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Pleiotropic Effects of Regulatory *ros* Mutants of *Agrobacterium radiobacter* and Their Interaction with Fe and Glucose

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Four *exo* mutants of *Agrobacterium radiobacter*, defective in the synthesis of acidic exopolysaccharide were complemented by a gene from that species, which is similar to the transcriptional regulator, *ros*, of *A. tumefaciens*. It was confirmed that this *A. radiobacter* gene, which we term *rosAR*, like *ros*, repressed its own transcription as well as that of *virC* and *virD*, two loci involved in tumorigenesis. The sequence of RosAR suggested that it might bind to a transition metal and its repressor abilities were shown to require Fe in the medium; repression was also enhanced with increasing levels of glucose. Certain *rosAR* mutants, in which its 3′ end was removed were dominant; i.e., when plasmids containing such mutant forms of the gene were introduced into wild-type *A. radiobacter*, the transconjugants were nonmucoid. Such effects were also seen in a wide range of bacteria, including *Escherichia coli* and *Xanthomonas*. Several mutants that were complemented by *rosAR* also accumulated protoporphyrin, suggesting a defect in haem synthesis.

Additional keywords: Agrobacterium, gene regulation, iron-dependent protein, porphyrin.

Gram-negative bacteria synthesize various forms of high molecular weight exopolysaccharides (EPS) that have roles in pathogenesis, symbiosis, attachment to solid surfaces, or resistance to desiccation.

Agrobacterium tumefaciens makes an acidic succinoglycan EPS which comprises a polymer of repeating octomeric units made up of glucose and galactose in a molar ratio of 7:1 (Hisamatsu et al. 1981). Cangalosi et al. (1987) isolated several nonmucoid mutants of a strain of *A. tumefaciens* and showed that these were in several different complementation groups. Only one group of mutants was defective in the ability to induce tumors, but this was probably not due to the failure to make the EPS per se; these mutations, in *exoC*, had pleiotropic effects, abolishing the ability of the bacteria to make a low molecular weight cyclic glucan whose role in tumor formation had been demonstrated (Uttaro et al. 1990). Another *A. tumefaciens exo* gene in which mutations have pleiotropic effects was described by Close et al. (1985) and by Cooley et al. (1991). Mutations in this gene, termed *ros* (*r*ough *o*uter *s*urface) caused a nonmucoid phenotype and caused elevated levels of transcription of *virC* and *virD*, two loci that augment tumorigenesis on dicotyledonous plants, indicating that *ros* was a negatively acting regulatory gene; using *virC–* and *virD–lacZ* fusions it was shown that *ros* repressed their transcription and was also shown to be autoregulatory, repressing its own transcription. More recently, D'Souza-Ault et al. (1993) showed that the Ros protein bound specifically to a region of dyad symmetry that precedes *virC* and *virD*. The sequence of *ros* showed no similarity to any other known regulatory bacterial gene (Cooley et al. 1991). *ros* mutant strains induced normal tumors on plants. Cooley et al. (1991) showed that strains of *Rhizobium* contained DNA that was homologous to *ros* and recently it has been shown that the sequence of *mucR* of *R. meliloti* is similar to that of *ros* (A. Puhler, personal communication). In *R. meliloti, mucR* is also a regulatory gene, being involved in the repression of genes involved in the synthesis of the "second" EPS of that species and, possibly, in the activation of transcription of the "conventional" *exo* genes of that species.

Previously (Aird et al. 1991), we obtained several *exo* mutants of a strain of *A. radiobacter* and isolated various recombinant plasmids from a gene bank of that strain (cloned in the wide host-range vector pLAFR1) which functionally complemented them. By doing complementation tests, hybridizations, and restriction mapping of the corresponding cosmids, it was shown that the *exo* loci were in at least five unlinked (i.e., each >30 kb apart) regions of the genome. This is in contrast to the situation in the closely related *Rhizobium meliloti* which makes a very similar EPS to that of *Agrobacterium* (reviewed in Leigh and Coplin 1992). In *R. meliloti*, most of the *exo* loci are located in a contiguous cluster on one of the megaplasmids of this species (Long et al. 1988b; Reuber and Walker 1993). Given the chemical similarity of the EPS's in these two genera it was surprising that only one of the *exo* alleles of *A. radiobacter*, in the *exoB* gene, was complemented by any of the cloned *exo* genes of *R. meliloti* (Aird et al. 1991). In contrast, approximately 50% of the *exo* mutants of *A. tumefaciens* were complemented by various of the cloned *R. meliloti exo* genes (Cangelosi et al. 1987).

