THE TRANSFER CAPABILITIES, MAINTENANCE AND CHROMOSOMAL INTEGRATION OF THE IncP-1 PLASMID pRD1 IN *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* PSSD220

By

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ABSTRACT

THE TRANSFER CAPABILITIES, MAINTENANCE AND CHROMOSOMAL INTEGRATION OF THE IncP-1 PLASMID pRD1 IN PSEUDOMONAS SYRINGAE pv. SYRINGAE PSSD220

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The IncP-1 antibiotic resistance plasmids have been successfully used to establish conjugal gene transfer systems in some bacterial genera. Plasmid pRD1 is a chimeric IncP-1 plasmid of RP4 that contains the Klebsiella pneumoniae histidine biosynthesis operon.

When pRD1 was transferred to Pseudomonas syringae pv. syringae PSSD220, the K. pneumoniae histidine genes complemented the histidine mutation of PSSD220. Plasmid pRD1 could not be conjugatively transferred from PSSD220 to other P. syringae strains, unlike other IncP-1 plasmids in PSSD220. Furthermore, PSSD220 (pRD1) was not susceptible to the IncP-1 plasmid specific bacteriophages PRR1 and PRD1.

Plasmid pRD1 could not be detected as an extrachromosomal element in agarose gels after P. syringae pv. syringae (pRD1) was successively transferred on a medium that selected for the pRD1-determined His^+ and Kan^r. Hybridization of the DNA from cleared lysates of PSSD220 (pRD1) to ^{32}P-labeled pSA30 and pRD1 indicated that pRD1 had integrated into the chromosome of PSSD220.

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Successive transfer of P. syringae PSSD220 (pRD1) on non-selective media produced kanamycin resistant variants which were unable to complement the histidine mutation of PSSD220. These variants contained a plasmid smaller than pRD1 which appeared to have been formed by an in vivo deletion of the K. pneumoniae histidine genes found on pRD1. Hybridization of ^{32}P-labeled pRD1 to the DNA from cleared lysates of PSSD220 cultures containing the pRD1 deletion plasmid or the IncP-1 plasmid RP1 indicated that these plasmids had not integrated into the chromosome of PSSD220. Restriction endonuclease analysis of these deletion plasmids isolated from PSSD220 indicated that they had not formed as a result of excision of integrated pRD1 from the PSSD220 chromosome.

DNA restriction analysis of total DNA from P. syringae PSSD220 (pRD1) in which pRD1 was not detected extrachromosomally and subsequent hybridization to ^{32}P-labeled pRD1 or RP4 indicated that integration of pRD1 into the PSSD220 chromosome resulted in a deletion within pRD1. This deletion included a region of pRD1 which was homologous to the region of RP4 where Tra functions had previously been mapped.

DEDICATED

to

my wife Susan,

my two children, Thomas and Michael,

and my parents, Betty and Warren.

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I. INTRODUCTION

Historically, the study of genetics in bacteria was impeded by a lack of knowledge of the mechanism of genetic exchange in bacteria. In fact, it was first thought that bacteria were sexless, that they had no mechanism for the sexual exchange of genetic information. It is now known that bacteria do indeed have mechanisms for genetic exchange that can be considered sexual. The ability to transfer genetic information in many bacteria generally depends on the presence of a conjugative plasmid capable of mediating the transfer of the host chromosome to the recipient cell. Since the discovery of plasmid-mediated genetic exchange in Escherichia coli, plasmids capable of infectious transfer among a diverse genera of Gram-negative bacteria have been discovered. Some of these wide host range plasmids have also been shown to be capable of mediating chromosomal gene transfer in a number of different genera of bacteria. The use of these wide host range plasmids has made possible the establishment of gene transfer systems in bacteria for which no known system for gene transfer existed. The wide host-range properties of these plasmids have been utilized in the construction of wide-host range cloning vectors which have also extended the possibilities for the transfer of DNA into bacteria lacking natural systems for genetic exchange.

The genetic study of the plant pathogenic pseudomonads is relatively far behind that of other bacterial genera (83). Perhaps this has been due to the number of investigators engaged in this area, or perhaps this has been due to the seemingly insignificance of bacterial diseases of plants when compared to the number and economic importance of plant diseases caused by fungi. Whatever the reason, no natural gene transfer system has been discovered among the plant pathogenic pseudomonads. The wide host-range and chromosome mobilizing properties of the IncP-1 antibiotic resistance plasmids have made them a logical choice for the establishment of gene transfer systems in these bacteria. IncP-1 antibiotic resistant plasmids have been transferred to plant pathogenic pseudomonads (for a review see Lacy and Leary (80)) and a partial genetic map of a strain of Pseudomonas syringae pv. glycinea has already been established using the IncP-1 plasmid R68 (41). Recently Staskawicz et al (132), using the IncP-1 plasmid derived wide host-range cosmid cloning vector pLAFRA1 (40), cloned an avirulence gene for the determination of race specificity from P. syringae pv. glycinea.

The long range purpose of this study was to identify the genetic determinants for pathogenicity in the wheat pathogen P. syringae pv. syringae. The first step in this goal required the establishment of a system for genetic exchange. Therefore, Chapter I is devoted to the conjugation and chromosome gene transfer capabilities of

wide host-range IncP-1 antibiotic resistance plasmids in P. syringae pv. syringae.

The E. coli fertility plasmid F is probably the best understood of the conjugative plasmids, ie. plasmids that contain the genetic information for their own conjugative transfer. The interaction of F with the host chromosome in the course of F-mediated chromosomal gene transfer has been a model by which plasmid-mediated gene transfer systems have been compared. This has led to a search for other chromosomally integrated conjugative plasmids which transfer chromosomal genes at high frequencies. Chapters II and III are focused on the integrative capability of an IncP-1 plasmid in P. syringae pv. syringae strain PSSD220. The preliminary experiments on the chromosomal integration of the IncP-1 plasmid pRD1 have already been published (138).

II. LITERATURE REVIEW

Conjugal gene transfer is mediated only by extrachromosomal DNA known as plasmids. These plasmids contain the genetic information for mechanisms which allow the vertical transmission of the conjugative plasmid in the bacterial population. Plasmids contain no genetic information that is essential to the growth of the host bacterium unless it confers an advantage to the bacterium under adverse conditions. An example of such an advantage may be resistance to antibiotics, in which the genes encoding resistance to the antibiotic are found on the plasmid. Some conjugative plasmids capable of plasmid transfer, also have chromosome mobilizing ability (Cma) whereby chromosomal DNA may be transferred during conjugation (49).

Conjugation was first discovered in Escherichia coli strain K12 by Lederberg and Tatum (87) as a result of observing genetic recombination involving auxotrophic mutants. In these experiments it was shown that direct contact between the bacterial strains was necessary for recombination, and therefore the genetic exchange could not be a result of transformation, in which cell-to-cell contact is not necessary. Within one year, two different research groups demonstrated sexual compatibility in E. coli K12 (54,

85). They suggested that a strain only able to donate genetic material be considered to be fertile (F^+) and a strain only able to receive genetic material be considered to be infertile (F^-). Therefore it required both an F^+ donor and an F^- recipient for transfer to occur. Lederberg's group (85) also showed that after conjugation with an F^+ donor strain, the F^- recipient became F^+; the fertility plasmid F was transmitted infectiously. Gene transfer of a particular chromosomal marker occurred at a frequency of 10^{-7} per donor cell, but the transfer of F^+ occurred at a frequency of 10^{-1} per donor cell. Hayes (54) concluded that the ability of an F^+ strain to complement mutations in an F^- strain was due to the transfer of donor chromosomal genes mediated by the fertility plasmid F.

Lederberg et al (84) and Hayes (54) also isolated donor strains that had the ability to promote a high frequency of recombination (Hfr) in recipient strains. The Hfr donor strains were able to produce recombinants for some markers at a thousand-fold greater frequency than the F^+ donor. Furthermore, the Hfr could only act as a donor and was not infectious, that is, the recipients were usually F^-. Unlike gene transfer with F^+ donors, gene transfer from an Hfr donor was not random and originated from a specific point, with that point being specific for each Hfr strain identified.

