that contained the intact *exoY* gene restored EPS synthesis to all the mutants on both C sources, the smallest such plasmid being pBIO918, which contained only *exoY* (Fig. 1). These *exo* mutants included

Fig. 3. Hydrophobicity profile of the product of *exoY* showing the sites of the *TnphoA* insertions. The sequence was analysed using the Kyte & Doolittle (1982) algorithm with a window of 12 amino acids.

Table 2. Effect of *ros* on expression of *exoY-phoA*

Plasmid	AP activities* in background strain:			
	T1305	Exo 81	Exo 53.1	Exo 66.1
pBIO21	22.1 ± 1.9	20.3 ± 2.6	11.6 ± 6.8	18.3 ± 6.5
pBIO72	264.3 ± 34.4	63.4 ± 18.7	75.9 ± 17.9	145.4 ± 64.1
pBIO92	365.2 ± 30.7	40.3 ± 10.5	100.4 ± 16.7	189.5 ± 70.1

* AP activities are expressed as a function of cellular protein concentration.

some (Exo 81, Exo 66.1, Exo 53.1 and Exo 63) which were complemented by both *exoY* and *ros*, and others (Exo 70.1 and Exo 73.1) which were complemented only by *exoY*. In contrast, none of the new constructs, nor pBIO60 itself, complemented the porphyrin over-production of the mutants Exo 53.1 and Exo 81. We conclude that *exoY* is indeed sufficient to complement all the *exo* mutants complemented by the original cosmid pBIO21 for EPS synthesis. This was substantiated by mapping the mutant derivatives of cosmid pBIO21 which no longer complemented the *exo* mutants.

Mapping of *exoY::phoA* mutations

Following mutagenesis of pBIO21 with *TnphoA* (Manoil & Beckwith, 1985), three mutant derivatives of pBIO21, termed pBIO69, pBIO72 and pBIO92, which no longer complemented any of the *exo* mutants that were complemented by pBIO21, were isolated (Aird *et al.*, 1991). These three fusions expressed AP activity in free-living cells (Aird *et al.*, 1991, and below). This suggested that the AP part of the fusion was localized in the periplasm although occasional anomalous results with *phoA* fusions have been reported when fusions are at the N-terminus of the protein (Prinz & Beckwith, 1994). However, in plasmids pBIO69, pBIO72 and pBIO92 this is not the case (see below). Interestingly, one of these alleles, *exoY1::TnphoA* was dominant; the mutant plasmid, pBIO92, which contained this allele caused both *R. leguminosarum* and *A. radiobacter* wild-type strains to become non-mucoid on media containing either glucose or succinate as C source. In contrast, wild-type strains containing pBIO69 or pBIO72 (containing the *exoY2* and the *exoY3* alleles, respectively) were fully mucoid. All these mutant plasmids expressed high levels of AP activity in *Agrobacterium* and in *R. leguminosarum* (see Table 2). Since the *phoA* gene in *TnphoA* lacks the signal sequence that guides AP to the periplasm (the subcellular compartment in which the enzyme has activity), this means that the AP part of the fusion protein must be in the periplasm, consistent with the proposed inner membrane location of *exoY* (Latchford *et al.*, 1991; Reuber & Walker, 1993).

Approximate restriction mapping located these insertions to the 4.5 kb *EcoRI*-*Bam*HI fragment of pBIO21 that had been sequenced and to that part of the DNA in which *exoY* had been found. Further, in all three cases, *phoA* in

the transposon was in the same orientation as *exoY*. To map the insertions precisely, a novel *Bam*HI fragment that had been created due to the insertion of *TnphoA* was cloned into plasmid pUC18; this cloned fragment contained part of the transposon plus the *Agrobacterium* DNA that lay immediately upstream of the *phoA*, i.e. which extended to the next genomic *Bam*HI site.

Using a primer that corresponded to the 5' region of the *phoA* gene, the junctions of the insertions into *exoY* were determined and are shown in Figs 2 and 3. The three inserts were close to each other, all being towards the 5' coding region of *exoY*. In fact, the sites of the insertions were no more than 33 bp apart, the dominant allele, *exoY1::TnphoA*, being the 3'-most of the three.

Why are some *exoY* mutants dominant?