Of the *exo* alleles of *A. radiobacter* which were isolated, four were of particular interest for two reasons. Firstly, they were each complemented by two cosmids, pBIO11 and

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pBIO21, which contained no cloned DNA in common as judged by hybridizations (Aird et al. 1991). Secondly, of the four, three had a "conditional" Exo phenotype, being EPS$^+$ on media containing glucose or glycerol as sole C source but were EPS$^-$ when succinate was the C source. By subcloning, it was shown that the relevant *exo* DNA was confined to regions of 4.0 and 4.1 kb in pBIO11 and pBIO21, respectively. Following the isolation of *exo–phoA* fusions, it was deduced that one or more of the *exo* genes in pBIO21 specified a polypeptide that was located in the bacterial membranes or periplasm (Aird et al. 1991). In *Rhizobium*, it has been shown that some of the proteins required for EPS synthesis are membrane-bound (Long et al. 1988a; Latchford et al. 1991; Reuber et al. 1991; see Leigh and Walker 1994).

Aird et al. (1991) proposed that these alleles could be complemented by two different, nonhomologous, regions of DNA due to their being in a regulatory gene whose presence was required for the activation of expression of unlinked regulatory *exo* "structural" genes. Such mutations could be complemented either by cloning the regulatory gene itself in (e.g.) pBIO11 or by cloning the structural "target" gene in such a way that it no longer required the regulatory gene (as, for example, would be the case if it were transcribed from a constitutive vector promoter in pBIO21).

In this paper, we confirm that pBIO11 indeed contains a regulatory gene which has some unusual properties.

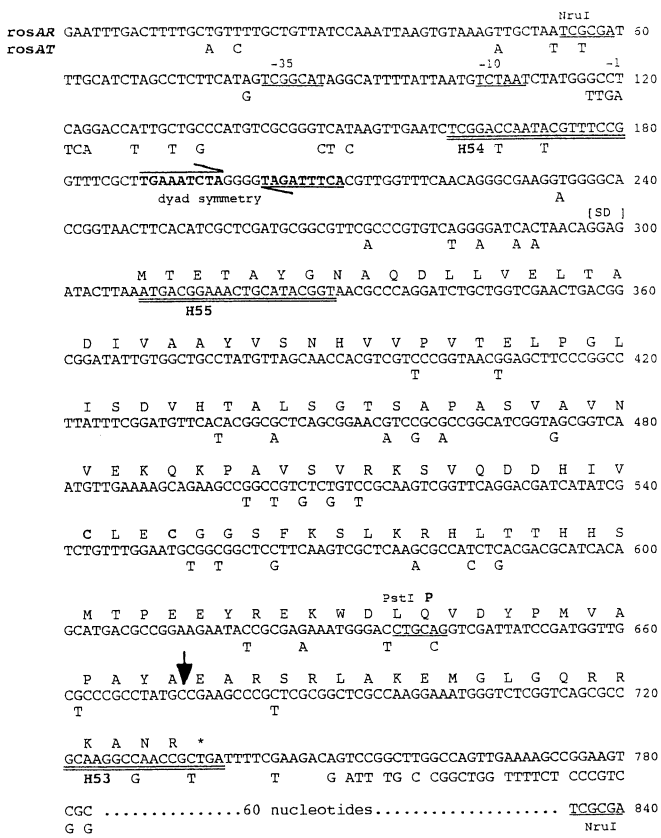

Fig. 1. The sequences of *rosAR* and its deduced peptide product are shown. The region of dyad symmetry is shown as are differences in nucleotide and the single amino acid between *rosAT* and *rosAR*. The two cysteines are highlighted. The vertical arrow shows the location of the insertion in the dominant mutant plasmid pBIO340 and the *Pst*I site used to clone the dominant *rosARΔ* fragment is also shown. The doubly underlined sequences represent the dimensions of the primers used for the amplification of *rosAR* with or without the 150 bp 5′ region and the *Nru*I sites used for the construction of pBIO355 are marked. A possible promoter region is also indicated. Accession number is X82941 ARROS in EMBL Database.

RESULTS

Identification and sequence of *rosAR*.

Aird et al. (1991) had shown that a 4-kb *Hin*dIII fragment, subcloned from the cosmid pBIO11 into pMP220 to form pBIO344 could complement all four *exo* alleles that were complemented by pBIO11 itself. A 2.5-kb *Hin*dIII–*Eco*RV fragment was subcloned from pBIO344 into pMP220 to form pBIO350. This plasmid was mobilized into the four *exo* mutants and was found to complement the EPS$^-$ defect of all of them. This *Hin*dIII–*Eco*RV fragment, obtained from pBIO350, was isolated from a gel, and, after filling in, was circularized by ligation and then, following sonication, fragments of approximately 600 bp's were filled in and ligated into the vector pGEM 3ZF(+), which had been cut with *Sma*I. From these clones, double-stranded DNA was sequenced, using forward and reverse "universal" primers. As the data were being compiled, we noted an ORF with a strong probability of encoding a polypeptide (with a deduced M_r of 15.5 kDa) which spanned the sites of the *exo* mutations in pBIO11 (Aird et al. 1991; Brightwell 1993). This ORF was cloned as an 0.8-kb *Nru*I fragment which extended, respectively, 260 and 55 upstream and downstream of it (Fig. 1). This fragment was cloned first into pUC18 and thence as a 0.8-kb *Nru*I fragment into pMP220 to form pBIO355. This plasmid was found to complement all four of the *A. radiobacter exo* mutants for EPS synthesis on both glucose and succinate. Since the cloning sites in the polylinker in pMP220 are not transcribed from any promoter in the vector (Spaink et al. 1987), these observations indicated that the region 5′ of this ORF which are present in pBIO355 also contained the promoter for this gene. Using the primers H53 and H54 (see Fig. 1) the ORF plus 150 bp's upstream of it was amplified and cloned into pCR and thence into the *Kpn*I/*Eco*RI sites of pMP220. The resultant plasmid, pBIO364, was mobilized into the original four *exo* mutant strains of *A. radiobacter*; in none of the cases were the transconjugants mucoid on media containing either glucose or succinate. This suggests that the promoter for the expression of the ORF lies between the *Nru*I site (position 54 in Fig. 1) and the region corresponding to the location of primer H54 (Fig. 1), consistent with the observations of D'Souza-Alt et al. (1993).

