DO WE REALLY UNDERSTAND CROWN GALL CONTROL BY AGROBACTERIUM RADIOBACTER STRAIN K84?

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INTRODUCTION

Control of crown gall disease by Agrobacterium radiobacter strain K84 has been commercially available for 15 years. Where it works, it works quite well. Where it does not work, it generally fails completely. In part, this mirrors the phenomenology of the biocontrol field. The difference is that with the K84 system we have some appreciation for how the organism controls the disease, and, perhaps even more importantly, why it may fail to do so. Our purpose here is to critically examine what is known about the system, to indicate current directions for research in the system, and, using the results generated by such research, to describe directions being taken to improve the system.

History

The discovery of strain K84 is, on the surface, an example of scientific serendipity. However, it was not just good luck; it was more the product of keen observational skills. During their studies of crown gall in the field, New and Kerr (1972) observed that the incidence of the disease was correlated with the ratio of pathogenic to non-pathogenic agrobacteria in the soils. To test whether the non-pathogen might be inhibiting the pathogen, these workers infected plants with mixtures of two isolates representative of each class. For the non-pathogen, a typical biovar 2 A. radiobacter strain, given the accession number K84, was chosen. That it prevented crown gall by the virulent A. tumefaciens isolate was, of course, the question being tested; that it proved to be the only A. radiobacter strain of a number that were tested that did so was a surprise; that it works on a commercial scale is an incentive to understanding the mechanism.

In a series of follow-up experiments, Kerr and his colleagues confirmed the usefulness of strain K84 and defined the optimum conditions for its use in the field (Htay and Kerr, 1974; Kerr, 1972; Kerr and Htay, 1974). Treatment is simple, effective, and safe to the environment (Moore, 1985; Moore and Warren, 1979; Moore et al., 1978). Strain K84 is commercially available in Europe, North America and Australia. Finally, a second generation derivative of strain K84 has been constructed by recombinant DNA techniques (Jones et al., 1988). This strain called K1026, is the first genetically

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engineered microorganism designed for release into the environment to become commercially available (Jones and Kerr, 1989).

It is not our purpose here to review the K84 system. The history of strain K84, its efficacy on the commercial scale, studies relating to how it may control crown gall disease, and recent genetic modifications to the bacterium have been the subjects of recent reviews (Farrand, 1990, 1991). We would rather concentrate on what we do not know about the K84 system. In this treatment we will critically examine the experimental results taken to support various notions concerning mechanisms of action of strain K84. Such an approach could be of considerable value. The K84 system works, and it works well. This allows us to identify the key components of a successful biocontrol agent using the techniques of genetics, molecular biology, biochemistry, and physiology. Information gained from such studies may form paradigms, and as such, could well be directly applicable to other biocontrol systems.

COMPONENTS OF THE SYSTEM

Production of Agrocin 84

Strain K84 expresses several properties that are believed to contribute to its success as a biological control agent. The first of these is the production of a novel antiagrobacterial agent called agrocin 84 (Kerr and Htay, 1974). This high specific antibiotic, first believed to be a bacteriocin, is a disubstituted fraudulent adenine nucleotide analogue (Roberts et al., 1977). Sensitivity to agrocin 84 is a property conferred on the pathogen by certain classes of Ti plasmids (Ellis and Murphy, 1981; Hayman and Farrand, 1991; Kerr and Roberts, 1976; Murphy and Roberts, 1979). The conclusion that agrocin 84 is required for successful biocontrol is based on two sets of observations. First, using a standard tomato stem assay (New and Kerr, 1972), disease caused by virulent strains of *A. tumefaciens* sensitive to agrocin 84 is generally preventable, while disease caused by resistant isolates is almost always refractory to control (Kerr and Htay, 1974). That is, only agrobacteria sensitive to agrocin 84 are controlled by strain K84 using this assay system. Second, a derivative of strain K84 which does not produce agrocin 84 no longer prevents disease under conditions in which wild-type strain K84 would normally be effective (Cooksey and Moore, 1982). Thus, failure to produce agrocin 84 results in an inability to control the disease caused by sensitive agrobacteria.

