Iron-Dependent Transcription of the Regulatory Gene *ros* of *Agrobacterium radiobacter*

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Transcription of the regulatory gene *ros* of *Agrobacterium radiobacter* requires growth in the presence of Fe although this regulation was not mediated by *ros* itself. The *ros* gene repressed its own transcription, independently of the Fe status of the growth media. It was shown that the two cysteine residues in the Ros protein were essential for the complementation of the exopolysaccharide synthesis defect of *ros* mutant strains. It was found that the mutation in one *exo* mutant strain that was complemented both by *ros* and by the “structural” *exoY* gene was not, in fact, in *ros* but is in some other, unknown gene. The two cysteines were also essential for the correction of this mutant. This mutant affected the expression of *exoY* but not of *ros*.

Additional keyword: autoregulation.

Bacteria of the genera *Rhizobium* and *Agrobacterium* interact with higher plants, inducing, respectively, N₂-fixing root nodules on legumes and tumors on dicotyledonous plants. These bacteria make acidic exopolysaccharides (EPS) that cause their mucoid colony appearance. The genetics of EPS synthesis have been studied in detail in *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*), in which the synthesis of the polymer has been shown to be essential for normal nodule development (see Leigh and Coplin 1992 and Leigh and Walker 1994) in which 22 “structural” genes were identified that specify enzymes for steps in the synthesis of EPS (see Reuber and Walker 1993).

There are two regulatory genes, *exoR* and *exoS*, that, if mutated, cause over-production of the succinoglycan EPS that is normally made by *S. meliloti* (Doherty et al. 1988; Reed et al. 1991). *S. meliloti* also has the potential of synthesizing a different polymer (EPSII) via the *exp* genes whose transcription is normally activated by *mucS* and repressed by at least two genes, *mucR* and *expR* (Astete and Leigh 1996; Keller et al. 1995).

A strain of the closely related *Agrobacterium radiobacter* makes an EPS that is very similar to the succinoglycan of *S. meliloti* (Sutherland 1985). We isolated several EPS[−] nonmucoid mutants of *A. radiobacter*, together with the corresponding, cloned, wild-type DNA (Aird et al. 1991). In contrast to *S. meliloti*, in which nearly all the *exo* genes are clustered on a megaplasmid (Finan et al. 1986), *exo* genes of *A. radiobacter* were in at least five separate loci (Aird et al. 1991). Despite

the chemical similarities of the EPSs of the two genera, with a single exception (*exoB*), *exo* mutants of *Agrobacterium* were not complemented by *exo* genes of *S. meliloti* nor vice versa. Similar observations had been made regarding several *exo* mutants of *A. tumefaciens* (Cangelosi et al. 1987).

Of the *exo* mutants of *A. radiobacter* that were isolated by Aird et al. (1991), some were of interest for two reasons. First, some had a “conditional” phenotype, being EPS[−] on media with dicarboxylic acids as carbon source but EPS⁺ with sugars or sugar alcohols. Further, the conditional EPS⁺ phenotype also required the addition of exogenous iron to the growth medium. Second, some of these conditional mutants, as well as one that was unconditionally EPS[−], were complemented by two cosmids from an *A. radiobacter* gene library that had no cloned DNA in common. The relevant gene in one of these cosmids was *exoY* (Tiburtius et al. 1996), which specifies a glycosyltransferase that acts at an early stage in the synthesis of the EPS monomeric subunits (Reuber and Walker 1993).

The gene in the other cosmid that complemented the unusual *exo* alleles of *A. radiobacter* corresponded to a gene termed *ros* that had been identified in *A. tumefaciens* (Brightwell et al. 1995; Close et al. 1985; Cooley et al. 1991). The *ros* gene product is a DNA-binding transcriptional regulator that represses its own transcription and that of two loci, *virC* and *virD*, that are involved in tumorigenesis (D’Souza-Alt et al. 1993). Repression of *virC* and *virD* required the presence of Fe in the medium (Brightwell et al. 1995) and it was suggested that the Ros might bind to a metal via two cysteines that are separated by two residues (Cooley et al. 1991; Brightwell et al. 1995). The role of metal binding in the function of bacterial regulators has been reviewed by Hennecke (1990). In *S. meliloti*, a homologue of *ros*, termed *mucR*, represses transcription of the *exp* genes that are required for the synthesis of the “second” EPS of this species (Keller et al. 1995). Recently, a gene, *rosR*, was identified in *Rhizobium etli* (Bittinger et al., 1997). Mutations in *rosR* did not abolish EPS production but did result in cells with hydrophobic surfaces and severely affected competitiveness for nodulation.