Similarity of the *A. radiobacter* ORF to *ros*, an *A. tumefaciens* regulatory gene.

We compared the sequence of the ORF identified here and its deduced protein product to those in databases and this revealed one significant match. As shown in Figure 1, the region in and around it was very similar to the regulatory gene *ros* which had been identified in a strain of *A. tumefaciens* (Cooley et al. 1991). In the coding sequences of the *A. radiobacter* ORF and *ros* of *A. tumefaciens,* there are only 27 differences at the nucleotide level, leading to one change in their deduced polypeptide products (at position 110 a Q in our sequence and a P in Ros). Further, the sequences 5′ of the two genes are also conserved, 23 differences in 260 bp's. This

conserved upstream region includes the sequence of dyad symmetry to which the Ros protein has been found to bind (D'Souza-Alt et al. 1993) However, 10 base pairs downstream of the coding regions, the two sequences diverge. (Fig. 1). For the purposes of this paper, we refer to the gene identified here as *rosAR* and that in *A. tumefaciens* as *rosAT.*

To show that *rosAR* and *rosAT* are functionally equivalent, the cloned *rosAR* gene (in pBIO355) was mobilized into the nonmucoid *rosAT* mutant strain LBA 4301R of *A. tumefaciens* and, reciprocally *rosAT*, in plasmid pCR8, was transferred to all four *rosAR* mutants of *A. radiobacter*. In all cases, the transconjugants were mucoid on MOD media showing that, for EPS synthesis, *rosAR* and *rosAT* are functionally interchangeable. Further, the cloned *rosAR* gene was introduced by transformation into *E. coli* strain JM103 (Lac$^-$) containing the *virC–lacZ* (pSM365) or *virD–lacZ* (pSM344) fusion plasmids. In qualitative tests for β-galactosidase, in which strains were streaked on LB medium supplemented with X-Gal, it was clear that the *rosAR* gene of *A. radiobacter* reduced transcription of both the *virC–* and *virD–lacZ* fusions; i.e., colonies of strains containing the cloned *rosAR* stained much more palely than those lacking this regulatory gene. Thus in *E. coli*, *rosAR*, like *rosAT*, can act as a transcriptional repressor of these two *vir* loci. In addition, the *virC-lacZ* fusion plasmid was mobilized into wild-type *A. radiobacter*, into four *rosAR* mutant derivatives and into a 'control' *exo* strain (Exo 87.1) and β-galactosidase activities were determined on cells grown in MOD-D2 (glucose) media. As shown in Table 1, the expression of the fusion was substantially elevated in the *rosAR* mutants relative to the wild-type or the Exo 87.1 mutant strain. (The background β-galactosidase in TI305*lac* is lower because this strain lacks the native enzyme).

Some *rosAR* mutant alleles are dominant.

Aird et al. (1991) found that some transposon insertions into pBIO11 generated "dominant" mutants; i.e., such