A third type of donor was identified by Adelberg and Burns (1) in which the recombinants formed from a mating

with an Hfr donor also acquired the capacity to act as a donor. This was unlike the usual condition in which the recipients from an Hfr mating were unable to act as donors of F. Furthermore, when the recombinants acted as donors, they transferred the genetic markers in the same orientation as the parent Hfr strain. This ability to act as a donor was perpetuated through a series of crosses with F^- recipients. The ability to transfer F resembled that of F^+ donors, but the frequency of gene transfer resembled that of Hfr donors.

The Campbell model (18) for the integration of lysogenic bacteriophages into host chromosomal DNA can be used to explain the three types of donors studied in E. coli. The fertility plasmid F, a small circular loop of double stranded DNA, is normally maintained as an extrachromosomal element in donor strains of E. coli. Within F are insertion sequences that recognize a limited number of sites on the chromosome and it is at these sites that chromosomal integration of F takes place. When F integrates into the chromosome, it behaves as an Hfr donor strain. In this state it is capable of linearly transferring to the recipient regions of the donor chromosome located proximal to a specific plasmid encoded origin of transfer. The higher frequency of gene transfer observed in matings using an Hfr donor, compared to the lower frequency of gene transfer observed with an F^+ donor is indicative of the frequency of integration of F into the

chromosome. The ability of an Hfr strain to be restored to an F^+ strain is the result of the excision of F from the chromosome. If F does not excise within the same region in which it had integrated, then it has the potential to carry with it some of the host chromosome. It is by this manner of imprecise excision that the third type of donor is formed. When this occurs and F contains chromosomal DNA it is known as F-prime (F'). If an F' carries a recognizable chromosomal marker the associated marker may transfer at a frequency that is indicative of an Hfr donor but at the same time allowing the recipient to act as a donor.

The fertility plasmid F, like all bacterial plasmids, exists in the bacterium as double-stranded covalently closed circles of DNA (39). The large size of F, about 100 kilobases, is fairly typical of conjugative plasmids since the genes that encode their transfer ability encompass a large portion of their total DNA. The transfer, (tra), genes of F have been identified and mapped through the isolation of F' factors incapable of transfer (for a review see Willets and Skurray (148)). The 19 known tra genes of F form a single linkage group that comprises roughly one-third of F. These genes encode pilis formation, plasmid replication, plasmid transfer, and surface exclusion functions. The genes for pilis formation, traA, L, E, K, B, V, W, C, U, F, H, G, are also involved in recipient recognition and mating pair formation. Mutations in these genes phenotypically result in resistance to male-specific

bacteriophages which attach to the F-pilis for infection. The other three groups of tra genes encode for mating pair stability, conjugal DNA metabolism, and a presumptive regulatory region, (traJ), in addition to the genes for surface exclusion properties which are not required for plasmid transfer. The regulation of F transfer is somewhat unique in that in its usual state F is not repressed for transfer. Other plasmids similar to F are normally repressed for transfer (100). The result of non-repressed transfer for F is its high frequency transfer to F^- recipients compared to other normally repressed conjugative plasmids.

The conjugal transfer of F begins at a specific origin of transfer, oriT, located on the plasmid (120, 146). Presumably F plasmid DNA transfer is preceeded by a specific, but yet unidentified, endonuclease mediated nicking of the DNA within oriT. Subsequent single-stranded DNA transfer is then followed by DNA synthesis and recircularization in both donor and recipient to produce a covalently closed circular plasmid molecule (148).

The plasmid FP2 of Pseudomonas aeruginosa strain two (PAT), a fertility plasmid similar to the F plasmid of E. coli, has also been studied in great detail (56, 57, 60, 62). Like F, FP2 was capable of transferring chromosomal markers from the donor to the recipient at a frequency of 10^{-3} - 10^{-7}, depending on the marker selected. In addition, FP2 conferred resistance to mercury salts, an easily

selectable phenotype (89). Unlike F, $FP2^+$ X $FP2^+$ matings are fertile, however at a frequency less than 10^{-8} per donor cell. The ability of FP2 to transfer independent of chromosomal DNA was a condition of the donor strain. In P. aeruginosa strain one (PAO), the transfer of FP2 occurred at a much lower frequency, although there was no difference in the frequency of chromosome transfer. Unlike F, it is not known whether FP2 must be integrated into the chromosome in order to mediate chromosomal gene transfer. The presence of FP2 in a cell did not appear to be associated with the appearance of sex pili, raising the question of the role of sex pili in FP2 mediated conjugation.

The use of the fertility plasmids F of E. coli or FP2 of P. aeruginosa in the establishment of genetic systems in other bacterial genera has been limited by the host range of these plasmids. The host range of F is almost exclusively limited to the Enterbacteriaceae, including bacteria of the genera Salmonella, Shigella, and Erwinia. The exception being the transfer of F-prime plasmids from E. coli to P. fluorescens (96). The use of FP2 in bacterial genetics has similarly been limited to to its natural host, P. aeruginosa.

The first step in overcoming the problem of limited host range fertility plasmids in gene transfer systems came about as a result of the discovery of antibiotic resistance (R) plasmids. These plasmids contain the genetic determinants that confer upon the host cell resistance to

one or several specific antibiotics. Antibiotic resistance plasmids were first indentified in Japan in the early 1960's in the genus Shigella, an enteric bacterium that causes human dysentery. It was found that these bacteria became resistant to more than one antibiotic in a single step (2). It was later found that this multiple drug resistance was due to the presence of a plasmid that itself was capable of conjugal transfer to almost every member of the Enterobacteriaceae (103, 140). In spite of the wide host range among the Enterbacteriaceae, these R plasmids did not appear to mediate chromosomal gene transfer.

Isolates of P. aeruginosa that were resistant to high levels of the antibiotic carbenicillin were discovered in hospital burn patients (90). Later studies showed that this high level of antibiotic resistance was due to the presence of R factors similar to the ones found in Enterobacteria isolated from dysentery patients (42). These R-factors were also capable of intergeneric transfer of the carbenicillin resistance from P. aeruginosa to E. coli (133). Since then, a number of Pseudomonas R factors have been isolated (20, 131) which have been classified into groups based on host range and replication factors (15). Those plasmids that could be transferred to E.coli and other Gram negative bacteria were considered group P-1. Group P-1 plasmids generally had a size of 45-78 kilobases and conferred upon their hosts susceptibility to the bacteriophages PRR1, PRD1, and PR4 (69). Group P-2 plasmids were only transmissible to

strains of P. aeruginosa, had a size of 34-37 kilobases, and did not confer susceptibility to bacteriophages PRR1, PRD1, and PR4. The R plasmids have also been classified into groups based on whether or not they are capable of simultaneous replication in a given cell. Those plasmids which were incapable of such replication were thought to have similar replication systems and were said to belong in the same incompatibility group. Thus the Pseudomonas R plasmids have been also placed into eight incompatibility groups (68, 127).

The incompatibility group P-1 (IncP-1) antibiotic resistance plasmids, especially RP1, RP4, R68, and RK2, have the unique capability of transferring themselves to a wide range of Gram negative bacteria (10, 32, 33, 80, 112). The IncP-1 plasmids have also been shown to mobilize the host chromosome. Stanisich and Holloway (130) showed that R68 and R91 could mobilize chromosomal markers from P. aeruginosa PAT at frequencies comparable to that mediated by FP2. These plasmids were not, however, equally efficient at mobilizing chromosomal markers in P. aeruginosa PAO, where frequencies of recombination for chromosomal markers was 10^{-7} to 10^{-9} per donor cell (131). In searching for R plasmids capable of mediating a higher frequency of chromosome transfer in P. aeruginosa, Haas and Holloway (49, 50) found a variant of R68 which was capable of producing recombinants at a frequency of 10^{-3} to 10^{-5} per donor cell. Using the variant plasmid R68.45 and the plasmids R68, R91, and FP2,

Watson and Holloway (141) were able to establish a linkage map and demonstrate that PAT chromosome was circular, as is the E. coli chromosome (142). By similar techniques, linkage data for chromosomal markers in P. aeruginosa PAO were established (63), and eventually the circularity of the PAO chromosome was also established (125).