The finding that at least one *exoY* mutant of *A. radiobacter* was dominant is reminiscent of a similar situation in *Rhizobium*. In *R. meliloti*, *exoY* is transcribed divergently from another gene, *exoX*, which also encodes a membrane-bound protein. When a recombinant plasmid containing both genes is present in a strain of wild-type *R. meliloti*, the bacteria appear normal. However, mutant plasmid-derivatives carrying mutations in *exoY* cause a non-mucoid phenotype in wild-type strains. This is due to a surfeit of the ExoX protein relative to ExoY, and is thought to disrupt the normal interaction between these two proteins in the inner membrane, which somehow results in the impairment of EPS synthesis (Reed *et al.*, 1991a). A similar situation was described by Gray *et al.* (1990) for *Rhizobium* strain NGR234. An analogous though slightly different phenomenon has also been described in a strain of *R. leguminosarum* biovar *phaseoli* (Borthakur *et al.*, 1985; Borthakur & Johnston, 1987). Here, a gene termed *psiA*, when cloned, also caused wild-type *Rhizobium* to become non-mucoid. The deduced products of *psiA* and *exoX* have very similar sizes, both are located in the inner membrane and their hydrophobicity plots are almost superimposable (see Gray *et al.*, 1990); strikingly, though, the two proteins have extremely limited identity at the amino acid sequence level.

Thus, it seemed possible that the dominant effect of the *exoY1::TnphoA* mutation in pBIO92 could be due to the presence of an *exoX/psiA*-like gene in the parental cosmid pBIO21. To test if this proposed gene was in the DNA sequences here, plasmid pBIO60 which contains the

region of DNA sequenced here, was mutagenized with *TnphoA*, and mutant derivatives that caused *Rhizobium* and *Agrobacterium* to be non-mucoid were isolated. Mapping the sites of these insertions showed that the *TnphoAs* had inserted towards the 5' end of *exoY*. Thus, if the dominant effects were due to the inhibitory effects of an *exoX*-like gene being revealed when *exoY* was mutated, this hypothetical gene must be located in the cloned DNA in pBIO60. However, this was shown not to be the case in two different ways.

Firstly, the sequenced region was scanned for sequences with similarity to ExoX and PsiA; none was found. Since PsiA and ExoX themselves have little sequence identity to each other (Gray *et al.*, 1990) but have similar sizes (10 kDa) and near-identical and extremely characteristic hydrophobicity plots (a very hydrophobic N-terminal half and an extremely hydrophilic C-terminal half), every ORF in the sequenced region was examined for any that had the same similar features; again, none was found.

If, nevertheless, there is a gene in pBIO60 which has the same properties as *exoX*, it should have been possible to identify it by subcloning regions of this plasmid which lack the intact *exoY* gene. Therefore, plasmids containing regions spanning the whole of the cloned DNA in pBIO60, but which lacked intact *exoY*, were subcloned into pMP220 to form plasmids pBIO903 and pBIO917 (see Fig. 1). These two plasmids were mobilized into wild-type *A. radiobacter* T1305; for both, the transconjugants were mucoid. This provided further evidence that pBIO60 did not contain a gene that was functionally equivalent to *exoX*.

ros regulates expression of *exoY*

We had speculated (Aird *et al.*, 1991) that the reason that certain *exo* alleles could be complemented by two different cosmids was that they were in a positively acting regulatory gene and so could be restored either by cloning that gene itself or by cloning the target structural gene such that it no longer needed the regulator. It subsequently transpired that the gene in one of the cosmids corresponded to *ros*, a known regulator (Brightwell *et al.*, 1995), and here we have shown that the other cosmid, pBIO21, indeed contains a structural *exo* gene that encodes a glycosyltransferase.

To test if *ros* regulated expression of *exoY*, two *exoY::phoA* mutant plasmids pBIO72 (containing the recessive *exoY2::TnphoA* allele) and pBIO92 (containing the dominant *exoY1::TnphoA* allele) were transferred into the wild-type and each of the *ros* mutants Exo 81, Exo 53.1 and Exo 66 of *A. radiobacter*. These three mutants could all be complemented for EPS synthesis by both *ros* and *exoY*, but they differed in that Exo 81 accumulated large amounts of porphyrin and was EPS⁻ on media with either glucose or succinate as C source; in contrast, Exo 66.1 and Exo 53.1 were mucoid on the former medium. Cells were grown in minimal media and assayed for AP. As seen in Table 2, the levels of AP activity were consistently higher in the wild-type background than with any of these three mutants. There was a slight

increase, though, even in Exo 81, when the fusion plasmids were present. This could be due to the fact that the defective *phoA* gene had been supplied with a ribosome-binding site and with the promoter of *exoY* expressed at a low basal level. These data suggest that transcription of *exoY* is normally activated by *ros* and that this is defective in the three mutants tested here. The data in Table 2 refer to the activities obtained with cells grown in normal media in which glycerol was the C source. The assays were also done on these strains that had been grown with succinate as sole C source and in media that were deficient in iron; the results were essentially the same as those presented in Table 2.