Table 1. Strains and plasmids used in this study

Species/strain	Characteristics	Source
Agrobacterium radiobacter		
T1305 lac^9	Wild type for EPS, also *str* β-galactosidase deficient	Aird et al. 1991
Exo53.1		
Exo63	Exo$^-$, complemented by pBIO11 and pBIO21	
Exo66.1		Aird et al. 1991
Exo73		
Exo81		
Exo87.1	Exo$^-$, not complemented by pBIO11 or pBIO21	Aird et al. 1991
A. tumefaciens		
LBA4301	Wild type	Close et al. 1985
LBA4301R	*rosAT* mutant of LBA4301	Close et al. 1985
Rhizobium leguminosarum		
8401	Str^r; cured of sym plasmid	Lamb et al. 1985
Escherichia coli		
803	Used for routine transformation	Wood 1966
DH1	Recombination deficient	Hanahan 1983
JM101	Host for pUC and M13 clones	Messing et al. 1983
Xanthomonas campestris		
8001R	Wild type *X. campestris* also rif^r	M. J. Daniels
R. sphaeroides	Wild type; Nal^r	
WS8		W. Sistrom
Erwinia amylovera	Wild type	Bennet and Billing1978
OT1		
Plasmids		
pUC18/pUC19	Amp^r	Messing et al. 1983
pGEM3ZF(+)	Amp^r; used for sequencing	Promega
pCR	Used for cloning PCR products	Invitrogen
pTrc99	Expression vector	Pharmacia
pBR328	Amp^r Tet^r	Boehringer Manaheim
pISR407	*rcsA* gene of *E. amylovora* cloned in pBR322	Coleman et al. 1990
pMP220	Wide host-range cloning vector	Spaink et al. 1987
pRK2013	Mobilizing plasmid	Figurski and Helinski 1979
pCR8	Cloned *rosAT* gene of *A. tumefaciens* in wide host-range vector	Cooley et al. 1991
pSM365	*virC-lacZ* fusion plasmid	Stachel et al. 1985
pSM344	*virD-lacZ* fusion plasmid	Stachel et al. 1985
pBIO11	pLAFR1-based cosmid; contains *rosAR*	Aird et al. 1991
pBIO21	pLAFRI-based cosmid; contains *A. radiobacter exoY*	Aird et al. 1991
pBIO350	2-kb *Eco*RV/*Hin*dIII fragment in pMP220; contains *rosAR*	This work
pBIO355	0.8-kb *Nru*I fragment in pMP220	This work
pBIO364	*rosAR* plus 150 bp's 5′ of *rosAR* in pMP220	This work
pBIO340	pBIO11 *rosAR*::Tn5; *lac* confers dominant phenotype	This work
pBIO386 and pBIO387	6-kb *Hin*dIII fragment of pBIO340 containing Tn5*lac* Z junction in pBR328	This work
pBIO385	Contains deleted *rosAR*(*rosARΔ*) *Hin*dIII-*Pst*I fragment in pMP220	This work
pBIO391	*rosARΔ* fragment from pBIO385 cloned in pUC18	This work
pBIO353	PCR *rosAR* fragment in pCR	This work
pBIO380	PCR *rosAR* fragment from pBIO353 in pTrc99	This work
pBIO344	4-kb *Hin*dIII fragment in pMP220; contains *rosAR*	Aird et al. 1991

pBIO11::Tn5*lac* mutant plasmids, when transferred into wild-type *A. radiobacter*, caused the transconjugants to be markedly reduced in their production of EPS. These dominant mutations had been located in or near to the *rosAR* gene (Aird et al. 1991; Brightwell 1993).

Plasmid pBIO340 is a derivative of pBIO11 containing one such dominant Tn5*lac* insertion. Restriction mapping showed that the transposon was in or near *rosAR*. To locate the insertion precisely, pBIO340 was digested with *Hin*dIII (which has a single site in Tn5*lac* but none in *lacZ*) and fragments were ligated to *Hin*dIII-cut pBR328 prior to transformation into *E. coli* strain JM103. Transformants were plated on media containing X-Gal. Two blue colonies, presumed to contain the *lacZ* gene of Tn5*lac* plus pBIO11 DNA extending 5′ to the next *Hin*dIII site were obtained. Plasmids, termed pBIO386 and pBIO387 were isolated from these two transformants and in both cases were shown to have inserts of approximately 6.5 kb. Double-stranded templates of pBIO386 and pBIO387 DNA were prepared and were sequenced, using a fluorescent primer which corresponds to the 5′ end of *lacZ*. In pBIO386 and pBIO387, the Tn5*lac* was shown to be inserted into the 3′ region of *rosAR*, 64 bp's from the end of the gene (see Fig. 1).

Within *rosAR*, 38 bp's upstream of the site of insertion of the dominant Tn5*lac* mutation in pBIO340 is a *Pst*I site, unique to the *rosAR* region. A 1-kb fragment, extending from this *Pst*I site to a *Hin*dIII site upstream of *rosAR* was cloned into pMP220 to form pBIO385. When mobilized into *A. tumefaciens* strains, pBIO385 failed to complement any of the mutants that were complemented by the intact *rosAR* and, like pBIO340, caused wild-type *A. radiobacter* to be reduced in its ability to make EPS. It is thus clear that the removal of the 3′ region of *rosAR* results in a mutant form that inhibits EPS synthesis in *Agrobacterium*.

Plasmids pBIO340 and pBIO385 were also each mobilized into *R. leguminosarum* strain 8401. On TY medium, both sets of transconjugants were strikingly less mucoid than the wild type. We had noticed earlier that the deleted form of *rosAR* (termed *rosARΔ*) when cloned in pUC18 (to form pBIO391) caused the *E. coli* transformants to be smaller and slightly less shiny—though the essentially nonmucoid nature of the laboratory working strains of *E. coli* made it difficult to show definitely that EPS synthesis was inhibited. Therefore we obtained a derivative of *E. coli*, DH1, which contained the *Erwinia amylovora rcsA* gene cloned in pBR322 (pISR407). Overproduction of *rcsA* activates colonic acid synthesis and so colonies of such strains are slimy. Both pBIO385 (*rosARΔ*) and pBIO350 (wild-type *rosAR*) were mobilized into this *E. coli* strain and also into *Xanthomonas campestris* strain 8001R and *Erwinia amylovora* strain OT1, both of which are naturally mucoid. Remarkably, pBIO385, but not pBIO350, inhibited EPS synthesis in all three genera.