The *ros* and *mucR* genes were subsequently shown to activate transcription of the *exoY* genes of *A. radiobacter* and *S. meliloti*, respectively (Tiburtius et al. 1996). This could explain why certain *exo* mutants of *A. radiobacter* were complemented by both the cloned *ros* and *exoY* genes. It was proposed that such mutations were in *ros* itself and so could be complemented by cloning that gene. Since *ros* enhanced transcription of *exoY*, it might be that by cloning *exoY* in a multicopy plasmid vector there would be sufficient constitutive

transcription of *exoY* to suppress the EPS⁻ phenotype of the *ros* mutant.

Here, we describe further features of the regulation of and by the unique *ros* transcriptional regulator (apart from that of *mucR*, the sequence of the *ros* gene product has no similarity to any other known regulators).

RESULTS

The *exo81* allele is in *ros* but the *exo73.1* mutation is not.

Aird et al. (1991) found that several of the nitrosoguanidine-induced *Exo* mutant strains of *A. radiobacter* (e.g., *Exo73.1*) that were complemented by both the cloned *exoY* and the *ros* genes were EPS⁻ on media containing dicarboxylic acids as sole C source but were EPS⁺ on media with sugars or sugar alcohols. Other mutants (e.g., *Exo81*) were nonmucoid on both types of media. To establish if this difference in phenotype is due to different types of mutation within *ros* itself, we sequenced the *ros* gene in a "conditional" mutant (*Exo73.1*) and an "unconditional" one (*Exo81*). With the primers described in Materials and Methods, the *ros* gene plus 585 bp upstream (which contains the *ros* promoter; see below) were amplified by polymerase chain reaction (PCR) from the genomes of both the mutant strains and the wild-type T1305. To reduce the risk of introducing a mutation during PCR, for each strain, two independent PCR products were cloned, first directly into pCRTMII and then into the wide-host-range plasmid pRK415 to form pBIO628a and b (*Exo73.1* products), pBIO626a and b (*Exo81* products), and pBIO609a and b (T1305 products). These six plasmids were each mobilized into the mutants *Exo81* and *Exo73.1* and the colony mor-

phologies were examined on media with either glycerol or succinate as sole carbon source. Neither recipient was phenotypically complemented by pBIO626, indicating that *Exo81* contained a mutant, nonfunctional *ros* gene. In contrast, plasmids pBIO628a and b and pBIO609a and b complemented both the *Exo81* and *Exo73.1* mutants on both media.

Sequence analysis of the three cloned PCR products was consistent with these observations. The sequences of the products derived from *Exo73.1* and T1305 were identical to those of the wild type. In contrast, the PCR product from *Exo81* had two single-base-pair substitutions in the coding sequence of *ros* that led to a Thr to Arg and a Lys to Glu substitution at positions 49 and 87, respectively, in the amino acid sequence of *ros* presented by Brightwell et al. (1995), and also had a "-1" frameshift 30 nucleotides from the 5' end of the *ros* coding region that would have disrupted the coding frame of the majority of the gene. Taken together, these observations show that the *Exo81* mutant is defective in *ros* itself whereas in *Exo73.1* the mutation responsible for the conditional phenotype must be in another, unknown gene even though the mutant phenotype can be complemented by *ros*.

Regulation of the *ros* gene of *A. radiobacter*.

To study the functional expression and regulation of *ros* of *A. radiobacter*, we constructed a series of fusion plasmids in which expression of a promoterless reporter gene *lacZ* in pMP220 would be under the control of the sequences upstream of *ros*. Some of these fusion plasmids contained the intact *ros* gene and others did not (see Figure 1).

As indicated in Figure 1, plasmid pBIO607 phenotypically complemented the EPS⁻ defects of *Exo81* and of three other *exo* mutants (*Exo53.1*, *Exo66.1*, and *Exo73.1*) that had been shown by Brightwell et al. (1995) to be complemented by the

Fig. 1. Representation of *Agrobacterium radiobacter* genomic DNA carrying the *ros* gene (hatched box). Horizontal line represents a 2-kb *EcoRV/HindIII* chromosomal fragment cloned into pMP220 (pBIO350) with relevant restriction sites as shown. A series of fragments, carrying different regions, shown as closed boxes, upstream of *ros* were cloned into the *lac* expression vector pMP220. Abilities of each of fusion plasmid to complement the EPS⁻ defect of the *Exo81* mutant strain are indicated. Bold horizontal arrow denotes proposed location of *ros* promoter. Approximate locations of Cys to Ser substitutions in pBIO650 and pBIO651 are shown.

