Plasmid and Chromosomal Determination of *Agrobacterium* Adherence Specificity

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INTRODUCTION

To initiate tumours *Agrobacterium tumefaciens* must adhere to a wound site via a process which requires considerable specificity on the part of the bacterium (Lippincott & Lippincott, 1969; Whatley *et al.*, 1976). Because this site binding ability and the presence of a large plasmid (Zaenen *et al.*, 1974) are both essential for tumourigenicity, it was of interest to determine if site binding ability was a plasmid-borne trait. Whatley *et al.* (1976) showed that lipopolysaccharide (LPS) of the outer membrane of the *Agrobacterium* cell envelope is the bacterial adherence component. Two avirulent strains of *Agrobacterium* derived from virulent strains and reported to be plasmid-free were found to retain site binding ability (Whatley *et al.*, 1976). It was proposed, therefore, that chromosomal DNA determined the specificity of LPS essential for adherence (Whatley *et al.*, 1976).

Van Larebeke *et al.* (1975) reported that introduction of a virulence plasmid into *Agrobacterium radiobacter* strain s1005 converted this strain to a tumourigenic state. However, whole cells and LPS of strain s1005 lack the ability to adhere to tumour initiation sites (Lippincott & Lippincott, 1969; Whatley *et al.*, 1976). Thus, either the strains of s1005 tested in the two laboratories differed in their site binding ability, or the presence of the plasmid in s1005 must affect LPS structure. To resolve these possibilities we determined the localization of genes governing adherence by testing non-site binding strains to which a virulence plasmid was added and additional site binding strains after curing of all plasmids.

METHODS

Characteristics of *A. tumefaciens* tumourigenic strains B6 and 181 and non-tumourigenic *A. radiobacter* strains s1005, TR1 and ATCC 6467 have been described (Lippincott & Lippincott, 1969). Strain NT1, obtained from E. Nester, University of Washington, Seattle, U.S.A., was derived from *A. tumefaciens* strain c58 by growth at 37 °C and lacks only the large plasmid for virulence (Watson *et al.*, 1975). Plasmid-cured strains c58c9 (virulent parental strain c58) and ACH5C3 (virulent parental strain ACH5) were also obtained by this method. Strain B91Q5 is a plasmid-cured isolate of virulent strain B91 selected by its failure to utilize octopine (Petit & Tourneur, 1972). *Agrobacterium radiobacter* strain K84, source of agrocin 84 (Kerr & Htay, 1974) was obtained from A. Kerr, University of Adelaide, Australia. Strain B6s3 is a single colony isolate from an ultraviolet-irradiated, mitomycin C-treated B6 culture (Zaenen *et al.*, 1974).

Tumourigenic strain s1005(pTiK14) was obtained by *in planta* transfer of the plasmid of *A. tumefaciens* strain K14 to *A. radiobacter* strain s1005 (Van Larebeke *et al.*, 1975). The plasmid from strain B6s3 was transferred to non-site binding *A. radiobacter* strains s1005, TR1 and ATCC 6467 from three donor strains, B6s3, c58c9(pTiB6S3) and LBA52 (an auxotrophic mutant of B6s3), following *in vitro* conjugation as described by Genetello *et al.* (1977). Chromosomal genes have not been observed to transfer in this process. Plasmid content was determined by the methods described by Zaenen *et al.* (1974) and Currier & Nester (1976a).

Washed bacteria were lyophilized and LPS was obtained by treating the cells with 45 % (w/v) phenol in water as described by Westphal & Jann (1965). LPS was purified by two ultracentrifugations or by chromatography on columns of Sepharose 2B, dialysed and lyophilized as described by Whatley *et al.* (1976). The

dry LPS was weighed and dissolved in distilled water by heating at 55 °C for 5 min for use in infectivity assays.

Viable or heat-killed bacteria or their LPS were mixed with viable virulent strain B6 and applied with carborundum wounding to 7-d-old primary pinto bean leaves (Lippincott & Heberlein, 1965). Samples and controls were each inoculated on 16 to 20 leaves and the number of tumours initiated was counted 7 d later. Data are reported as a percentage of the number of control tumours, values of 80 % or less providing evidence for the ability of the test materials to compete with strain B6 for attachment sites. The controls were portions of the same culture of virulent bacteria used in the experimental samples inoculated at the same concentrations.

Bacterial sensitivity to agrocin 84 was used to select for cured non-infectious derivatives of strains s1005(pTiK14) and 181 (Engler *et al.*, 1975; Watson *et al.*, 1975).

RESULTS AND DISCUSSION

The plasmid from strain B6S3 was transferred to three strains of *A. radiobacter*, 6467, TR1 and s1005, previously found to be non-site binding (Lippincott & Lippincott, 1969). Rifampicin-resistant colonies with a plasmid were selected by their capacity to grow on minimal medium with octopine as sole nitrogen and carbon source, a character determined by the strain B6S3 plasmid (Genetello *et al.*, 1977). Three different donor strains, each carrying the B6S3 plasmid, were used to supply the plasmid to the *A. radiobacter* strains. All transconjugants were tumourigenic, suggesting plasmid gain carried site binding ability similar to that observed in transconjugant strain s1005(pTiK14). When whole cells and LPS from these transconjugant strains were tested for their effect on tumour initiation, they proved inhibitory as predicted by their virulence (Table 1).