The IncP-1 R plasmids have been used in Pseudomonas to mobilize chromosomal DNA and in a number of different genera of bacteria where they also effect Cma (59). The plasmids RP4 and R68.45 were used to generate a genetic map in Rhizobium meliloti and R. leguminosarum. In both R. meliloti and R. leguminosarum, RP4 and R68.45 both were capable of mediating chromosomal gene transfer (11, 12, 66, 76, 95). Plasmid RP4 was only capable of mediating chromosomal gene transfer at low frequencies while plasmid R68.45 exhibited enhanced chromosome mobilizing ability (76). A similar situation was also observed in Erwinia chrysanthemi where the transfer frequency of chromosomal markers using RP1 to mobilize the chromosome was lower (77) than when R68.45 was used (23).

It first appeared that the mechanism responsible for high frequency recombination in P. aeruginosa (R68.45) was comparable to Hfr formation in E. coli. That is, R68 integrates into the chromosome as a result of homologous sequences found on both R68 and the chromosome. The ability of R68.45 to promote a higher frequency of chromosomal gene transfer was then speculated to be a result of R68 excision

from the chromosome and subsequent R' formation (50). The result of this chromosomal DNA acquisition would then have explained the property of Enhanced Chromosome Mobilization (ECM) in R68.45 (59, 61). The fact that R'-plasmid formation was found using R68.45 in P. aeruginosa (58) and Rhizobium meliloti (70) further supported the role of exogenous DNA in ECM by R68.45. However, restriction endonuclease analysis of the DNA of R68.45 showed that this DNA was not of chromosomal origin, but was rather a spontaneous duplication of a region of R68 itself (28, 88, 122, 147). Furthermore, R-plasmid variants of R68 with ECM formed in E. coli instead of P. aeruginosa also contained a direct repeat of R68 DNA, although in a different location than that found in R68.45 (104). When R68.45 containing donor strains have lost Cma, there is a subsequent loss of the duplicated DNA (29, 51).

Molecular and genetic studies on the IncP-1 antibiotic resistance plasmids RP1, RP4, and RK2 have shown that these plasmids are not like other conjugative plasmids in the distribution of their conjugative transfer (tra) and replication genes. Unlike the fertility plasmid F of E. coli, whose tra genes are found as one large linkage group, the tra genes of RP1, RP4, and RK2 are located in three separate regions of the plasmid. The identification of these tra genes came about through the isolation of transfer-deficient mutant plasmids by either in vitro deletion formation (38, 144), or transposon mutagenesis (7,

8). Mutations which mapped in the Tra 1 region phenotypically resulted in the lack or reduction in the self-transmissibility of the plasmid while retaining resistance to the bacteriophages PRR1 and PRD1 (8, 144). Transfer-deficient mutants which mapped in the Tra 2 region, however, were resistant to these bacteriophages (8). The origin of transfer of these plasmids, oriT, has been mapped to the Tra 1 region (8, 137, 144). The replication genes have also been found to be located at different regions around these plasmids, including the origin of vegetative replication oriV (37, 137). Both origins are thought to play a role in the wide host-range properties of these plasmids.

The role of plasmid integration in Cma mediated by R68.45 is still unknown. However, the IncP-1 plasmids considered to be identical to R68, the parent plasmid of R68.45, contain unique restriction endonuclease sites and have been used to generate in vitro R-primes (67, 97). When DNA from R. leguminosarum or Proteus mirabilis was cloned into the single EcoR1 site on RP4, no increase in efficiency of gene transfer was noted above that of RP4 itself (66). However when foreign DNA was cloned into other unique restriction endonuclease sites found on RP4, high frequency gene transfer was obtained in a polarized orientation (6, 9, 46, 71, 143). Furthermore, since this high frequency chromosome mobilization was dependent on a functional recA gene in the host, it appeared that these RP4-hybrid plasmids

were capable of integration into the chromosome. As a result of that integration, these R-prime plasmids were able to mediate high frequency gene transfer. That RP4 itself was capable of integration into the chromosome of E.coli was demonstrated by the use of mutants of RP4 that were temperature-sensitive for maintenance (31). When these temperature-sensitive RP4 mutants had integrated into the chromosome, they too were capable of high frequency chromosomal gene transfer (52, 53). Therefore, while it is apparent that members of the IncP-1 antibiotic resistance plasmids are capable of high frequency chromosomal gene transfer when integrated into the chromosome, it is not known whether or not this method is responsible for Cma.

The role of plasmid integration in the establishment of plasmid-mediated gene transfer systems has lead to the search for conjugative and integrative plasmids among cryptic plasmids found in plant pathogenic Pseudomonas spp. Recently a large naturally occurring plasmid of Pseudomonas syringae pv. phaseolicola has been shown to integrate into the host chromosome (27, 134). The conjugative properties of this plasmid, pMC7105, have not yet been demonstrated. However, pMC7105 appeared to integrate and excise from the P. syringae pv. phaseolicola chromosome in a manner analogous to the insertion sequence-mediated integration and excision of F from the E. coli chromosome (136). The insertion and excision of pMC7105 appeared to occur at unique sites on the plasmid and chromosome and suggested

that insertion sequences may also have been involved here (136).

Although the wide host-range IncP-1 plasmids have been used as cloning vectors, their large size (about 56 kb, (99)), and low copy number (4-7 per host chromosome, (37)), have limited their widespread applicability. The small, high copy number, non-conjugative plasmid RSF1010 (4, 104) has been shown to be mobilized by RK2 replicons and as a result has been shown to be a suitable cloning vehicle in non-E. coli systems where the ColE1 replicons are not functional (4, 114). These systems include plant pathogenic pseudomonads. The separation of the conjugative functions from the replicative functions of the wide host-range plasmid RK2 has now been achieved (35). The result of this has been that small highly transformable plasmids can transfer cloned DNA into a wide variety of bacteria where the wide host-range replication and maintenance properties of RK2 stably maintain the introduced plasmid. Similarly, a wide host-range cosmid cloning vector has also been constructed (40).

Summary. Plasmid mediated gene transfer systems have played an essential role in the genetic development of many different genera of bacteria. The discovery of IncP-1 plasmid-mediated gene transfer extended the range of bacteria suitable for genetic study beyond that of the Enterobacteriaceae. Furthermore, studies on the replication and maintenance functions of the IncP-1 plasmids have

resulted in the construction of wide host-range cloning vectors that have been used in a number of different genera of bacteria, including plant pathogenic pseudomonads. Therefore, the potential for genetic study of the plant pathogenic pseudomonads is now greater than ever before, as exemplified by the recent cloning of an avirulence gene from P. syringae pv. glycinea (132).

The purpose of all studies in plant pathology is the reduction of crop losses due to plant disease. The purpose in studying the genetics of plant pathogenic bacteria is no different. Paramount among the goals of plant pathology is an understanding of the mechanism of pathogen specificity. The relative ease of genetic manipulation of prokaryotic organisms compared to eukaryotic organisms makes plant pathogenic bacteria the ideal model system for studying plant-parasite interactions. This is especially true for the plant pathogenic pseudomonads for which gene-for-gene systems determining race specificity are already established. It is hoped that an understanding of the genetics of pathogenicity would lead to the control of plant diseases.

CHAPTER I

The transfer of IncP-1 plasmids to *Pseudomonas syringae* pv. *syringae* strains and the monitoring of plasmid pRD1 in *P. syringae* PSSD220 (pRD1).

CHAPTER I

The transfer of IncP-1 plasmids to *Pseudomonas syringae* pv. *syringae* and the monitoring of plasmid pRD1 in *P. syringae* PSSD220 (pRD1).

Abstract

The IncP-1 antibiotic resistance plasmids R68.45, RP1, and pRD1 were conjugatively transferred to *Pseudomonas syringae* pv. *syringae* strains. *P. syringae* donor strains harboring R68.45 were able to transfer R68.45 to *P. syringae* recipients 100 to 10,000 times more frequently than *Escherichia coli* K12 or *P. aeruginosa* PAO donor strains. No plasmid-mediated chromosomal gene transfer was detected between *P. syringae* strains using R68.45 or pRD1-containing donors. Plasmid pRD1, containing the *Klebsiella pneumoniae* histidine biosynthesis operon, complemented histidine mutations in histidine requiring *P. syringae* strains. *P. syringae* PSSD220 (pRD1) was unable to conjugatively transfer pRD1 to other bacteria and was not susceptible to the IncP-1 plasmid specific bacteriophages PRR1 and PRD1.