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TCGACGCTGGCTCGGGCGCTCGCGGTTTCTTCITTTTTCGTTCTAGCAATGGCTGCGCA 60
CAAGGCAGGTTATCCGCTTTTCGTTAAATCGGTAGTTGATGACCTCAAGAAATTTGAAG 120
TTGAATTACCCCTAATAATAGGGGGTGGCGGTTTTCATTAATCTACTAGGCATAGTATTT 180
TATTACCCGTAATTTTGTATTCCGATTAGACTGTTTTGAACATAAAATTCACAGCAG 240
GAATTTGACTTTTGTCTGTTCCTGTTTATCCAAATTAAGTCTAAAGTTGCTAATCGCGAT 300
                                     -35 -10 +1
                                     NruI
TTGCATCTAGCCTCTTCATAGTCGGCATAGGCATTTTATTAATGTCTAATCTATGGCCT 360
CAGGACCATTTGCTGCCCATGTCGGGGTCTAAGTTGAATCTCGACCAATACGTTTCCG 420
GTTTCGCTTGAAATCTAGGGGTAGATTTCACGTTGTTTCAACAGGGGGAAGTTGGGCA 480
                                     dyad symmetry
CCGTAATTCACATCGCTCGATGCGGCGTTTCGCCCGTGTGAGGATCACTAACAGGAG 540
                                     [SD]
                                     Sau3A
M T E T A Y G N A Q D L L V E L T A
ATACTTAATGACGGAACGTCATACGGTAACGCCAGGATCTGCTGGTCAACTGACGG 600

D I V A A Y V S N H V V P V T E L P G L
CGGATATTGTGGCTGCCTATGTTAGCAACACGTCGTCGCCGTAACGGAGCTTCCCGGCC 660

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Fig. 2. DNA sequence of the *Agrobacterium radiobacter* *ros* promoter region and the start of *ros* gene extending from the 5' cloning site in pBIO350. Relevant restriction sites are shown; proposed -10 and -35 promoter sequence homologous to an *Escherichia coli* σ^{70} promoter consensus sequence upstream of *NruI* site is boxed. Putative Ros-binding site identified by Cooley et al. (1991) is indicated by inverted arrows. The sequence shown is part of that published by Brightwell et al. (1995), accession number X82941 ARROS in the EMBL data base.

cloned *ros* gene. However, pBIO605, which also contained an intact *ros* gene, did not complement any of these four mutants. This suggested that expression of *ros* required sequences upstream of the *NruI* site that are present in pBIO607 but absent from pBIO605. Brightwell et al. (1995) had reported that a plasmid, pBIO355, that was thought to be identical to pBIO605 did complement these *exo* alleles and suggested that the *ros* promoter lay between the *NruI* site and the start of the gene. However, more detailed subsequent examination of pBIO355 revealed that it inadvertently contained an extra piece of DNA of unknown origin upstream of the *NruI* cloning site; this fragment may have directed the spurious expression of *ros* in that plasmid.

To confirm that the *ros* promoter was upstream of the *NruI* site, the plasmids shown in Figure 1 (and the vector pMP220) were mobilized into wild-type strain T1305 and into the Exo81 *ros* mutant derivative. Each of the strains was grown in MOD minimal growth medium with glucose as sole carbon source, and the cells were assayed for β -galactosidase activity (Table 1). In the wild-type background, plasmids pBIO647 and pBIO643 gave high levels of β -galactosidase activity whereas that with pBIO605 was barely above background. This confirms that the *ros* promoter is upstream of the *NruI* site. As shown in Figure 2, there is a putative *Escherichia coli* σ^{70} promoter-like sequence (TTGCTG.N₁₆.TAAAGT) in this region (McClure 1985). However, it was not confirmed directly that this sequence represented the *ros* promoter.

Plasmid pBIO607 also led to expression of β -galactosidase but the levels were lower (by a factor of two- to threefold) than those found with pBIO643 and pBIO647. Since pBIO607, unlike pBIO643 or pBIO647, contains an intact and expressed *ros* gene, this result is consistent with the autoregulatory properties of *ros* that had been demonstrated by Cooley et al. (1991) and Brightwell et al. (1995). The repressive effects of *ros* on its own transcription were further demonstrated by the fact that the levels of β -galactosidase with pBIO607, pBIO643, and pBIO647 were, as expected, consistently and significantly higher in the *ros* mutant Exo81 than in the wild-type strain since, in this mutant, a functional, chromosomal copy of *ros* is also missing (Table 1).

Effect of Fe and carbon source on the expression of *ros*.

Brightwell et al. (1995) showed that the ability of *ros* to repress *virC-lacZ* and *virD-lacZ* fusions was greatly enhanced by the presence of Fe in the medium. It was not established if this was due to a direct role for the metal for Ros protein to

function or if it was mediated via an effect on the regulation of transcription of *ros*.