Are there any reasons to doubt that production of agrocin 84 is a necessary component to biocontrol by strain K84? Perhaps. First, there is no definitive proof that strain K84 produces agrocin 84 *in situ*. While this does not argue against a role for the antibiotic, it would certainly be of value to show that agrocin 84 is produced by strain K84 when it is associated with plants. To this end we are constructing gene fusions to act as reporters for the *in situ* expression of determinants involved in agrocin 84 biosynthesis. Second, many field isolates of *Agrobacterium* spp. produce agents toxic to other agrobacteria. A number of these agrocinogenic strains have been tested for biocontrol properties and none seem to prevent crown gall at anything near the efficiency shown by strain K84 (Cooksey and Moore, 1980; Ellis et al., 1979; Kerr and Panagopoulos, 1977). This indicates that production of antagonistic compounds *per se* is not sufficient for successful biocontrol. However, it does not argue against a role for agrocin 84. Third, transfer of the genes encoding agrocin 84 production to other agrobacterial isolates confers on these strains the capacity to produce the antibiotic (Ellis et al., 1979; Shim et al., 1987). However, very few of these genetic constructs control crown gall. This also does not argue *sensu stricto* against a role for agrocin 84. As above, it only indicates that antibiotic production is not sufficient for successful biocontrol. Finally,

a derivative of strain K84 that no longer produces agrocin 84 can control agrocin 84-sensitive and -resistant A. tumefaciens strains, but only under certain conditions of inoculation (see below).

This last point may be key to understanding factors other than agrocin 84 production important to the control process. Lopez et al. (1989) recently reported studies showing that strain K84 could control disease caused by agrocin 84-resistant agrobacteria under field conditions. However, the degree of protection was not as high as that seen when the pathogen population was sensitive to the antibiotic. Similarly, these workers found that a derivative of strain K84 unable to produce agrocin 84 also controlled disease caused by sensitive and resistant A. tumefaciens isolates. Again the level of control was not as high, at least for sensitive pathogens, as that seen with wild-type strain K84. However, the levels of control against resistant isolates were significant, and probably acceptable in the field. Furthermore, in the cases where agrocin 84 was not a factor, the incidence of plants with only single galls was substantially increased indicating that disease, when it occurred, was less severe.

It seems, then, that there are two conflicting sets of data. On the one hand are experiments showing that strain K84 only controls crown gall induced by challenge strains sensitive to agrocin 84. On the other are the studies such as those of Lopez et al. (1989). The difference between the two may lie in the methodologies used to assess disease control. In those studies showing control only of agrocin-sensitive pathogens, the plants were artificially inoculated on their stems with both bacteria (Kerr and Htay, 1974). Furthermore, ratios of pathogen to strain K84 were generally in the order of 1:1. In studies by New and Kerr (1972), this was the lowest ratio of sensitive pathogen to control agent giving maximum control. In the study by Lopez et al. (1989), the soil was inoculated to give populations of sensitive or resistant pathogens of around 10^6/g. Prior to transplanting into this infested soil, plants were dipped in a suspension of strain K84 containing about 10^9 bacteria per ml. Considering the strain K84 colonizes plant roots extremely well (Macrae et al., 1988), this probably resulted in a ratio of pathogen to strain K84 well in favor of the control agent.

These results suggest that production of agrocin 84 may not be essential per se, but that it is required for maximum control by strain K84. Alternatively, production of agrocin 84 may be a key factor in biocontrol when the ratio of pathogen to control strain is relatively high. The results discussed above suggest that at lower ratios production of agrocin 84 may play only a minor role. However, a variety of different sensitive and resistant tumorigenic strains were used in these studies and differences between these strains other than their agrocin phenotypes may contribute to control by strain K84. Furthermore, production of agrocin 84, on top of other factors intrinsic to strain K84 (see below), may be what makes this bacterium a consistently effective biocontrol agent.

The role for agrocin 84 production in biocontrol by strain K84 should be determined in definitive, controlled experiments. First, a standard bioassay approximating the conditions by which strain K84 is used in the field should be developed. The assay should involve inoculation of seeds or plant roots with the control strain at titers similar to those used in the commercial application of strain K84. Seeds should then be sown into infested soils, or, as an alternative, plant roots could be dipped into suspensions of the test pathogen before planting to soil. Second, standard pathogens, sensitive and resistant to agrocin 84 should be used. Included in this set should be a sensitive strain such as C58 for which near isogenic, agrocin 84-resistant mutants are available. In this strain the genes necessary for sensitivity have been localized to the Ti plasmid and have been precisely mapped and analyzed (Hayman and Farrand, 1988). The sensitivity locus

encodes catabolism of the crown gall opines agrocinopines A and B, and has been named acc (Hayman and Farrand, 1988). Furthermore, precise mutations in acc which abolish sensitivity to agrocin 84 have been engineered into pTiC58 (Von Bodman et al., submitted for publication). Strains harboring this Ti plasmid are indistinguishable from the parent C58 except for exhibiting resistance to the antibiotic.

