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

Anne Tiburtius, Nicola G. de Luca, Haitham Hussain and Andrew W. B. Johnston

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 (Fiman et al., 1986), and their transcription is controlled by two negatively acting transcriptional genes, exoA 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, 1935) 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. radio bacter (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. radio bacter 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. radio bacter 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.

The GenBank/EMBL accession number for the sequence reported in this paper is X95394.
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 accumulated protoporphyrin IX, the immediate precursor including the conditional ones, were complemented by of haem. Lastly, it was found that certain mutations, again 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 I, but had no effect on transcription of the exp genes that encode EPS II, although mucR mutants made less EPS I than did the wild-type.

The second cosmid, with cloned A. radiobacter DNA that complemented those exp mutants that were also complemented by ros, was termed pBIO21. Aird et al. (1991) localized the exp 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 exp-phaA fusions in pBIO21 expressed alkaline phosphatase (AP), indicating that the product of the exp gene(s) is associated with the bacterial periplasm or membrane(s), something that has been established for several exp genes (Latchford et al., 1990; Long et al., 1988; Reuber et al., 1991), and is consistent with the hydrophobicity pattern of Exo Y and homologous proteins (e.g. Borthakur et al., 1988). We therefore speculated (Aird et al., 1991) that the exp 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 exp 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 Bichinchoninic 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 Bioresearch Cyclone oligonucleotide synthesizer (University of East Anglia, Norwich) 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).

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 expY region of A. radiobacter**

The relevant exp region of cosmid pBIO21 region had been localized to a 4.5 kb EcoRI-BamHI 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 exp 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

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics</th>
<th>Reference/source</th>
</tr>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>803</td>
<td>Met-, used as a recipient in routine transformations</td>
<td>Wood (1966)</td>
</tr>
<tr>
<td>JM101</td>
<td>F- traD36 proA+ proB+ lacIq lacZAM15 supE thi K(lac-proAB)</td>
<td>Messing et al. (1983)</td>
</tr>
<tr>
<td><strong>A. radiobacter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1305</td>
<td>Wild-type; Str&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Aird et al. (1991)</td>
</tr>
<tr>
<td>Exo 51.1</td>
<td>Exo&lt;sup&gt;e+&lt;/sup&gt;, complemented by pBIO11 and pBIO21</td>
<td>Aird et al. (1991)</td>
</tr>
<tr>
<td>Exo 53.1</td>
<td>Exo&lt;sup&gt;e+&lt;/sup&gt;, complemented by pBIO11 and pBIO21</td>
<td>Aird et al. (1991)</td>
</tr>
<tr>
<td>Exo 63</td>
<td>Exo&lt;sup&gt;e+&lt;/sup&gt;, complemented by pBIO11 and pBIO21</td>
<td>Aird et al. (1991)</td>
</tr>
<tr>
<td>Exo 66.1</td>
<td>Exo&lt;sup&gt;e+&lt;/sup&gt;, complemented by pBIO11 and pBIO21</td>
<td>Aird et al. (1991)</td>
</tr>
<tr>
<td>Exo 70.1</td>
<td>Exo&lt;sup&gt;e+&lt;/sup&gt;, complemented by pBIO21 only</td>
<td>Aird et al. (1991)</td>
</tr>
<tr>
<td>Exo 73.1</td>
<td>Exo&lt;sup&gt;e+&lt;/sup&gt;, complemented by pBIO21 only</td>
<td>Aird et al. (1991)</td>
</tr>
<tr>
<td>Exo 81</td>
<td>Exo&lt;sup&gt;e+&lt;/sup&gt;, complemented by pBIO11 and pBIO21</td>
<td>Aird et al. (1991)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<tr>
<td>pUC18</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Vieira &amp; Messing (1984)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, used for mobilizing P1-group plasmids</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pLAFR1</td>
<td>Tet&lt;sup&gt;R&lt;/sup&gt;, wide-host-range P-group cloning vector</td>
<td>Friedman et al. (1982)</td>
</tr>
<tr>
<td>pMP220</td>
<td>Tet&lt;sup&gt;R&lt;/sup&gt;, lacZ, wide-host-range P-group cloning vector</td>
<td>Spaink et al. (1987)</td>
</tr>
<tr>
<td>pBIO21</td>
<td>pLAFR1-based cosmid, contains A. radiobacter exoY</td>
<td>Aird et al. (1991)</td>
</tr>
<tr>
<td>pBIO60</td>
<td>4.5 kb EcoRI-BamHI fragment in pMP220, contains exoY</td>
<td>Aird et al. (1991)</td>
</tr>
<tr>
<td>pBIO903</td>
<td>3.1 kb SmaI-deletant of pBIO60</td>
<td>This work</td>
</tr>
<tr>
<td>pBIO912</td>
<td>1.6 kb PstI-BamHI fragment in pMP220, contains exoY</td>
<td>This work</td>
</tr>
<tr>
<td>pBIO917</td>
<td>1.2 kb SmaI fragment in pMP220, exoY not intact, contains 700 bp upstream of exoY</td>
<td>This work</td>
</tr>
<tr>
<td>pBIO918</td>
<td>1.1 kb NdeI-BamHI fragment in pMP220, contains intact exoY and 300 bp upstream of exoY</td>
<td>This work</td>
</tr>
<tr>
<td>pBIO92</td>
<td>TnphoA pBIO21 mutant containing the dominant exoY&lt;sup&gt;1&lt;/sup&gt;: TnphoA allele</td>
<td>This work</td>
</tr>
<tr>
<td>pBIO69</td>
<td>TnphoA pBIO21 mutant containing the exoY&lt;sup&gt;2&lt;/sup&gt;: TnphoA allele</td>
<td>This work</td>
</tr>
<tr>
<td>pBIO72</td>
<td>TnphoA pBIO21 mutant containing the exoY&lt;sup&gt;3&lt;/sup&gt;: TnphoA allele</td>
<td>This work</td>
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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 BamHI (B), SmaI (Sm), NdeI (Nd), PstI (P) and EcoRI (RI). The ability of the plasmids to complement the EPS-defect of mutant Exo 81 is 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. melliloti (82% amino acid identity), pssA of R. leguminosarum (81%), rfbP of Salmonella (70%), gndD of Xanthomonas (71%) and epsD (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 gndD 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. melliloti, 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 transconjugants 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