Successive transfer of *P. syringae* PSSD220 (pRD1) on non-selective media produced kanamycin resistant variants which were unable to complement the histidine mutation of PSSD220.

Introduction

The IncP-1 antibiotic resistance plasmids are capable of transfer to a wide variety of Gram-negative bacteria (32, 112). The most familiar of these plasmids, RP1, RP4, R68, R68.45, and RK2, confer resistance to the antibiotics ampicillin (Ap^r), tetracycline (Tc^r), and kanamycin (Km^r) to the host in which they reside. In addition, bacteria that harbor IncP-1 antibiotic resistance plasmids are normally sensitive to the bacteriophages PRR1 (113) and PRD1 (111). IncP-1 antibiotic resistance plasmids have been transferred to phytopathogenic xanthomonads (81), pseudomonads (78, 97, 115), erwiniae (24, 26, 77), and agrobacteria (32). For a review see Lacy and Leary (80). Some members of the IncP-1 antibiotic resistance plasmids can promote chromosomal gene transfer (see Holloway (59)). Among the plant pathogenic pseudomonads, IncP-1 plasmid-mediated chromosomal gene transfer has been detected in Pseudomonas glycinea using plasmids R68 and R68.45 (41, 79).

R-prime plasmids are antibiotic resistance plasmids that have acquired additional DNA. Spontaneous R-prime formation has been observed using IncP-1 antibiotic resistance plasmids in Pseudomonas aeruginosa (58, 124), Escherichia coli (110), and Rhizobium meliloti (70, 73). Additionally, in vitro R-primes have been created by the insertion of chromosomal DNA into one of the unique restriction endonuclease sites found on the IncP-1 plasmid RP4 (6, 9, 46, 66, 71). Some of these R-prime plasmids

have also shown the capability to promote chromosomal gene transfer at frequencies higher than that obtained by the parental plasmids (6, 9, 46, 71).

The plasmid pRD1 (formerly called RP41) (36) is an R-prime plasmid isolated by Dixon et al after in vivo recombination between the IncP-1 plasmid RP4 and an F-prime plasmid carrying Klebsiella pneumoniae DNA (36). As a result, pRD1 contains the K. pneumoniae genes for nitrogen fixation, histidine biosynthesis, gluconate-6-phosphate dehydrogenase, and shikimate permease, along with the antibiotic resistance genes normally associated with RP4 which confer resistance to ampicillin, tetracycline, and neomycin/kanamycin. The R-prime plasmid pRD1 has previously been transferred to E. coli (36), Agrobacterium tumefaciens (36), Rhizobium meliloti (36), Salmonella typhimurium (116), Enterobacter cloacae (75), and P. fluorescens (96), with the expression of plasmid-determined histidine biosynthesis and kanamycin resistance. Plasmid pRD1 could not, however, be conjugatively transferred to P. aeruginosa (36). When pRD1 was conjugatively transferred from E. coli JC5466 (pRD1) to Agrobacterium tumefaciens 544 (36), Rhizobium meliloti A1 (36), Salmonella typhimurium strains (116), or Enterobacter cloacae MF10 (75), subsequent segregation of the histidine biosynthesis or nitrogen fixation genes from the antibiotic resistance genes occurred frequently. In E. coli K12, this segregation was shown to be the result of recA dependent spontaneous degradation of plasmid pRD1 in

the host cell (117).

The purpose of the work presented in this section was to determine the intergeneric and intrageneric frequency of transfer of plasmids R68.45 and pRD1 in the wheat pathogen *P. syringae* pv. *syringae*, and to determine whether these plasmids could mediate chromosomal gene transfer in *P. syringae* pv. *syringae*.

P. syringae PSSD220 (pRD1) was also monitored for pRD1-determined kanamycin resistance and the ability of the *Klebsiella pneumoniae* histidine biosynthesis genes to complement the histidine mutation of PSSD220. The conditions under which segregation of the pRD1-determined phenotypic histidine prototrophy in PSSD220 (pRD1) from the kanamycin resistance were also determined.

Materials and Methods:

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are listed in table 1.

Media. Cultures of P. syringae and P. aeruginosa were grown in complete medium (84) or Davis minimal medium (84). The complete broth consisted of casamino acids (Difco) 10 g/l, Bacto yeast extract (Difco) 5 g/l, K_2HPO_4 3 g/l, and KH_2PO_4 1 g/ml. When nalidixic acid was included in the complete medium, KH_2PO_4 was omitted and the quantity of K_2HPO_4 was increased to 6 g/l to raise the pH of the medium above 7.0 to prevent the precipitation of the nalidixic acid within the medium. Davis minimal broth consisted of K_2HPO_4 7 g/l, KH_2PO_4 2 g/l, sodium citrate 0.5 g/l, $MgSO_4$ heptahydrate 0.1 g/l, ammonium sulfate 1 g/l, D-glucose 10 g/l (added to the salts solution from a 50% stock solution after autoclaving of the salts solution). Nitrogen-free minimal medium consisted of Davis minimal medium with the ammonium sulfate omitted. When nalidixic acid was included in the minimal medium, the KH_2PO_4 was reduced to 1 g/l. Escherichia coli was grown in L medium (101) or Davis minimal medium. L broth consisted of Bacto tryptone (Difco) 10 g/l, Bacto yeast extract (Difco) 5 g/l, and NaCl 10 g/l. Solidified media was obtained by the addition of Bacto agar (Difco) to 1.5%. Amino acid supplements were added to Davis minimal medium to a final concentration of 20 ug/ml. Kanamycin sulfate (Sigma) and streptomycin sulfate (Sigma) were added to a final concentration of 50 ug/ml from stock

solutions of 1 mg/ml. Nalidixic acid (Sigma) was added to a final concentration of 200 ug/ml from a 100 mg/ml stock solution, pH 12.0 (101). Rifampicin (Sigma) was added to a final concentration of 100 ug/ml. Due to the relative insolubility of rifampicin in water, it was first dissolved in 2 ml of methanol. Sterile water was then added to a final volume of 10 ml before the rifampicin solution was added to the medium (145).

Mutant Isolation. Auxotrophic mutants of P. syringae were isolated through ethyl methanesulfonate (EMS) mutagenesis followed by penicillin enrichment for auxotrophs (101). Single colonies of P. syringae were transferred to complete broth (CB) and grown overnight at room temperature with continuous shaking. After overnight incubation, the culture was divided into 10 aliquots and 1 drop of stock EMS was added to each aliquot and vortexed vigorously. The cultures were then incubated for 4-5 hours without shaking at room temperature at which time the EMS treated cultures were diluted 1:20 into fresh CB and allowed to grow overnight. After overnight growth, the cultures were washed twice in sterile saline (0.85% NaCl), brought up to the original volume with sterile saline, and diluted 1:50 into nitrogen-free minimal broth. The cultures were then incubated overnight at room temperature with continuous shaking. Following nitrogen starvation, the cultures were diluted 1:10 into parental minimal medium supplemented with penicillin G (Sigma), 10 mg/ml, and incubated overnight

Table 1. Bacterial Strains and Plasmids Used in This Study

Bacteria	Genotype	Source or Reference
Escherichia coli K12		
HB101	*hsd*R *hsd*M *rec*A13 *sup*E44 *lac*Z4 *leu*B6 *pro*A2 *thi*-1 Str^r	(13)
JC5466	*trp his rps*E *rec*A56	(36)
Pseudomonas aeruginosa		
PAO303	*arg*B18 *chl*-2	B. W. Holloway
PAO8	*met*-28 *ilv*-202 Str^r	B. W. Holloway
PAO25	*arg*F *leu*-10	R. H. Olsen
Pseudomonas syringae pv. *syringae*		
PSSD211	*nal*A *his*-1 *met*-1	D. W. Fulbright
PSSD220	*nal*A *his*-1 *met*-1	D. W. Fulbright
SB110	Str^r *phe*-1	this work
SB120	Str^r *ura*-2	this work
SB130	Str^r *his*-1	this work
SB140	Str^r *trp*-1	this work
SB150	Str^r *leu*-1	this work
SB160	Str^r *ser*-1	this work
Plasmids	**Genotype**	**Reference**
pRD1	*his nif gnd shiA* kan^+ tet^+ bla^+ Tra^+	(36)
R68.45	kan^+ tet^+ bla^+ Tra^+ Cma^+	(50)
RP1	kan^+ tet^+ bla^+ Tra^+	R. H. Olsen

Table 1 (cont'd)

1) Symbols in this table are according to Bachman (3) or Novick et al (106).