Therefore, the derivatives of wild type and the *ros* mutant Exo81 *A. radiobacter* carrying the *lac* fusion plasmids described above were grown in liquid minimal medium to which no Fe had been added and the cells were assayed for β -galactosidase activity. As shown in Table 1, those fusions (pBIO607, pBIO643, and pBIO647) with relatively high activity in cells grown in complete media had strikingly reduced levels of β -galactosidase when grown in the medium depleted of Fe. Note that this Fe-dependent expression from the *ros* promoter occurred with fusion plasmids pBIO643 and pBIO647 in both wild type and in the Exo81 mutant background. Since neither pBIO643 nor pBIO647 carries an intact *ros* gene, and Exo81 is mutant in that gene (see above), it was deduced that the Fe-dependent activation of *ros* expression is independent of *ros* itself. Further, since pBIO643 also displays this behavior, it is apparent that the Fe-mediated regulation involves the DNA upstream of the *NruI* site in which the regulatory sequences involved in *ros* autoregulation are also located.

In the absence of added Fe, in the strains lacking any functional *ros* gene (Exo81 containing pBIO643 or pBIO647), there is still a significant level of expression of the *lac* fusion. This is reduced when these plasmids are in the wild-type background, with its single chromosomal copy of *ros* and, when a functional copy of *ros* is present in the multi-copy plasmid pBIO607, expression of the *ros-lac* fusion is barely above that obtained from the vector alone (Table 1). Thus, *ros* appears to be able to repress its own transcription both in the presence and absence of Fe in the growth medium.

Brightwell et al. (1995) also showed that *ros*-mediated repression of *virC* and *virD* was greater in cells that had been grown in glucose than in those in which succinate was the sole carbon source. Derivatives of wild-type strain T1305 and *ros* mutant Exo81 containing each of the *lac* fusion plasmids described above were grown in Fe-replete and -depleted media in which Na succinate was the sole C source. As with the glucose-grown cells, expression from pBIO605 was barely above background. With the other, "active" fusions (pBIO607, pBIO643, and pBIO647) the β -galactosidase activities were significantly higher, by approximately 20%, in the succinate-grown cells than in those that had been grown in glucose. This slight but significant increase was found in both the wild-type and the mutant Exo81 backgrounds and occurred in both the presence and absence of added Fe (data not shown).

Table 1. β -Galactosidase activities of *ros*

<i>lac</i> fusion plasmid	β -Galactosidase activity ^a					
	T1305		Exo81		Exo73.1	
	Fe ⁺	Fe ⁻	Fe ⁺	Fe ⁻	Fe ⁺	Fe ⁻
pMP220	11 $\pm$ 2	10 $\pm$ 3	12 $\pm$ 3	11 $\pm$ 3	10 $\pm$ 3	9 $\pm$ 2
pBIO605	11 $\pm$ 4	10 $\pm$ 3	13 $\pm$ 3	11 $\pm$ 2	14 $\pm$ 3	10 $\pm$ 2
pBIO607	1,251 $\pm$ 104	75 $\pm$ 11	3,046 $\pm$ 111	48 $\pm$ 12	1,563 $\pm$ 69	65 $\pm$ 10
pBIO643	4,313 $\pm$ 122	344 $\pm$ 42	10,523 $\pm$ 506	955 $\pm$ 56	3,421 $\pm$ 166	605 $\pm$ 108
pBIO647	4,100 $\pm$ 161	409 $\pm$ 31	8,478 $\pm$ 416	1,221 $\pm$ 126	ND ^b	ND ^b

^a β -Galactosidase activities of *ros-lac* fusion plasmids in wild-type (T1305) and mutant (Exo81 and Exo73.1) backgrounds. Bacteria were grown in MOD (Aird et al. 1991) supplemented with or without added 0.5 mM FeCl₃. Activities are as defined by Miller (1972). Means and standard deviations of duplicate determinations are from at least three independently grown cultures.

^b Not determined.

Expression of *ros* in the Exo73.1 mutant.

Although the cloned *ros* gene corrected the EPS⁻ phenotype of the Exo73.1 conditional mutant on succinate growth medium, the lesion in Exo73.1 had been shown not to be in *ros* or its upstream sequences. One hypothetical explanation for this is that the gene that is mutated in Exo73.1 normally serves to activate expression of *ros*. Just as cloning of the target gene *exoY* can suppress the EPS⁻ defect of a *ros* mutant, so it might have been the case that cloning *ros* could suppress the phenotype of the Exo73.1 mutant. However, this was shown not to be the case.