Table 2. Effect of *rosAR* on repression of *virC–lacZ*

Background strain	Plasmid present	β-Galactosidase
T1305 (wild type)	None	0.185
T1305	pSM365 (*virC–lacZ*)	0.214
Exo66.1	None	5.33
Exo66.1	pSM365	37.9
Exo53.1	None	8.58
Exo53.1	pSM365	33.48
Exo81	None	7.87
Exo81	pSM365	31.14
Exo63	None	5.41
Exo63	pSM365	11.6
Exo87.1[a]	None	4.44
Exo87.1	pSM365	5.77

[a] Exo87.1 is an Exo- control which is not complemented by *rosAR*.

rosAR-mediated repression is Fe dependent.

Cooley et al. (1991) noted that the product of *rosAT* contained a sequence, "CLECX$_9$HLTTHH," which they suggested had a similarity to a zinc finger motif found in several eukaryotic regulatory proteins. Such a Zn-finger would be extremely unusual in a prokaryotic regulatory protein but the presence of two cysteines, separated by only two amino acids was suggestive of an interaction between RosAR and a transition metal. Since the metal Fe has been shown to bind to several regulatory bacterial proteins such as Fnr, Fur of *E. coli*, and NifA of *Rhizobium* (see Hennecke 1990) we decided to test if the availability of Fe affected the regulatory properties of *rosAR* and *rosAT*.

To do this, the two fusion plasmids, pSM365 and pSM344, containing the *virC–lacZ* and *virD–lacZ* fusions in wide host-range plasmids were each mobilized into wild-type strain T1305 and into the *rosAR* mutants of *A. radiobacter*. These strains, together with the parental controls lacking any fusion plasmid were grown in MOD-D1 minimal medium (glucose as C source) containing no added $FeSO_4$ and to the same medium to which different concentrations of $FeSO_4$ had been added. After 2 days growth, β-galactosidase activities were determined. As indicated in Table 2, two clear deductions could be made from the results obtained. Firstly, in the wild-type backgrounds the expression of the two *vir–lac* fusions fell progressively as the Fe^{2+} concentration increased. Secondly, this phenomenon did not occur in the *rosAR* mutant strains, with there being high level expression of β-galactosidase activities irrespective of the Fe^{2+} concentration. This confirms the observations of Cooley et al. (1991) that *rosAT* (and also, now, *rosAR*) represses *virC* and *virD*; what is novel, though, is that this repression is Fe dependent.

It had been shown that *rosAT* could repress its own transcription (Cooley et al. 1991). By Tn5 *lac* mutagenesis, we made two *rosAR-lac* fusion derivatives of pBIO11 in which *lacZ* was in the same orientation as *rosAR* (Brightwell 1993). These fusion plasmids were mobilized into the same strains as described above and, in essence, the same results were obtained, namely there was *rosAR*-mediated repression of *rosAR-lac*, but only in medium replete with Fe.

rosAR mutants can be corrected by the addition of Fe^{2+}.

We determined if the concentration of the metal affected the Exo phenotypes of *rosAR* mutants. Each of the four mutants, as well as the wild-type parent and a mutant (Exo87.1) with an *exo* mutation which was not complemented by *ros* were streaked out on MOD-D2 minimal medium with glucose or succinate as sole C source and containing different concentrations of added $FeSO_4$.

Three of the mutants (Exo63, Exo66.1, and Exo73) had previously been shown to be conditional, being EPS$^+$ on glu-

cose-containing medium and EPS$^-$ with succinate in normal MOD-D2 media in which the Fe concentration is 1.0 mM. As shown in Table 3, if no Fe was added to the media, with these three strains the colonies were nonmucoid even on glucose media, whereas the wild-type was fully mucoid. In contrast, Exo53 and Exo87 were not rescued by Fe on glucose and none of the mutants were EPS$^+$ on succinate-containing media.

***rosAR*-mediated repression is augmented by glucose.**

Since some *rosAR* mutants could be complemented by glucose, but only in the presence of Fe, we also examined the effects of glucose on *rosAR*-mediated repression of *virC* and *virD* in media replete with Fe. Strains of wild-type, *rosAT,* and *rosAR53* mutant strains of *Agrobacterium* containing the *virC* fusion (pSM365) and *virD* fusion (pSM344) were grown in media with 1.5 mM $FeSO_4$ and to which various concentrations of glucose were added. As shown in Table 4, in wild-type strains the expression of the fusions were reduced by the addition of glucose at 5 g liter^{-1}. However in the *rosAT* or *rosAR* mutant the levels of β-galactosidase progressively increased as the glucose concentration was raised. Thus the repressive ability of *rosAR* on *virC* is significantly enhanced in strains grown with glucose.