Certainly the influence of agrocin 84 production on biocontrol should be tested over a range of pathogen: control agent ratios. It may well be that at low ratios (<0.1) the role of agrocin 84 is minimal while at higher ratios it is essential. Using the types of assays described above, the proportion of pathogen to control agent easily can be varied. Furthermore, strain K84 and its non-agrocinogenic derivatives can be compared directly with each other with respect to their effectiveness against sensitive and resistant pathogens. In this manner the role of the antibiotic in biocontrol by strain K84 can be assessed.

Colonization of the Infection Site

Even if production of agrocin 84 is a required component, it clearly is not the sole determinant involved in control by strain K84. This is strikingly demonstrated by the observation that transfer of the genes for agrocin 84 biosynthesis to other A. radiobacter isolates, while conferring on these strains the capacity to produce the antibiotic, does not neccessarily make them efficient crown gall control agents (Ellis et al., 1979; Shim et al., 1987). Furthermore, the non-agrocinogenic derivative of strain K84 described above will control crown gall to a limited extent, but only when inoculated onto the test plants at high cell density, and before the pathogen (Cooksey and Moore, 1982). Interestingly, under such conditions, the strain controls agrocin 84-resistant pathogens as well as it controls those sensitive to the antibiotic.

Several studies suggest that strain K84 is quite efficient at colonizing plant roots, and that this colonization ability is important to the control process. Early on Kerr and Htay (1974) showed that strain K84 colonizes tomato seedlings, and in mixed inoculations, suppresses colonization by a sensitive A. tumefaciens isolate. However, strain K84 had no effect on the colonization ability of an agrocin 84-resistant pathogen. These results suggest that production of agrocin 84 provides strain K84 with an advantage when in competition with antibiotic-sensitive agrobacteria. In further experiments, Ellis et al. (1979) and Shim et al. (1987) showed that strain K84 colonized plant roots more efficiently than did other agrobacteria into which the agrocin 84 biosynthetic genes had been introduced. Significantly, these other strains failed to control disease caused by agrocin 84-sensitive A. tumefaciens isolates. However, differential colonization by the pathogen and the control agent in mixed inocula was not determined in either study. No conclusions could be made, therefore, concerning any competitive colonization advantages exhibited by strain K84. Finally, Macrae et al. (1988) showed that strain K84 colonized tomato roots more efficiently, and persisted at high cell numbers for a longer period, than did another agrocinogenic Agrobacterium strain, J73. Interestingly, strain K84 proved to be an effective biocontrol agent while strain J73 did not control pathogens sensitive or resistant to agrocin 73.

It should be noted that in the study by Macrae et al. (1988) the increased colonization potential of strain K84 was not exhibited until two weeks after infection. Similarly, in the report by Shim et al. (1987), strain K84 did not colonize almond roots to levels any higher than the non-controlling strains until at least 40 days after inoculation. In fact, at the earliest time point reported (39 days), some of the non-controlling

strains appeared to be the best root colonizers, exhibiting population sizes up to two orders of magnitude greater than those of effective strains at this time point. This is significant because it is generally thought that there is a fairly narrow temporal window for infection immediately following planting, and that K84 successfully controls the pathogen because it is effective during this early time period.

Clearly, the role of root colonization by strain K84 should be critically assessed. Parameters involved in bacterial colonization of roots are not well understood. In fact, there is no consensus even on a definition for colonization. Nevertheless, the ability of strain K84 to bind to roots and to maintain or increase its numbers as the roots grow should be quantified. Furthermore, it may be possible to isolate mutants of the bacterium deficient in these abilities. Attachment-defective mutants of A. tumefaciens are greatly attenuated for tumorigenicity. It would of considerable value to determine the effects of similar mutations affecting root colonization and persistence on the biocontrol properties of strain K84.