The “active” fusion plasmids (pBIO607 and pBIO643) that expressed β -galactosidase in the T1305 background were mobilized into the Exo73.1 mutant and the cells were grown in the presence or absence of Fe in the medium. The results of the β -galactosidase assays on these strains were essentially the same as in the wild-type strain T1305 background, i.e., activities were augmented by growth in the presence of Fe and were higher with pBIO643 than with the pBIO607 plasmid (Table 1).

Since the Exo73.1 mutant is conditional, being EPS⁻ on medium with succinate but EPS⁺ with glucose as C source, these assays were also done with cells that had been grown in media with either of these compounds as sole carbon source. Again, the levels of β -galactosidase obtained with each of these fusion plasmids were similar to those that had been found in strain T1305, rather than to the higher levels found in the Exo81 background; thus, the activities were significantly higher in the succinate-grown cells. As in the wild-type background, this was found with cells grown in both the presence and absence of added Fe (data not shown).

These sets of results with Exo73.1 confirm that, despite the apparent similarities, this mutant is significantly different from Exo81.

Importance of the two cysteine residues in the Ros protein.

Within the deduced Ros proteins of *A. tumefaciens* (Cooley et al. 1991) and *A. radiobacter* (Brightwell et al. 1995), which each have 142 amino acid residues, is a sequence, ₇₅HIVCLECGG₈₅ (in which subscript numbers refer to the location of the residues in the Ros protein sequence of Brightwell et al. [1995]), that Cooley et al. (1991) suggested might be a zinc finger or some other metal-binding motif. To establish the importance of this region and, specifically, that of these two cysteines, mutagenic primers were used separately to convert each of the cysteine residues to serine as described in Materials and Methods. The two mutated forms of *ros* were confirmed by sequencing and each was recloned into pMP220 to form plasmids pBIO650 and pBIO651 that, respectively, contain the C79S and C82S substitution mutations. These two plasmids were mobilized into the Exo81 and Exo73.1 mutants. In all cases, the transconjugants were completely nonmucoid, showing that both of these two cysteine residues are essential for Ros to function, at least in terms of correcting the defect in EPS synthesis.

Plasmids pBIO650 and pBIO651 were constructed in such a way as to form transcriptional fusions in which the *lacZ* of vector pMP220 was under the control of the *ros* promoter. To establish the effects of changing the two cysteines, these two plasmids were mobilized into the *ros* mutant strain Exo81 and β -galactosidase activities were determined. The activities were relatively low, being very similar to those obtained with

pBIO607 (which contains the intact *ros* gene) rather than to those of any other fusion plasmids (e.g., pBIO643) that lacked any part of *ros* (not shown). Our interpretation of these results is that although these two cysteine residues are needed for Ros to complement the EPS⁻ defect, they are dispensable for the autoregulation of transcription.

Brightwell et al. (1995) showed that the removal of 100 nucleotides at the 3' end of *ros* generated a “dominant” allele; i.e., when this deleted form of *ros* was transferred to wild-type *Agrobacterium* or to *Rhizobium leguminosarum*, the transconjugants became nonmucoid. Therefore, we mobilized pBIO650 and pBIO651 into wild-type *A. radiobacter*. It was found that the transconjugants were fully mucoid, showing that substitution of the two cysteines did not confer a dominant phenotype. In contrast, another *ros* deletion mutant plasmid that we constructed, pBIO641, contains a smaller deletion (extending downstream from a *StyI* site) that has lost 40 bp of the 3' *ros* coding region. pBIO641 was dominant, repressing EPS production in wild-type *A. radiobacter*.

DISCUSSION

The *ros* gene product of *Agrobacterium* (equivalent to that of *mucR* of *S. meliloti*) is a transcriptional regulator that is distinct from any known bacterial regulatory protein. It can repress its own transcription and that of the *virC* and *virD* loci, and mutations in it result in elevated expression of the structural gene *exoY*.