Some *rosAR* mutants accumulate protoporphyrin.

Since the ability of *rosAR* product to repress the expression of other genes depended on the presence of Fe in the growth medium, we tested to determine if *rosAR* mutants were defective in any other aspect of iron metabolism. During the course of this work, we noted that colonies of four of the *rosAR* mutants (carrying the alleles *81*, *63*, *73,* and *66.1*) fluoresced under long-wave UV light, emitting a pink color. For all four mutants, there was a pronounced absorption peak at 396.4 nm (Fig. 2), consistent with the fluorescent material being protoporphyrin IX, the immediate precursor of haem, but lacking the Fe ligand (Cox et al. 1973). Note that Exo53, one of the *rosAR* mutant which failed to be phenotypically complemented by the addition of Fe in glucose, did not fluoresce. The original *A. tumefaciens rosAT* mutant strain LBA4301R, like the Exo53 mutant of *A. radiobacter*, did not accumulate protoporphyrin IX. A mutant of *R. leguminosarum* which accumulated this molecule were referred to by Nadler et al. (1990) as being Pop$^-$ (porphyrin over-producer).

The cosmid pBIO11 and the subcloned *rosAR* gene in pBIO355 were each mobilized into the four mutants. In all cases, the transconjugants no longer fluoresced, showing that *rosAR* complemented both the Eps$^-$ and the Pop$^-$ phenotypes. In contrast, strains containing pBIO21, which also complements the Eps$^-$ phenotype still fluoresced under UV light.

DISCUSSION

The *ros* gene, initially identified in *A. tumefaciens* (Cooley et al. 1990) and now by us in *A. radiobacter,* represents a novel prokaryotic regulator, bearing no relation in its sequence to any other bacterial transcription factor. We could detect no helix-turn-helix motif, characteristic of DNA-binding proteins, yet it is clear that Ros does bind to specific sequences upstream of the genes that it regulates (D'Souza-Alt et al. 1992). Recently it has also been found that the Ros protein is similar in sequence to the product of the MucR gene of *R. meliloti* (A. Puhler, personal communication); *mucR* is also a regulatory gene, normally repressing genes that have the potential to specify a second EPS molecule in these bacteria.

At first sight, the phenotypes of *ros* or *mucR* mutants are contrary to what might be expected. If these genes specify a repressor, mutations in them would not be expected to lead to a loss of function; if anything, production of EPS might be predicted to rise, not to fall. Possible explanations for these observations are that *ros* normally represses another gene which, in turn, represses *exo* gene transcription. Alternatively, *ros* might have the ability to act as an activator at some, as yet unidentified promoters but as a repressor at others.

Cooley et al. (1990) suggested that the sequence $CLECX_9HLTTHH$ in Ros might represent a zinc finger motif; this has not been proved, but we have found a link between the *rosAR* gene of *A. radiobacter* and a transition metal, namely Fe. Firstly, the EPS$^-$ phenotype of some of the *rosAR*

Table 3. Effect of Fe on *rosAR* mediated repression of *virClacZ* and *virD–lacZ*

Strain	Fe concentration (mM)	β-Galactosidase activity
T1305 pSM365 (*virC-lacZ*)	0	22.4
	0.1	3.6
	1.0	0.2
T1305 pSM344 (*virD-lacZ*)	0	36.9
	0.1	4.0
	1.0	0.2
Exo53 pSM365 (*virC-lacZ*)	0	24.6
	0.1	21.8
	1.0	22.3
Exo53 pSM344 (*virD-lacZ*)	0	31.6
	0.1	32.3
	1.0	30.4
LBA4301 pSM365 (*virC-lacZ*)	0	45.3
	0.1	6.9
	1.0	0.2
LBA4301 pSM344 (*virD-lacZ*)	0	50.3
	0.1	3.4
	1.0	0.2
LBA4301R pSM365(*virC-lacZ*)	0	37.6
	0.1	34.7
	1.0	34.2
LBA4301R pSM344(*virD-lacZ*)	0	43.2
	0.1	31.3
	1.0	31.5

Table 4. Effects of Fe and carbon source on EPS production by *exo* mutants of *A radiobacter*[a]

	MOD-D2/glucose $FeSO_4$ concentration (mM)				MOD-D2/succinate $FeSO_4$ concentration (mM)			
Strain	0	0.5	1.0	1.5	0	0.5	1.0	1.5
T1305 (wild type)	++	++	++	++	+	+	+	+
Exo81*+	–	–	–	–	–	–	–	–
Exo63*+	–	+	++	++	–	–	–	–
Exo73.1*+	–	+	++	++	–	–	–	–
Exo66.1*+	–	+	++	++	–	–	–	–
Exo53+	–	–	–	–	–	–	–	–
Exo87.1	–	–	–	–	–	–	–	–