The Ros protein binds to specific regions preceding genes whose expression it represses. These sequences contain structures of dyad symmetry (D'Souza-Alt *et al.*, 1993); no sequences similar to such *ros* boxes were found in the region upstream of *exoY*, nor, indeed in the whole of the sequenced region. There is an imperfect inverted repeat [AAAATCCCA;N9;TCAAATTTT] 392 bp upstream of the translational start of *exoY* but there is no experimental evidence to show whether the Ros protein binds directly to that sequence or, indeed to any other region 5' of *exoY*.

Identification of two genes, *aldA* and *oatA*, upstream of *exoY*

Upstream of *exoY* we identified three other genes, two of whose products had significant homology to those of other bacterial genes with known functions (see Fig. 1). One of these, termed *aldA*, showed homology (46% identity) to a part of the *Escherichia coli* aldehyde dehydrogenase (ALDH) (see Heim & Strehler, 1991). Interestingly, the N-terminal region of AldA of *Agrobacterium* also shows conserved sequences that correspond to the α -helix-turn- α -helix motif (Dodd & Egan, 1990) representing the DNA-binding region of a range of prokaryotic transcriptional regulators, including Cro, Fnr, TrpR, PhoB and SinR. It seems possible that the AldA protein of *A. radiobacter* may be a DNA-binding protein that also has an extensive motif resembling part of the *E. coli* ALDH enzyme. It is conceivable that this latter motif responds to molecules that resemble aldehydes, or their enzymic products, and that such an interaction may determine whether the regulatory protein actively induces (or represses) transcription of its target gene(s) (Tiburtius, 1995).

Transcribed divergently from *aldA*, and separated from it by 530 bp, is an ORF (2425–3214); the product of this ORF (termed *oatA*) had a striking and extensive similarity to a family of bacterial ω -aminotransferases, the greatest being to pyruvate transaminase of *E. coli*; it was also similar to the product of the *avrD* gene of the phytopathogen *Pseudomonas syringae* (Kobayashi *et al.*, 1990). Downstream of *oatA* and transcribed in the same orientation was one other significant ORF (termed ORF A; Fig. 1). The deduced product of this ORF had no clear homology with any protein sequence in databases.

Unfortunately, the failure to conduct site-directed mutagenesis according to Ruvkun & Ausubel (1981) in *A.*

radiobacter (Aird *et al.*, 1991) precluded us from determining the phenotypic effects of mutation in any of these genes.

DISCUSSION

In this paper we have shown that certain *exo* mutants of *A. radiobacter* can be complemented for their failure to make EPS by the cloned *exoY* gene. Close homologues of this gene have been identified in various Gram-negative and -positive bacteria; in all cases, mutations lead to a defect in the synthesis of the particular EPS that is characteristic of the particular genus.

Some of the mutants that were complemented for EPS synthesis by *exoY* were also complemented by the regulatory gene *ros*. We propose that these mutants are defective in *ros*, whereas those mutants that were complemented for EPS synthesis by *exoY* but not by *ros* are mutated in *exoY* itself. For the mutant strains Exo 81 and Exo 53.1, their defects in haem synthesis, indicated by the accumulation of protoporphyrin, were not complemented by *exoY*, but have been shown to be complemented by *ros* (Brightwell *et al.*, 1995). Taken together, we propose that *ros* acts as a positively acting transcriptional regulator for *exo* genes and for genes involved in haem biosynthesis. Thus, *bona fide* *ros* mutations can be complemented by *ros* for both phenotypes, whereas the cloned *exo* target gene can complement only the EPS defect. It is possible that the *ros* mutations in Exo 53.1 and in Exo 81 abolish the ability of the protein to recognize or activate the promoters at either the *exo* or the haem gene loci, whereas with Exo 66.1, the defective activation is confined to the recognition of the *exo* gene(s).