Upstream of *virC* and *virD* are identical, inverted repeats, termed *ros* boxes (Cooley et al. 1991), and in the case of *virD* it was shown in vitro that the Ros and MucR proteins could bind to sequences that spanned and included this region of dyad symmetry (Bertram-Drogatz et al. 1997; D'Souza-Alt et al. 1993). Cooley et al. (1991) noted that a near-identical sequence of dyad symmetry also preceded the *ros* gene of *Agrobacterium* but that the repeats were “inside out” compared with those in the *ros* boxes preceding *virC* and *virD*. By implication, this would mean that if these sequences were important for the recognition and binding of the Ros protein, each of the two repeated elements of the inverted repeat would have to be recognized independently, an unusual feature for an interaction between a bacterial regulatory protein and its cognate DNA recognition sequence. However, recognition of such protein-binding “half-sites” has been proposed for the product of the *narL* gene of *E. coli* (see Stewart 1993). Nevertheless, the results presented here indicate that the “inside out” *ros* box preceding *ros* is not involved in autoregulation of this gene. We found that *lac* fusion plasmid pBIO643, which lacked this motif, was autoregulated, its expression being higher in the *ros* mutant Exo81 than in the wild-type background. Within the 300-bp sequence upstream of *ros* that is present in pBIO643, there is no sequence similar to that of the *ros* box nor any sequence of significant dyad symmetries. Further, pBIO643 lacked the putative -10 and -35 consensus sequences that Cooley et al. (1991) suggested might be the *ros* promoter of *A. tumefaciens*. This 300-bp region does, however, contain a potential (though not experimentally confirmed) *E. coli* σ^{70} promoter sequence. It may be that *ros* in *A. radiobacter* acts to repress its own transcription in an indirect manner by, for example, repressing the expression of an activator of *ros* transcription. Recently, Bertram-Drogatz et al.

(1997) showed that the homologous MucR protein of *S. meliloti* also bound to a region that contained an inverted repeat just upstream of *mucR*. Just as we found here, they found that the *mucR* transcriptional start site was upstream, by approximately 250 bp, of this potentially regulatory sequence. Thus, the autoregulatory properties of *ros* and *mucR* would appear to be most unusual, with at least one of their binding sites being so far downstream of the transcriptional starts of these genes.

Brightwell et al. (1995) showed that *ros*-dependent repression of *virC* and *virD* was greatly diminished when the bacteria were deprived of Fe. Given the suggestion that *ros* might be a metallo-protein (Cooley et al. 1991; Brightwell et al. 1995), binding a metal via the two closely linked cysteines (whose importance for *ros* gene function has been demonstrated here), it was thought possible that this dependence on Fe might be due to a direct interaction between the metal and the Ros protein (see, for example, Hennecke 1990). However, this may not be the case. We found that transcription of *ros* was significantly higher in cells whose growth medium was replete with Fe and that this was independent of a functional copy of *ros*. Thus, the simplest explanation for the Fe-dependent repression of *virC* and *virD* by *ros* is that, in the presence of Fe, transcription of *ros* is greatly enhanced. This observation points to the presence of another regulatory gene whose product either activates *ros* transcription in the presence of Fe or represses it under low Fe conditions. Since pBIO643, containing only 300 bp of the region upstream of *ros*, exhibits this Fe effect, it appears that this region also contains sequences that respond to Fe-dependent regulation. In bacteria, many genes have been shown to be controlled, in response to the availability of Fe, by the regulatory *fur* gene (see Hennecke 1990), whose product binds to a characteristic *fur*-box motif (ATAATGAT-N1-ATCATTAT). No such consensus sequence was found upstream of the *ros* gene.

Brightwell et al. (1995) also showed that *ros*-dependent repression of *virC* and *virD* was greater in cells grown in glucose than in those grown in succinate. However, this could not be attributed to enhanced transcription of *ros* in the former conditions. In fact, we found that in the presence or absence of a functional *ros* gene, and in the presence or absence of added Fe, transcription of *ros* was significantly higher with succinate-grown cells than with those grown with glucose. At present, the molecular basis of the effect of the C source on *ros*-mediated repression of *virC* and *virD* is unknown.

Some of the mutants that can be complemented by both the cloned *ros* and *exoY* genes are conditional, being EPS⁺ on glucose medium and EPS⁻ on medium with succinate as sole C source, providing that exogenous Fe is present in the growth medium (Aird et al. 1991). Here, we showed that in one such mutant, Exo73.1, the *ros* gene and its upstream regulatory sequences are the same as in the wild type and so the mutation that leads to the loss of EPS synthesis must be in some other, unknown gene. Expression of *ros* was not affected by the mutation in Exo73.1, indicating that its mutated gene acts independently of *ros*. At present, though, we have no clear model to describe the basis of the carbon-source dependent, conditional EPS phenotype of mutants such as Exo73.1. It may be that in Exo73.1, the primary defect can somehow be substituted if the *ros* gene is expressed at higher than normal levels, as would be the case when it is cloned on a multi-copy

plasmid. The requirement for Fe in the medium for the EPS⁺ phenotype to occur can be explained simply in terms of the higher expression of *ros* under such growth conditions. Whatever the explanation for the ability of the cloned *ros* gene to correct Exo73.1, it is clear that the two cysteines in the Ros protein are also needed for this complementation.