[a] +; complemented by pBIO11, pBIO121 and cloned *rosAR*. *; porphyrin-accumulating mutant.

mutants isolated here depended on the presence of Fe in the growth medium. These mutants were conditional, being EPS$^-$ on media with succinate but EPS$^+$ when glucose was the C source. However, even on the latter medium, the colonies were EPS$^-$ unless exogenous Fe was added. It is possible that Fe binds to the Ros protein and that this is required for its function, but this has not been proved. It was shown, though, that the ability of *ros* to repress transcription of *virC, virD,* and itself was enhanced with increasing levels of Fe in the medium. Given that some of the *rosAR* mutants were conditional on the growth medium, it was of interest to note that the presence of glucose, compared to succinate also markedly increased the extent of *ros*-mediated repression of transcription of all the fusions tested. The biochemical or physiological basis of this observation is not known.

A striking effect of the cloned *rosAR* gene is that when its 3′ end was removed by deletion or when it contained a transposon insertion, the mutant form of the gene was dominant, causing a reduction in the amount of EPS that was made in a taxonomically diverse range of Gram-negative bacteria. The simplest explanation for this phenomenon is that RosAR acts as a multimer and that a form of the protein lacking its C terminus interacts with the less abundant wild-type form such that it can still form a complex but that this is inactive. What was surprising was that this dominant phenotype was not confined to *Agrobacterium* or its close relative *Rhizobium* but extended to *Xanthomonas* and even *E. coli.* If the explanation for the dominant phenotype of the deleted form of *rosAR* is correct, this result suggests that (for example) *E. coli* contains a protein that is required for EPS synthesis in that species, that it acts as a multimer and that it can interact with mutant, but not wild-type RosAR to form an inactive complex.

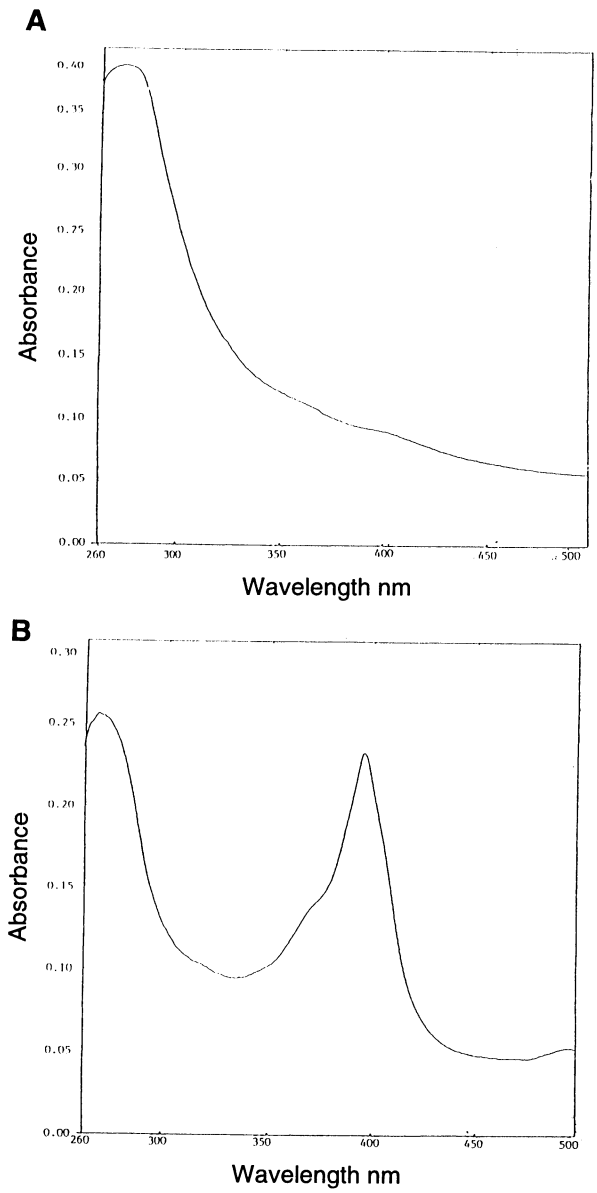

Fig. 2. Absorption spectra of extracts of wild-type *Agrobacterium radiobacter* (A) and mutant Exo63 (B).

Three of the *rosAR* mutants had a pleiotropic phenotype, accumulating large amounts of the haem precursor protoporphyrin IX and this phenotype could also be complemented by the cloned *rosAR* gene. We do not understand why some of the *rosAR* mutants were defective in haem metabolism, whereas others appeared to be unaffected; the *rosAT* mutant of *A. tumefaciens* also appeared to be unaffected in the accumulation of protoporphyrin. It is possible that *ros* activates transcription of a gene or genes involved in the maturation of haem and that certain alleles alter the protein product in such a way as to abolish the proposed induction of *exo* genes but to leave the induction of the *pop* (*p*rotoporphyrin *o*verproduction) genes relatively unaffected. If so, such mutants would be EPS$^-$ but would not accumulate porphyrin accumulators. We are unaware of any obvious biochemical or physiological link between the biosynthesis of EPS and of haem, but the fact that the two properties can be affected by a mutations in the same regulatory gene indicates that there may be one.