It is also not clear if general root colonization is the important parameter. For example, it may be that strain K84 is efficient at recognizing and colonizing, as a subset of sites, root wound sites which are believed to constitute the A. tumefaciens infection court. In this scenario, the control agent acts at a specific microhabitat, and could inhibit the pathogen by specific blockage of the primary infection site (Lippincott and Lippincott, 1969), by production of agrocins, or by a combination of the two. If this is the case, application of strain K84, or its agrocin non-producing derivatives to wounded roots in high numbers and before challenge with the pathogen should result in protection against sensitive and resistant agrobacteria. In fact, this is exactly what is observed (Cooksey and Moore, 1982). It would be interesting to search for mutants of strain K84 deficient in this ability. Such mutants should fail to control, but should colonize roots as effectively as the parent strain.

Production of Other Agrocins

While the bulk of the work with strain K84 has focused on the production of agrocin 84, there is some evidence indicating that this bacterium produces more than one antiagrobacterial activity. Some years ago Cooksey and Moore (1981) reported that strain K84 produces a substance inhibitory to several fast growing rhizobia. We confirmed this observation, but showed by genetic studies that the antirhizobial agent is not agrocin 84. In another set of experiments, Dhanvantari (1983) reported that biovar 3 strains of A. tumefaciens, which were resistant to agrocin 84 when assayed on standard media, were inhibited by strain K84 when the assay medium was amended with a different nitrogen source. Dhanvantari assumed that the antiagrobacterial activity was agrocin 84. However, from what is known about the mechanism of sensitivity to this agent (Hayman and Farrand, 1987; Murphy and Roberts, 1979), it is unlikely that the agent active against the biovar 3 strains is agrocin 84. Finally, Clare has shown that a derivative of strain K84 lacking pAgK84, the plasmid which encodes the biosynthesis of agrocin 84, still produces an inhibitory substance (Clare, personal communication). This agent, called agrocin 434, is active against a range of Agrobacterium isolates including some that are intrinsically resistant to agrocin 84.

The nature of agrocin 434, its relationship to the antirhizobial factor, the conditions under which it is produced, and its role in biocontrol by strain K84 are only now being addressed (Clare, personal communication). However, it is clear that this agent may be a component in the strain K84 control process, especially against isolates resistant to agrocin 84.

OTHER CONSIDERATIONS

If, as suggested by the work of Lopez et al. (1989), strain K84 can control disease caused by agrocin 84-resistant A. tumefaciens isolates, why then does the strain fail to control disease in some field applications? One can only speculate. Perhaps strain K84 does not colonize as well as the A. tumefaciens strains indigenous to these sites. Perhaps the indigenous agrobacteria produce substances antagonistic to strain K84. Kerr and Htay (1974) observed that A. tumefaciens strain K108, the only agrocin 84-sensitive pathogen strain K84 did not control, itself produces an agrocin that inhibits growth of the control strain. It is also possible that agrocin 434 plays a significant role, and uncontrollable pathogens are also resistant to this factor. Furthermore, we have considered here only the biological components of the system. Perhaps variables such as soil types, pH, moisture content or other abiotic factors influence the effectiveness of control by strain K84. These are parameters that certainly should be investigated.

SUMMARY AND FUTURE DIRECTIONS

Although there is no question that strain K84 is a commercially successful biocontrol agent, careful analysis of the literature indicates that we really have no good evidence as to what makes this organism so effective. However, the fact that strain K84 does work at the commercial level makes it a particularly attractive system in which to identify the relevant components. In this case we can perturb the system, for example by mutation, such that it no longer functions. Determining the nature of the perturbation at the biochemical and physiological levels should allow us to identify the important features of the system. We have presented several possibilities including production of agrocin 84, and perhaps agrocin 434, and the capacity to colonize plant roots in general, or specific infection sites. However, further experimentation is necessary to firmly establish these characteristics as being important to the biocontrol process. The effect of other biotic and abiotic factors must also be assessed. Furthermore, it is conceivable that the various characteristics may contribute to varying degrees depending upon the circumstances of the application. Before the K84 system can be improved we must determine what paremeters limit its current effectiveness. Once these are understood, rational approaches to overcoming these limitations can be developed, and the necessary traits engineered into strain K84.

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