2) Strain PSSD220 resulted from the spontaneous loss of the resident plasmid from PSSD211.

Abbreviations: hsdR = endonuclease R; hsdM = DNA methylase M; recA = general recombination; supE = amber suppressor; lacZ = Beta-D-galactosidase; leuB = Beta-isopropylmalate dehydrogenase; proA = Gamma-glutamyl phosphate reductase; thi = thiamine requirement; Str^r = streptomycin resistance; trp = tryptophane requirement; his = histidine requirement; rpsE = 30s ribosomal subunit protein S5; argB = acetylglutamate kinase; chl = nitrate reductase, formate dehydrogenase, and biotin sulfoxide reductase activities; met = methionine requirement; ilv = isoleucine-valine requirement; argF = ornithine carbamoyltransferase; leu = leucine requirement; nalA = high level nalidixic acid resistance; phe = phenylalanine requirement; ura = uracil requirement; ser = serine requirement; Rif^r = rifampicin resistance; gnd = gluconate-6-phosphate dehydrogenase; shiA = shikimate and dehydroshikimate permease; kan = kanamycin resistance; tet = tetracycline resistance; bla = Beta-lactamase; Tra = mediating conjugation; Cma = chromosome mobilizing ability.

with continuous shaking to enrich for auxotrophic mutants. The cultures were then serially diluted, spread onto complete agar (CA), and incubated at 27 C until single colonies were apparent. Single colonies were transferred onto CA and parental minimal medium. Those colonies that did not grow on parental minimal medium but did grow on CA were then transferred to pool plates to determine their nutritional requirements (101).

Spontaneous mutants for streptomycin and nalidixic acid resistance were isolated by the gradient plate method of Bryson and Szybalski (16), while spontaneous rifampicin resistant mutants were identified by the method of Weller and Saettler (145).

Plate Mating and Selection. Plasmid transfer was accomplished by growing the donor and the recipient strains in either complete broth or minimal broth supplemented with the appropriate amino acids and antibiotics. After overnight growth, the cultures were washed twice and resuspended in sterile saline. One-tenth ml samples of the washed donor and recipient cultures were added to the surface of a sterile Millipore filter (0.45 um pore size) on an agar plate and incubated overnight at 27 C. After overnight incubation, the filters were removed from the plates and placed in 50 ml sterile glass centrifuge tubes containing 10 ml of sterile saline. The bacteria were then washed off the filters by vortexing, diluted, and spread onto selective minimal agar (MA) plates. The

transconjugants from the mating were then transferred to a selective medium and incubated at 27 C. In order to be certain that there were no E. coli contaminants among the transconjugants, the transconjugants were also transferred to a complete medium and incubated at 37 C, a temperature selective for E. coli.

Prior to R68.45 transfer from P. aeruginosa PAO8 to P. syringae strains, high frequency P. aeruginosa donors were first identified by their ability to transfer the argB locus to argB$^-$ P. aeruginosa PAO recipients (50). One-tenth ml aliquots from washed overnight cultures of PAO8 (R68.45) were spotted onto a lawn of stationary phase PAO303 recipient cells on a minimal medium that would select for the transfer of arg$^+$. The donor culture that produced the greatest number of recombinant colonies, PAO8-83 (R68.45), was then used as the donor for R68.45 transfer to P. syringae.

Monitoring of Plasmid pRD1 Markers. The maintenance of plasmid pRD1 markers in P. syringae PSSD220 was monitored in the transconjugants obtained after mating with E. coli JC5466 (pRD1). The transconjugants were successively transferred from the minimal plates used to select for the transconjugants onto CA plus kanamycin, MA plus kanamycin supplemented with methionine, and CA. Bacterial transfers were accomplished using sterile brass templates to mediate replica plating.

Bacteriophage Sensitivity. The sensitivity of IncP-1

plasmid containing bacteria to the bacteriophages PRR1 and PRD1 was determined by a cross-streaking method (R. H. Olsen, personal communication). Loopfuls of the bacteriophage, at a concentration of about 10^{10} pfu/ml, were streaked in a line across a CA plate. Across this line of bacteriophage, a dilute suspension of the bacteria to be tested was streaked. After incubation for 48-72 hours, those bacteria that were sensitive to the bacteriophage were lysed from the point where they crossed the line of bacteriophage.

Results:

The intergeneric transfer of the IncP-1 antibiotic resistance plasmid R68.45 from P. aeruginosa PAO8 (R68.45) to P. syringae strains occurred at frequencies which ranged from 9.2×10^{-7} to 1.4×10^{-5} per donor cell, depending on the recipient strain used (Table 2). The subsequent transfer of R68.45 between P. syringae strains occurred at frequencies which ranged from 5×10^{-2} to 6×10^{-4} per donor cell, depending on the strains involved (Table 3).

Table 2. Transfer of R68.45 from Pseudomonas aeruginosa to P. syringae.

Donor	Recipient	Frequency [1]
PAO8-83 (R68.45)	SB110 Str^r phe-1	1.4×10^{-5}
	SB120 Str^r ura-2	9.1×10^{-6}
	SB130 Str^r his-1	1.7×10^{-5}
	SB140 Str^r trp-1	9.2×10^{-7}
	SB150 Str^r leu-1	3.2×10^{-6}
	SB160 Str^r ser-1	3.6×10^{-5}

1) Per donor cell after selection for kanamycin resistance in the transconjugants.

Matings were performed on Millipore filters on complete agar by mixing 0.1 ml of donor and recipient on the filter and incubating overnight at 27 C.

Table 3. Transfer of R68.45 between Pseudomonas syringae strains.

Donor	Recipient	Frequency [1]
SB140 (R68.45)	SB120	6.6×10^{-2}
SB140 (R68.45)	PSSD211	5.1×10^{-2}
PSSD210 (R68.45)	PSSD230	6.1×10^{-4}

1) Per donor cell after selection for kanamycin resistance in the transconjugants.

Matings were performed on Millipore filters on complete agar by mixing 0.1 ml of donor and recipient on the filter and incubating overnight at 27 C.

Table 4. Transfer of pRD1 from Escherichia coli to Pseudomonas syringae strains.

Donor	Recipients	Selection	Frequency [1]
JC5466 (pRD1)	SB130	Kan^r His^+	3×10^{-6}
	SB130	Kan^r	8×10^{-6}
	PSSD211	Kan^r His^+	5×10^{-6}
	PSSD211	Kan^r	6×10^{-6}
	PSSD220	Kan^r His^+	5×10^{-6}
	PSSD220	Kan^r	6×10^{-6}

1) Frequency determination is per donor cell.

Matings were performed on Millipore filters on complete agar by mixing 0.1 ml of donor and recipient on the filter and incubating overnight at 27 C.

Plasmid pRD1 was transferred from E. coli JC5466 (pRD1) to histidine requiring P. syringae strains by selection for either kanamycin resistance or histidine prototrophy and kanamycin resistance, indicating that the Klebsiella pneumoniae histidine genes carried on pRD1 complemented the histidine mutations in the recipients (Table 4). The frequency of pRD1 transfer from E. coli to P. syringae strains was the same whether selection was for kanamycin resistance or for kanamycin resistance and the ability to complement histidine mutations (Table 4). In either case the frequency of transfer of pRD1 from E. coli JC5466 to P. syringae, 5×10^{-6} per donor cell, was approximately the same as when plasmid R68.45 was transferred from P. aeruginosa to P. syringae. Subsequent transfer of pRD1 from PSSD220 (pRD1) to PSSD241, as determined by the acquisition of kanamycin resistance, was not detected at frequencies up to 1×10^{-9} per donor cell.