We now have direct evidence that *ros* acts positively on the expression of *exoY* of *A. radiobacter*. Since this was deduced from studies on the *exoY*::*TnpA* fusions, we did not formally show that this regulation was at the level of transcription; the levels of AP activity measured here would be a function of the transcription, translation and 'targeting' of the ExoY::PhoA fusion protein to the membrane. However, given the previously identified effects of *ros* on other genes (Brightwell *et al.*, 1995; D'Souza-Alt *et al.*, 1993; Cooley *et al.*, 1991), it seems inherently likely that its effect on *exoY* expression is at the level of transcription. However, in previous studies, *ros* and the related *R. meliloti* gene *mucR* (Keller *et al.*, 1995) had only been shown to act as repressors of transcription, not activators as is the case with its effect on *exoY*. There are, though, many examples of bacterial DNA-binding transcriptional regulators acting positively at some promoters but negatively at others. It is also conceivable that *ros* might normally function as a repressor of a gene which, in turn, represses expression of *exoY*. Thus, mutations in *ros* would cause that repressor to be active and so depress the transcription of *exoY*.

The DNA sequence to which the Ros protein of *A. tumefaciens* binds and which includes a region of dyad symmetry known as the *ros* box has no precise counterpart in the region of DNA up to 3.2 kb upstream of *exoY*. If the Ros protein of *A. radiobacter* does bind to DNA

upstream of *exoY* (something that has not been proven), it must therefore have alternative binding sites to the *ros* box. Certainly, even in *A. tumefaciens*, the sequence of dyad symmetry 5' of *virC* is inverted relative to that preceding *ros*, suggesting that there may be more than one sequence that is recognized by the Ros protein (D'Souza-Alt *et al.*, 1993).

Three closely linked *exoY*::*TnpA* mutant alleles were isolated and mapped precisely. All three expressed AP, indicating that the AP part of the fusion proteins was, in all cases, exposed to the periplasmic space. The fusion junctions were located in a region of the protein that contained charged residues, consistent with this region of the protein not being buried in the bacterial membrane, but rather being exposed to the periplasmic space.

Strikingly, one of these mutations caused a dominant phenotype whereas the other two were recessive. Other studies in *Rhizobium* had shown that such dominant phenotypes caused by mutations in *exoY* were due to the presence of the 'extra' copies of *exoX* on the same recombinant plasmid (Gray *et al.*, 1990; Reed *et al.*, 1991a). However, this does not appear to be the explanation for the phenomenon described here. By both sequence and functional analyses, there does not appear to be a gene corresponding to *exoX* in the vicinity of *exoY* which is present in pBIO60. A tentative explanation for the observation points to a possibly trivial explanation. Since the three mutations studied here all express AP, they must all generate fusion proteins between part of ExoY and PhoA. It is likely that the various proteins that engage in EPS synthesis form complexes with each other at or near the bacterial membrane (see model by Leigh & Walker, 1994). It is possible that if a fusion protein between one of these *exo* gene products is formed, depending on the exact position of the fusion, the mutant protein might be able to participate in the formation of the complex but, depending on the exact position and thus the spatial orientation of the AP part of the fusion protein, this may or may not prevent other proteins of the complex from functioning properly or, indeed from being able to participate in it at all. If, as is the case here, the fusion protein is encoded by a plasmid, it will be present in larger amounts since the vector for the cloned *exoY*::*TnpA* fusion has a copy number of approximately eight per cell (unpublished observations).

The precise interactions between proteins in the EPS-forming complex may also explain another somewhat surprising result, namely the failure of the *exoY* genes of *Rhizobium* to correct *exoY* mutants of *Agrobacterium* and vice versa (Aird *et al.*, 1991), despite the near-identity of the EPSs that are made by the two genera and the >80% identity of the amino acid sequence of the two ExoY proteins. It seems reasonable to suggest that although the ExoY proteins of these two genera may carry out exactly the same catalytic functions (i.e. the initial glycosylation step), because they each act as part of a protein complex, the heterologous combination in which the ExoY protein of one of the genera is required to act in the context of the Exo proteins of the other genus does not result in a functional complex.

Future work is designed to understand better the regulatory function of *ros*. It has been shown to be a repressor and, here, also to have activating abilities. The *ros* and *mucR* genes encode a new class of bacterial regulators with no sequence similarities to any other DNA-binding regulatory proteins, although Cooley *et al.* (1993) suggested that the Ros protein contains a motif with some similarity to a zinc-finger. We wish to know in more detail how it activates expression of *exoY* and how it is involved, apparently, in the biosynthesis of haem in *Agrobacterium*.

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