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**Fig. 2.** Sequence of the deduced ExoY product of A. radiobacter (r), compared with the corresponding gene in R. melliloti (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.

**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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>AP activities* in background strain:</th>
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<tbody>
<tr>
<td></td>
<td>T1305</td>
</tr>
<tr>
<td></td>
<td>Exo 81</td>
</tr>
<tr>
<td></td>
<td>Exo 53.1</td>
</tr>
<tr>
<td>pBIO21</td>
<td>22.1±1.9</td>
</tr>
<tr>
<td>pBIO72</td>
<td>264±3±44</td>
</tr>
<tr>
<td>pBIO92</td>
<td>365±2±30.7</td>
</tr>
</tbody>
</table>

*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 TnpphoA (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, exoY::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 TnpphoA 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 & Johnston, 1987).

Approximate restriction mapping located these insertions to the 4.5 kb EcoRI–BamHI 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 BamHI fragment that had been created due to the insertion of TnpphoA 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 BamHI 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, exoY::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 surplus 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 (Reid 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 exoY::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 in pBIOGO. However, this was shown not to be the case in an exox-like gene being revealed when emY was mutated, if the dominant effects were due to the inhibitory effects of T'nphaA, and mutant derivatives that caused large amounts of porphyrin and was EPS- on media with either glucose or succinate as source; in contrast, Exo 81 accumulated large amounts of porphyrin and was EPS+ on media with either glucose or succinate as source; consequently transpired that the gene in one of the cosmids 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 [AAAAATCCCA;N9;TCAAATTTT] 392 bp upstream of the transcriptional 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 Agrobacterium may also have 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 enzymatic 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.
radio bacter (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. radio bacter* can be complemented for their failure to make EPS by the cloned exo Y 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 exo Y were also complemented by the regulatory gene ros. We propose that these mutants are deficient in ros, whereas those mutants that were complemented for EPS synthesis by exo Y but not by ros are mutated in exo Y 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 exo Y, 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 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 exo Y of *A. radio bacter*. Since this was deduced from studies on the exo Y::Tnpbo A 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 exo Y expression is at the level of transcription. However, in previous studies, ros and the related R. meliloti gene murR (Keller et al., 1995) had only been shown to act as repressors of transcription, not activators as is the case with the effect on exo Y. 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 exo Y. Thus, mutations in ros would cause that repressor to be active and so depress the transcription of exo Y.

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 exo Y. If the Ros protein of *A. radio bacter* does bind to DNA upstream of exo Y (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 vir C 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 exo Y::Tnpbo A 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 exo Y were due to the presence of the 'extra' copies of exo X 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 exo X in the vicinity of exo Y which is present in pBI060. 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 Exo Y and Pho A. 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 exo Y::Tnpbo A 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 exo Y genes of Rhizobium to correct exo Y 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 Exo Y proteins. It seems reasonable to suggest that although the Exo Y 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 Exo Y 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 mnrR 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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REFERENCES


products of *Rhizobium* genes, *psi* and *psr*, which affect exopolysaccharide production, are associated with the bacterial cell surface. *Mol Microbiol* 5, 2107–2114.


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