The IncP-1 plasmid RP1 was transferred from P. aeruginosa PAO and E. coli (RP1) to P. syringae PSSD220 by selection for kanamycin resistance in the recipients and counterselecting against the donors with nalidixic acid. The frequency of RP1 transfer from E. coli HB101 (RP1) to P. syringae occurred at a frequency comparable to that of the other IncP-1 plasmids tested, 5×10^{-6} per donor cell (Tables 2 and 4), while P. aeruginosa PAO25 (RP1) was only able to transfer RP1 at a frequency of 5×10^{-8} per donor cell (Table 5).

Table 5. Transfer of RP1 to Pseudomonas syringae PSSD220

Donor	Recipient	Frequency [1]
PAO25 (RP1)	PSSD220	5×10^{-8}
HB101 (RP1)	PSSD220	4×10^{-6}

1) Per donor cell after selection for kanamycin resistance in the transconjugants.

Matings were performed on Millipore filters on complete agar by mixing 0.1 ml of donor and recipient on the filter and incubating overnight at 27 C.

Donor strains of P. syringae (R68.45) or P. syringae (pRD1) strains were used as donors to determine whether or not these plasmids were capable of mediating chromosomal gene transfer in P. syringae. In matings with auxotrophic P. syringae recipients, no gene transfer was detected at frequencies up to 1×10^{-9} per donor cell.

When P. syringae strain PSSD211 nalA his-1 met-1 (pRD1) was maintained on minimal agar (MA) supplemented with methionine, or MA supplemented with methionine and kanamycin, the pRD1-determined K. pneumoniae histidine biosynthesis genes complemented the requirement for histidine in PSSD211, making it phenotypically His^+. The genes for resistance to kanamycin, ampicillin, and tetracycline found on pRD1 also conferred resistance to these same antibiotics when pRD1 was present in PSSD211.

However, when PSSD211 (pRD1) was grown on a complete medium without kanamycin, or if PSSD211 (pRD1) cultures were recovered from storage at -20 C in sterile 0.85% NaCl, three different phenotypes could be recovered. Bacteria of the first phenotype could still grow on a minimal medium without histidine supplementation and were resistant to kanamycin (His^+ Kan^r). Those bacteria with this phenotype apparently still contained pRD1 which complemented the histidine requirement of PSSD211 and conferred resistance to kanamycin. Bacteria of the second recovered phenotype could not grow on a minimal medium without histidine supplementation and were sensitive to kanamycin (His^- Kan^s). Those bacteria of this phenotype apparently were cured of plasmid pRD1 and were phenotypically the same as the parent PSSD211. Bacteria of the third recovered phenotype were resistant to kanamycin, but could not grow on a minimal medium without histidine supplementation (His^- Kan^r).

To determine the stability of pRD1 in P. syringae PSSD220, 150 His^+ Kan^r transconjugants of PSSD220 (pRD1) were selected after mating E. coli JC5466 (pRD1) with PSSD220. The transconjugants were then successively transferred to media to monitor the plasmid-encoded His^+ and Km^r determinants. After three transfers on CA, 15% of the clones had lost the His^+ determinant and 11% had lost the Km^r determinant (Table 6). After the fourth transfer on CA, 43% had lost the His^+ determinant and 35% had lost the Km^r determinant. When the transconjugants were successively

Table 6. Maintenance of pRD1 markers in Pseudomonas syringae PSSD220.

Transfer medium	Phenotype[1]	No. of clones growing after each transfer[2]			
		1	2	3	4
CA + kanamycin	Kan^r His^+	150	150	150	149
	Kan^r His^-	0	0	0	1
	Kan^{s3}	0	0	0	0
CA	Kan^r His^+	150	149	110	33
	Kan^r His^-	0	0	23	64
	Kan^{s3}	0	1	17	53

1) The Kan^r His^+ phenotype was determined by the ability to grow on CA, CA plus kanamycin, and MA plus kanamycin plus methionine. The Kan^r His^- phenotype was determined by the ability to grow on CA and CA plus kanamycin, but not on MA plus kanamycin plus methionine. The Kan^s phenotype was determined by the ability to grow on CA, but not on CA plus kanamycin or MA plus kanamycin plus methionine.

2) Of 150 colonies originally transferred.

3) Those clones unable to grow on media containing kanamycin were not checked for histidine prototrophy.

transferred on a medium that selected only for Kan^r, more than 99% of the clones were still His^+ and Kan^r after four transfers, ie. only one clone had lost the His^+ determinant. None of the clones maintained on CA plus kanamycin became Kan^s. When the transconjugants were repeatedly transferred on a medium that selected for either His^+ or His^+ Kan^r, the clones could be maintained with repeated transfers.

When P. syringae PSSD220 (pRD1), E. coli HB101 (RP1), P. syringae PSSD220 (RP1), P. aeruginosa PAO25 (RP1), E. coli JC5466 (pRD1) were tested for sensitivity to the IncP-1 plasmid specific bacteriophages PRR1 and PRD1, it was found that PSSD220 (pRD1) was resistant to lysis by both PRR1 and PRD1 (Table 7) while the rest were sensitive to the and were subsequently lysed (Table 7).

Table 7. Sensitivity of IncP-1 plasmid-containing bacteria to the bacteriophages PRR1 and PRD1.

Bacterial Strain	Sensitivity to PRR1	PRD1
P. aeruginosa PAO25 (RP1)	S	S
E. coli HB101	R	R
E. coli HB101 (RP1)	S	S
E. coli JC5466 (pRD1)	S	S
P. syringae PSSD220	R	R
P. syringae PSSD220 (RP1)	S	S
P. syringae PSSD220 (pRD1)	R	R

1) R = resistant to lysis by the bacteriophage.

2) S = susceptible to lysis by the bacteriophage.

Susceptibility to bacteriophages PRR1 and PRD1 was determined by streaking a loopful of the bacteria to be tested across a line of bacteriophage (about 10^{10} pfu/ml). Bacteria were considered susceptible if they were lysed at the point where they crossed the line of bacteriophage.

Discussion. The ability of plant pathogenic pseudomonads to act as recipients of wide host range antibiotic resistance plasmids was previously established by Panopoulos et al (115), and Lacy and Leary (78). They demonstrated that plant pathogenic Pseudomonas spp. acquired the IncP-1 plasmid RP1 from E. coli donors at frequencies of 2 X 10^{-5} to 1.6 X10^{-2} per donor cell. Lacy and Leary were, however, unable to detect any RP1 transfer from a P. aeruginosa donor, suspecting that the inability to transfer RP1 was a result of bacteriocin production in the P. aeruginosa donor. In the present study, IncP-1 plasmids were transferred to P. syringae strains at frequencies ranging from 5 X 10^{-8} per donor cell for RP1 transfer from P. aeruginosa PAO25 to 3.6 X 10^{-5} per donor cell for R68.45 transfer from P. aeruginosa PAO8. When P. syringae strain PSSD220 was used as the recipient for RP1 transfer from either E. coli or P. aeruginosa, the frequency of transfer was 100-fold higher from the E. coli donor than from the P. aeruginosa donor. That IncP-1 plasmid transfer to P. syringae strains from P. aeruginosa was detected in these experiments where it could not be detected by Lacy and Leary was probably the result of using a different donor strain of P. aeruginosa. The differences in ability of the P. syringae strains used in this study to act as recipients for IncP-1 plasmid transfer may indicate differences in the DNA restriction and modification systems of the P. syringae strains themselves, or may indicate the ability of the different strains to

maintain the mating pairs formed during conjugation.