Brightwell et al. (1995) found that 3' deletions of *ros* destroyed its ability to complement *exo* mutants and that such deletions, removing up to 100 nucleotides of the coding region, had a dominant phenotype, causing wild-type strains of *Agrobacterium* to become nonmucoid. Here, we showed that a 3' deletion of as little as 40 bp conferred the same dominant phenotype but that substitution of the two Ros cysteine residues did not. In *R. etli*, a frameshift mutation at the 3' end of *rosR* also conferred a dominant phenotype. Brightwell et al. (1995) suggested that the Ros protein acted as a multimer and that the dominant alleles specified a polypeptide that could participate in such a multimer but, by so doing, inactivated the whole complex even though it contained wild-type forms of the protein. If this is true, it is apparent from the results obtained here that the substitution of the cysteines does not lead to the formation of a mutant protein with such "blocking" properties.

Given the novel nature of the Ros/MucR proteins, found so far only in *Agrobacterium*, *Rhizobium*, and *Sinorhizobium*, it will be of interest to analyze them at a structural level since, at present, no obvious DNA-binding motifs are evident. In particular, the finding that the substitutions of the two cysteine residues in Ros abolish its ability to correct the EPS⁻ defects of Exo81 and Exo73.1 but not, apparently, to autoregulate, offers the prospect of generating other site-directed mutants in *ros* in order to dissect the functions of different domains within the protein. The relatively small size of Ros (15.5 kDa) makes it a potentially attractive candidate for such future studies, although these might be hampered by the reported relative insolubility of the over-expressed Ros protein (D'Souza-Alt et al. 1993; H. Hussain, unpublished observations).

MATERIALS AND METHODS

Strains and microbiological procedures.

Bacterial strains and plasmids used in this work are listed in Table 2. *E. coli* was grown in LB medium at 37°C and *Agrobacterium* at 28°C in MOD medium (Aird et al. 1991). Recombinant plasmids based on pMP220 or pRK415 were mobilized into *Agrobacterium* by triparental mating with pRK2013 as a helper (Figurski and Helinski 1979).

β-Galactosidase assays.

Cultures of *Agrobacterium* were grown for 2 days in MOD medium at 28°C and then diluted 1:50 into MOD medium supplemented with 0.4% glucose or succinate and in the presence or absence of 0.5 mM FeCl₃. Cells were then grown to an OD₆₀₀ of approximately 0.4 to 0.8. Cells were permeabilized and β-galactosidase activities were determined by the method of Miller (1972). All strains, together with the controls, were assayed in duplicate on two or more occasions.

DNA manipulations.

Standard recombinant DNA techniques were used throughout this work as described in Sambrook et al. (1989). Oligo-

Table 2. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference / source
<i>Escherichia coli</i>		
803	Met ⁻ ; used as a recipient in routine transformations	Wood 1966
JM101	F ⁻ traD36 proA ⁺ proB ⁺ lac ^q lacZAM15 supE thi Δ(lac-proAB)	Messing et al. 1983
<i>Agrobacterium radiobacter</i>		
T1305	Wild type; Str ^r	
Exo73.1	Exo ⁻ , complemented by <i>ros</i> and <i>exoY</i>	Aird et al. 1991
Exo81	<i>ros81</i> , Exo ⁻ , complemented by <i>ros</i> and <i>exoY</i>	Aird et al. 1991
Plasmids		
pUC18	Amp ^r	Vieira and Messing 1984
pBluescript	Amp ^r	Stratagene (La Jolla, CA)
pRK2013	Kan ^r , used for mobilizing P1-group plasmids	Figurski and Helinski 1979
pMP220	Tet ^r , <i>lacZ</i> , wide-host range P-group cloning vector	Spaank et al. 1987
pCRTMII	Amp ^r , Kan ^r , used for direct cloning of polymerase chain reaction (PCR) product	Invitrogen (Abingdon, England)
pRK415	Tet ^r , wide-host range P-group cloning vector	Keen et al. 1988
pBIO350	2-kb <i>EcoRV</i> / <i>HindIII</i> fragment in pMP220; containing <i>ros</i>	Brightwell et al. 1995
pBIO605	0.8-kb <i>NruI</i> fragment in pMP220	This work
pBIO606	0.9-kb PCR <i>ros</i> in pBluescript	This work
pBIO607	0.9-kb PCR <i>ros</i> from pBIO606 in pMP220	This work
pBIO608	0.9-kb PCR <i>ros</i> from T1305 in pCR TM II	This work
pBIO609	0.9-kb <i>EcoRI</i> fragment of pBIO608 in pRK415	This work
pBIO626	0.9-kb <i>EcoRI</i> fragment of pBIO610 in pRK415	This work
pBIO628	0.9-kb <i>EcoRI</i> fragment of pBIO612 in pRK415	This work
pBIO641	0.9-kb <i>EcoRI</i> / <i>SstI</i> of pBIO606 in pRK415	This work
pBIO643	0.3-kb <i>EcoRI</i> / <i>KpnI</i> fragment from pBIO642 in pMP220	This work
pBIO647	0.55-kb <i>EcoRI</i> / <i>PstI</i> fragment from pBIO645 in pMP220	This work
pBIO650	0.9-kb <i>EcoRI</i> fragment of pBIO630 in pMP220	This work
pBIO651	0.9-kb <i>EcoRI</i> fragment of pBIO631 in pMP220	This work