Nadler et al. (1990) isolated a mutant, Pop116, of *R. leguminosarum* bv. *viciae* (which nodulates peas) which was defective in Fe uptake and symbiotic N_2 fixation and accumulated protoporphyrin IX. A recombinant plasmid, pKN16, which complemented Pop116 was isolated (Nadler et al. 1990). Recently, we found that subcloned genes from pKN16 also complemented the Pop$^-$ and EPS$^-$ phenotypes of those *A. radiobacter* mutants that were defective in both characters.

One of the reasons for our initial interest in the four *exo* alleles described here is that Aird et al. (1990) showed that they could be complemented by two different, non-overlapping cosmids. We had suggested that an explanation for this phenomenon is that these mutations are in a regulatory gene which activates the transcription of a "structural" *exo* gene. Thus, the phenotype could be complemented by cloning the regulatory gene itself or by cloning the "target" gene such that it no longer needed activation if, for example, it was transcribed constitutively from a vector promotor. We have certainly shown that one of the original cosmids, pBIO11, does contain a regulator, *ros*, but, to date, it has only been shown to repress the transcription of other genes. However, as discussed above, it may have activating abilities at other promoters.

Recently, the other cosmid (pBIO21) which complemented these mutations was found to contain a gene with homology to the structural gene *exoY* of *R. meliloti* which specifies an early step in the biosynthetic pathway of EPS synthesis in that species. The four *exo* mutants described here could all be complemented by *exoY* or a gene very closely linked to it.

The studies described here show that the properties of the *ros* gene of *A. radiobacter* (and presumably that of *A. tumefaciens* and the *mucR* of *R. meliloti*) are subject to several environmental influences and that the properties of the mutants described here reveal some surprising and complex pleiotropic links. It is apparent, then, that this novel series of transcriptional regulators may lead to the further identification of regulatory circuits in the *Rhizobia* and, perhaps, other bacterial groups.

MATERIALS AND METHODS

Strains and plasmids.

Bacterial strains and plasmids used in this work are listed in Table 5.

Media culture conditions and *in vivo* genetic manipulation.

For *Agrobacterium*, media and culture conditions were as described by Aird et al. (1991). In triparental matings, pRK2013 (Figurski and Helinksi 1979) was used as the helper plasmid. Quantitative β-galactosidase assays were performed as described by Rossen et al. (1985), except that activities were expressed as a function of cellular protein concentration which was determined by the Bicenchonic Acid Protein Assay Kit (Sigma) according to the manufacturer's instructions.

DNA manipulation.

For routine analysis and isolation of DNA (transformation, restriction mapping, etc.), experiments were done using the protocols (or minor variants thereof) described in Sambrook et al. (1989). Oligonucleotides were synthesized by Richard James on a Bioresearch Cyclone Oligonucleotide Synthesizer according to the manufacturer's instructions and the PCR products were prepared on an LEP Scientific cycler. DNA was sequenced using the dideoxy chain termination method and gels run on an ALF (Pharmacia) automated sequencer which was used according to the manufacturer's instructions.

Table 5. Effect of glucose on *rosAR*-mediated repression of *virC–lacZ* and *virD–lacZ*

Strain	Glucose (g liter^{-1})	β-Galactosidase activity
T1305 pSM365 (*virC-lacZ*)	0.05	66.08
	0.5	8.28
	5.0	0.15
T1305 pSM344 (*virD-lacZ*)	0.05	99.2
	0.5	7.3
	5.0	0.34
Exo53 pSM365 (*virC-lacZ*)	0.05	14.1
	0.5	28.4
	5.0	66.1
Exo53 pSM344 (*virD-lacZ*)	0.05	25.6
	0.5	62.4
	5.0	131.6
LBA4301 pSM365 (*virC-lacZ*)	0.05	65.8
	0.5	16.2
	5.0	1.1
LBA4301 pSM344 (*virC-lacZ*)	0.05	114.9
	0.5	18.5
	5.0	0.2
LBA4301R pSM365 (*virC-lacZ*)	0.05	20.0
	0.5	92.5
	5.0	134.7
LBA4301R pSM344 (*virD-lacZ*)	0.05	29.2
	0.5	53.2
	5.0	120.4

Spectrophotometric analysis of protoporphyrin IX.

Cultures of *Agrobacterium* strains were grown to late-log phase for 3 days on MOD-D2 glucose medium. Cells were harvested and suspended in 1 ml of 3:1, vol/vol, of ethyl acetate solution and shaken overnight at 37°C. The cells were removed by centrifugation and the absorption spectra of the supernatants between λ230 nm and λ800 nm were determined.

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