Intrageneric and interspecific transfer of IncP-1 antibiotic resistance plasmids among the plant pathogenic pseudomonads has received little attention in the literature. Lacy and Leary (78) reported that the transfer of RP1 from P. glycinea PG9 to P. phaseolicola HB36 occurred at a frequency of 4.9×10^{-3} to 5.8×10^{-2} per recipient cell in vitro and 4.2×10^{-3} to 5.5×10^{-1} per recipient cell in planta. While the P. syringae strains used in this study were only able to acquire IncP-1 plasmids during intergeneric transfer at a frequency of about 5×10^{-6} per donor cell, they were capable of subsequent intrageneric transfer of R68.45 at much higher frequencies. The transfer of R68.45 among P. syringae strains occurred at frequencies between 6.1×10^{-4} and 6.6×10^{-2} per donor cell, a 100 to 10,000-fold higher frequency than the transfer of R68.45 to P. syringae strains from P. aeruginosa PAO.

Based on restriction endonuclease and heteroduplex mapping, the IncP-1 plasmids R68, RP4, RP1, and RK2 are considered to be identical (17, 28). Therefore, the parental plasmids of pRD1 and R68.45, RP4 and R68 respectively, are probably identical to the IncP-1 plasmid RP1. When the frequency of intergeneric transfer of RP1 to P. syringae strains is compared to the intergeneric transfer frequencies of plasmids R68.45 and pRD1 to P. syringae strains, it was observed that they all transferred at about the same frequency of 5×10^{-6} per donor cell.

The plasmid R68.45 has been a useful tool in the establishment of gene transfer systems in a number of different genera of bacteria where it has been shown to be capable of mediating high frequency chromosomal gene transfer (59). Lacy and Leary were able to show that RP1 was able to mediate chromosomal gene transfer in P. glycinea race 6 (79). In the present studies using P. syringae donor strains containing IncP-1 plasmids, no chromosomal gene transfer was observed.

When plasmid RP4 was modified by the insertion of foreign DNA into one of its unique restriction sites, the resulting R-prime plasmid was often capable of mediating chromosomal gene transfer (6, 9, 46, 71). It had been hypothesized that the presence of DNA homologous to both the RP4-primes and the chromosome allowed recombination between plasmid and chromosome. The result of this recombination produced Hfr-like gene transfer. The IncP-1 plasmid pRD1 is an R-prime plasmid of RP4 containing K. pneumoniae DNA inserted in vivo. Since the K. pneumoniae histidine biosynthesis genes on pRD1 complemented histidine mutations in P. syringae, it was assumed that there was sufficient homology on pRD1 with P. syringae chromosomal DNA to allow Hfr-like formation and subsequent chromosome mobilization. Unlike other reports where RP4-primes were capable of chromosomal mobilizing ability, no gene transfer between P. syringae PSSD2 strains was detected when P. syringae PSSD220 (pRD1) was used as a donor. Furthermore, the ability of

pRD1 to transfer itself out of PSSD220 (pRD1) was not detected when selection was for either kanamycin resistance or histidine prototrophy in the recipients.

P. syringae PSSD220 (pRD1) was not susceptible to the R-specific bacteriophages PRR1 and PRD1. These bacteriophages are thought to attach to the pilis produced by the bacterium as a result of R-plasmid infection. Since cell-to-cell contact is required during conjugation (87), the pili produced by bacteria as a result of sex plasmid infection are thought to be involved in mating pair formation and DNA transfer during conjugation. The inability of the bacteriophages PRR1 and PRD1 to lyse PSSD220 (pRD1) indicates that PSSD220 (pRD1) may be defective in sex pili normally produced as a result of R plasmid infection. Furthermore, since the R plasmid R68.45 was capable of transfer in P. syringae PSSD2, it appears that the lack of transfer ability of pRD1 in PSSD2 is not due to the repression of R plasmid transfer ability in PSSD2.

The IncP-1 plasmid pRD1 isolated by Dixon et al (36) is a large R-prime plasmid. Since pRD1 was formed by in vivo recombination between RP4 and an F-prime plasmid containing K. pneumoniae DNA, pRD1 also contains the genetic determinants for resistance to the antibiotics ampicillin, tetracycline, and kanamycin. When pRD1 was transferred from E. coli JC5466 (pRD1) to P. syringae strains SB and PSSD, the K. pneumoniae histidine genes found on pRD1 complemented

the histidine mutations in the recipients in addition to conferring resistance to kanamycin, ampicillin, and tetracycline.

After conjugative transfer of pRD1 from E. coli JC5466 (pRD1) to Agrobacterium tumefaciens 544 (36), Rhizobium meliloti A1 (36), Salmonella typhimurium strains (116), or Enterobacter cloacae MF10 (75), subsequent segregation of the histidine biosynthesis or nitrogen fixation genes from the antibiotic resistance genes occurred frequently. In E. coli K12, this segregation was shown to be the result of recA dependent spontaneous degradation of plasmid pRD1 in the host cell (117). P. syringae PSSD220 (pRD1) also segregated the K. pneumoniae biosynthesis genes from the antibiotic resistance genes. The rate of loss of the histidine genes depended on whether PSSD220 (pRD1) was grown on media that selected for any of the other phenotypic markers found on pRD1 or whether PSSD220 (pRD1) was grown on a non-selective medium. No segregation of pRD1 markers was observed when PSSD220 (pRD1) was maintained on media which selected for histidine complementation and kanamycin resistance. The segregation of pRD1 markers in P. syringae PSSD220 is apparently comparable to that observed in other bacteria and may be indicative of E. coli recA-like activity in P. syringae.

CHAPTER II

The Monitoring of pRD1 in *Pseudomonas syringae* and Evidence for its Integration Into the Chromosome.

CHAPTER II

The monitoring of pRD1 in *Pseudomonas syringae* and Evidence for its Integration into the Chromosome.

Abstract

The IncP-1 antibiotic resistance plasmid pRD1 could not be detected as an extrachromosomal element in agarose gels after *Pseudomonas syringae* pv. *syringae* (pRD1) was successively subcultured on a medium that selected for the pRD1-determined His^+ and Kan^r. Hybridization of the DNA from cleared lysates of PSSD220 (pRD1) to ^{32}P-labeled pSA30 and pRD1 indicated that pRD1 had integrated into the chromosome of PSSD220. When PSSD220 (pRD1) was maintained on non-selective media, variants appeared which were unable to complement the histidine mutation of PSSD220. These variants contained a plasmid smaller than pRD1 which appeared to have been formed by an *in vivo* deletion of the *Klebsiella pneumoniae* histidine genes found on pRD1. Hybridization of ^{32}P-labeled pRD1 to the DNA from cleared lysates of PSSD220 cultures containing the pRD1 deletion plasmid or the IncP-1 plasmid RP1 indicated that these plasmids had not integrated into the chromosome of PSSD220.

Introduction. The IncP-1 antibiotic resistance plasmid pRD1 was isolated by Dixon after in vivo recombination with an F-prime plasmid and RP4 (36). As a result, pRD1 contains the Klebsiella pneumoniae genes for nitrogen fixation, histidine biosynthesis, gluconate-6-phosphate dehydrogenase, and shikimate permease along with the antibiotic resistance genes normally associated with RP4. Plasmid pRD1 has been transferred to Escherichia coli (36), Agrobacterium tumefaciens (36), Rhizobium meliloti (36), Salmonella typhimurium (116), and Enterobacter cloacae (75) with the expression of both His^+ and Kan^r. Among pseudomonads, pRD1 has been conjugatively transferred to Pseudomonas fluorescens (96), and P. syringae pv. syringae (138) with expression of both His^+ and Kan^r. Plasmid pRD1 could not, however, be conjugatively transferred to P. aeruginosa PAO (36), and was only introduced into P. putida by transformation (21). It has previously been shown that in some bacteria pRD1 spontaneously degrades with the subsequent loss of some or all of the plasmid markers (36, 75, 116). In E. coli, this spontaneous degradation has been shown to be recA dependent (117). After transfer of pRD1 to Enterobacter cloacae, pRD1 could no longer be detected as an extrachromosomal element after agarose gel electrophoresis, even though the pRD1-determined antibiotic resistance and nitrogen fixation genes were still expressed (75). It was speculated that pRD1 had integrated into the chromosome of E. cloacae

In this section P. syringae PSSD220 (pRD1) was monitored for the presence of pRD1 by both agarose gel electrophoresis of cleared lysates and hybridization of ^{32}P-labeled pRD1 to the DNA from the cleared lysates. The results provide evidence that pRD1 had integrated into the chromosome of PSSD220. The chromosomal integrating capability of the IncP-1 antibiotic resistance plasmid RP1 and deletion plasmids of pRD1 was also checked by similar hybridization experiments.