The virulent strain s1005(pTiK14), formed by introduction of the strain K14 plasmid into the avirulent non-site binding strain s1005, induced tumours on pinto bean leaves and both whole cells and LPS derived from this strain inhibited tumour formation by strain B6 (Table 1). The plasmid from strain K14 also carries genes that confer nopaline utilization and sensitivity to agrocin 84 on strain s1005(pTiK14). Three agrocin-resistant derivatives of strain s1005(pTiK14) were selected and all were found to be avirulent and unable to utilize nopaline. Both whole cells and LPS from these plasmid-cured strains were non-inhibitory when tested in tumour initiation assays, and no plasmid DNA was detected in them by direct analysis.

Agrobacterium tumefaciens strain 181 is tumourigenic, sensitive to agrocin 84 and contains two plasmids with molecular weights of 114×10^6 and 158×10^6 (Currier & Nester, 1976b). Two agrocin 84-resistant isolates of this strain were selected and found to be avirulent (Table 1). However, whole cells of these strains and their LPS were still inhibitory in infectivity tests. These strains lack one of the two strain 181 plasmids and do not utilize nopaline, a trait present in the parental strain (Lippincott *et al.*, 1973) and determined by the virulence plasmid (Watson *et al.*, 1975; Bomhoff *et al.*, 1976).

These data show that plasmid transfer conveying virulence to non-tumourigenic non-site binding strains of *Agrobacterium* also conveys site binding ability in the case of two plasmids (pTiK14 and pTiB6S3) in three strains of bacteria (s1005, TR1, 6467). Plasmid-cured variants obtained from one of these strains, s1005(pTiK14), showed loss of site binding ability as well as loss of virulence and nopaline utilization. Thus, in addition to such traits as virulence, octopine or nopaline utilization (Watson *et al.*, 1975; Bomhoff *et al.*, 1976), agrocin 84 sensitivity (Engler *et al.*, 1975; Watson *et al.*, 1975; Kerr & Roberts, 1976) and exclusion of phage AP1 (Van Larebeke *et al.*, 1975), virulence plasmids may also contain information governing one or more features of LPS structure.

Many avirulent agrobacteria, however, show site binding ability and LPS from these strains is inhibitory (Lippincott & Lippincott, 1969; Whatley *et al.*, 1976). The plasmid content of most of these strains is unknown. Agrocin 84-resistant strains derived from virulent strain 181 have lost a plasmid essential for virulence and nopaline utilization but retain site binding ability. Plasmid-free strain NT1 retained site binding ability in the absence

Table 1. *Effect of the introduction and curing of plasmids on the ability of cells and lipopolysaccharide of Agrobacterium to compete for host sites*

Bacterial strain	Tumourigenicity and site binding ability before plasmid change	Plasmid change*	Tumourigenicity† after plasmid change	Tumour initiation after plasmid change (% tumours relative to B6 control)‡	
				Whole cells + B6	LPS + B6
6467	—	None	—	96	102
6467	—	pTiB6S3 (3D)	+	35–73	38–53
TR1	—	None	—	106	90
TR1	—	pTiB6S3 (2D)	+	33–51	49–63
s1005	—	None	—	93	91
s1005	—	pTiB6S3 (2D)	+	34–47	34–51
s1005	—	pTiK14 (1D)	+	48	48
s1005(pTiK14)	+	(3C)	—	98–102	87–102
181(pTi181)	+	(2C)§	—	38–62	45–58

* Designations in parentheses indicate the number of different strains each formed using a different plasmid donor strain (D) or the number of separate cured isolates (C).

† Tested on peas or beans.

‡ Test cells were heat inactivated at 80 °C for 20 min and inoculated at 5×10^8 ml⁻¹ with 5×10^8 ml⁻¹ viable strain B6; LPS was inoculated at 100 µg ml⁻¹ or, in last two lines, 40 µg ml⁻¹.

§ Retain one of two plasmids of the parental strain.

of the plasmid (Whatley *et al.*, 1976) and three additional plasmid-cured strains (c58c9, ACH5C3 and B91Q5) also retained site binding activity (data not shown). Similarly, *A. radiobacter* strain ATCC 4718 showed site binding ability (Lippincott & Lippincott, 1969) but does not contain a plasmid (Zaenen *et al.*, 1974).

We conclude that the ability of agrobacteria to adhere to host sites may be determined by either chromosomal or plasmid genes which affect LPS structure. Because the structural features of LPS essential for adherence are unknown and the degree of variation consistent with adherence is unknown, the effect of the plasmid and chromosomal determinants may or may not be identical, although they could have equal relevance to the infection process. It seems probable that most virulent agrobacteria have both chromosomal and plasmid genes which can determine adherence, since both the K14 and B6S3 virulence plasmids confer site binding ability on non-site binding recipient strains and since four 'naturally' virulent strains (c58, ACH5, B91 and 181) when cured of their virulence plasmid retain site binding activity, as does avirulent plasmid-free strain ATCC 4718. Determination of the structure of LPS which is essential for adherence in different strains of agrobacteria and the discovery of means to cure additional strains of their plasmids will resolve these relationships.

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