nucleotides were synthesized by the Genosys Co. (Cambridge) and PCR products were amplified on an Omnigen thermal cycler. For DNA sequencing, the mutant forms of *ros* were recloned into plasmid vector pUC18 and the sequences of the entire fragment determined by the dideoxy-chain termination method (Sanger et al. 1977). The reactions were run on an ALF automated sequencer (Pharmacia, Uppsala, Sweden).

Plasmid constructions.

Plasmid pBIO350 contains a 2-kb *EcoRV*/*HindIII* fragment that includes *ros* plus sequences upstream and downstream of the gene, cloned in the wide-host-range vector pMP220 (Brightwell et al. 1995). This plasmid was used as a template to generate a PCR product that contained *ros* plus 585 bp upstream of the start of the gene. The primers used (5'-CTATG GATATCTCGACGCTGGCTC and CGCAAGCTTCAGCG GTTGGCCTTC-3') also introduced *EcoRV* and *HindIII* restriction sites at the 5' and 3' ends of the PCR product. This product was digested with *EcoRV*/*HindIII* and cloned into pBluescript IISK digested with *EcoRV*/*HindIII*. The resulting plasmid, pBIO606, was used to create four *lac* fusion derivatives of pMP220, as follows (Fig 1).

pBIO607. This plasmid contains the entire *ros* gene, including its termination codon plus 585 bp of upstream DNA. This fragment was created by inserting the *EcoRV*/*HincII* fragment of pBIO606 into pMP220 digested with *EcoRI* and treated with the Klenow fragment of DNA polymerase. This plasmid therefore contained exclusively a *lac* transcriptional fusion.

pBIO643. This plasmid contains 0.3 kb of DNA upstream of the *NruI* site shown in Figure 1. It was created by deleting the *NruI*/*HincII* fragment from pBIO606 to form pBIO642 and then cloning an 0.3-kb *EcoRI*/*KpnI* fragment of pBIO642 into pMP220 digested with the same enzymes to make pBIO643.

pBIO647. This plasmid contains 565 bp of DNA upstream of *ros* (extending to the *Sau3A* site). It was created by cloning the 0.55-kb *EcoRI*/*Sau3A* fragment of pBIO606 into pUC18 (pBIO645) and then into pMP220 as an *EcoRI*/*PstI* fragment.

pBIO605. This plasmid contains the entire *ros* gene plus 250 bp immediately upstream of it (extending to the *NruI* site; see Figure 1). It was constructed by cloning the 0.8-kb *NruI* fragment of pBIO350 into pMP220 digested with *EcoRI* and treated with the Klenow fragment of DNA polymerase.

Sequence analysis of Exo mutants.

Genomic DNA was isolated from wild-type strain T1305 and from mutant strains Exo73.1 and Exo81. The oligonucleotides described above were used to amplify a PCR product of the *ros* gene from these three strains. These were cloned into the pCRTMII vector to generate pBIO610, pBIO612, and pBIO608 (from Exo81, Exo73.1, and T1305, respectively).

These 0.9-kb fragments were recloned into pUC18 and their entire DNA sequences determined. They were also inserted into the wide-host-range vector pRK415 to generate pBIO626, pBIO628, and pBIO609, respectively.

Site-directed mutagenesis.

Plasmid pBIO350 DNA was used as the template for mutagenesis of each of the two codons specifying the cysteine residues in the Ros protein to convert them into serine codons. Site-directed mutagenesis was performed by the methods of Marini et al. (1993), using a PCR method for internal site-directed mutagenesis of large amplified molecules (megaprimer) and two-step PCR amplification. The final PCR products that contained the mutations with appropriate substitutions were then cloned directly into pCRTMII with a TA cloning kit (Invitrogen, Abingdon, England), and their nucleotide sequences were confirmed. Then the resulting plasmids were

digested with *Eco*RI and the fragments containing the mutant *ros* were cloned into pMP220 to form the transcriptional *lac* fusion plasmids pBIO650 and pBIO651.

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