Materials and Methods:

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are listed in Table 1, Chapter 1. In addition, purified plasmid pSA30 (19) was a gift of Dr. Barry Chelm.

Media. The media used to grow bacterial cultures was previously described in Chapter 1.

Plate Mating and Selection. Plate matings and selection for transconjugants was as previously described in Chapter 1.

Monitoring of Plasmid pRD1 Markers. The condition for monitoring plasmid pRD1 markers in P. syringae PSSD220 was described in Chapter 1.

Plasmid Detection. The detection of plasmids in bacterial cultures was by the procedure of Kado and Liu (72). Bacteria from 3 ml of an overnight culture in CB or MB supplemented with the appropriate amino acids and antibiotics were pelleted by centrifugation and resuspended in 1 ml of E buffer (40 mM Tris, 2 mM EDTA, 5 mM sodium acetate, pH 7.9). The bacterial suspension was then lysed by the addition of 2 ml of filter sterilized lysis buffer (3% Sarkosyl, 50 mM Tris, pH 12.6), and heated in a 55 C water bath for 30 minutes. The lysate was extracted with two volumes of distilled phenol:chloroform (1:1) until the aqueous phase was clear. Fifty ul of a solution of 50 ul of the aqueous phase and 15 ul tracking dye (5% SDS, 25% glycerol, 0.025% bromphenol blue) was loaded on a 3 mm thick

0.7% agarose (Sigma type II or Bethesda Research Laboratories) vertical gel and electrophoresed in E buffer at a constant current of 40 mA until the tracking dye had reached the bottom of the gel. The DNA in the gel was visualized after ethidium bromide staining (1 ug/ml) on a transilluminator (UV Products), and photographed through a Wratten No. 4 filter using Polaroid type 55 film.

Plasmid Isolation. Purified plasmid DNA was by the method of Currier and Nester (30). Cultures of bacteria containing the plasmid to be isolated were grown to stationary phase in CB supplemented with the appropriate antibiotics. The bacteria were pelleted by centrifugation and washed twice in TE buffer (0.05 M Tris-HCl, 0.02 M EDTA, pH 8.0). The resulting bacterial suspension was then adjusted to a Klett reading of 150 with TE buffer. To accomplish cell lysis, predigested pronase (Calbiochem) was added to a final concentration of 500 ug/ml and sarkosyl was added to 1% by the addition of 30% sarkosyl (ICN Pharmaceuticals, Inc.). The resulting suspension was then incubated in a 37 C water bath for 40 minutes or until lysis was complete. The pronase was predigested by incubation at 37 C for 30 minutes in TE Buffer. After lysis, the viscosity of the solution was reduced by shearing the DNA in solution by stirring the lysate at 100 rpm with a 7.6 cm Teflon-coated magnetic stir bar in a 600 ml to 2000 ml beaker, depending on the volume of the lysate, until the lysate was homogeneous. The DNA in the lysate was then

denatured by raising the pH of the lysate to 12.5 by the dropwise addition of 3 N NaOH. The alkaline lysate was stirred for 10 minutes at 100 rpm and 2 M Tris-HCl, pH 7.0 was added dropwise until the pH reached a value between 8.5 to 9.0. Following denaturation and partial neutralization, the lysate was adjusted to 3% NaCl by the addition of crystalline NaCl and extracted with an equal volume of distilled phenol equilibrated with an equal volume of 3% NaCl. The phenol extraction was accomplished by the addition of the 3% NaCl:phenol to the beaker in which the lysate was contained and stirring until the solution was emulsified. After phenol extraction the aqueous phase was collected by centrifugation and extracted with an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous phase was again collected after centrifugation and 0.7 volumes of cold ethanol were added followed by the precipitation of the nucleic acids by overnight incubation at -20 C. The ethanol precipitate was collected by centrifugation, resuspended in 0.1 M EDTA pH 8.0, and dialyzed overnight against repeated changes of TES (0.05 M Tris-HCl, 0.05 M NaCl, 5 mM EDTA, pH 8.0) at 4 C. The resulting dialysate was made 50% cesium chloride by the addition of 8 g of cesium chloride to 8 ml of the dialysate and 0.6 ml of a 10 mg/ml solution of ethidium bromide was added. The solution was then centrifuged at 35,000 rpm for 60 hours in a Beckman Type 40 rotor at 20 C to separate covalently closed circular (CCC) DNA from open-circle or

linear DNA (121). The lower CCC DNA band was then identified under long-wave ultraviolet light and removed from the tube by either collecting drops from the bottom of the tube or by withdrawing the CCC band by insertion of a hypodermic needle through the side of the tube. The ethidium bromide was removed from the CCC DNA by extraction with isoamyl alcohol and dialysis against 0.25 mM EDTA, pH 8.0 at 4 C removed the isoamyl alcohol. The DNA was then precipitated at - 70 C by the addition of 0.1 volume of sterile 3 M sodium acetate (pH 5.2) and an equal volume of isopropyl alcohol. The DNA-salt-alcohol solution was thoroughly mixed and allowed to stand at -70 C at least 2 hours. After precipitation, the DNA was pelleted by centrifugation for 10 minutes in a microfuge (Fisher), the alcohol removed and the pellet dried under forced air. The DNA pellet was resuspended in 10 mM Tris-1 mM EDTA, pH 8.0 and stored at 4 C. The DNA concentration was determined by A_{260} using 0.022 as the extinction coefficient.

DNA Transfer From Agarose Gels to Nitrocellulose Paper. The DNA was transferred from the agarose gel to nitrocellulose paper (Schleicher & Schuell, type BA85) by the method of Southern (129), using the modification of Wahl et al which allowed for the efficient transfer of large DNA fragments (139). The agarose gel was soaked in 0.25 M HCl for 15 minutes to achieve partial depurination of the DNA in the gel. the gel was then transferred to a solution of 1.5 M NaCl, 0.5 M NaOH for 30 minutes, changing the solution at

least once, to denature the DNA in the gel. Following denaturation, the gel was transferred to 3 M NaCl, 0.5 M Tris-HCl, pH 7.4 for 45 - 60 minutes for neutralization, changing the solution every 15 minutes. The agarose gel was then transferred to a stack of filter paper (Whatman 3MM) previously saturated in 20 X SSC (3.0 M NaCl, 0.3 M sodium citrate, pH 8.0) in a large baking dish for blotting.

A piece of nitrocellulose filter cut 1 mm longer and 1 mm wider than the gel to be blotted was floated on water until the filter was wetted. The filter was then soaked in 20 X SSC until use, at which time it was carefully layered on top of the gel to be blotted such that no air bubbles were trapped between the gel and the filter. Cut strips of Saran Wrap were then placed on the 3MM filter paper, around the edges of the gel, so that as 20 X SSC was drawn up from the reservoir it passed through the gel. On top of the filter was placed 6-8 pieces of 3MM filter paper cut 0.5 mm shorter and narrower than the nitrocellulose filter. A 6 cm stack of paper towels cut the same size as the 3MM filter paper was placed on top of the 3MM filter paper. A glass plate longer and wider than the gel was placed on top of the stack of paper towels and the stack, along with the glass plate, was wrapped in Saran Wrap to prevent dehydration. A 500 g weight was placed on the stack and the blotting was allowed to continue until the stack of paper towels was soaked, up to 24 hours. More 20 X SSC was added to the baking dish as necessary.

After the DNA transfer had been completed, the 3MM filter paper and the paper towels were removed and the nitrocellulose filter was placed on a dry sheet of 3MM filter paper with the gel side facing up. The wells of the gel were then marked on the nitrocellulose with a blue ball-point pen. The gel was removed from the nitrocellulose filter, restained with ethidium bromide and visualized under ultraviolet light to ensure that the DNA transfer was complete. The nitrocellulose filter was then transferred to 6 X SSC and allowed to soak 5 minutes after which time the filter was placed onto paper towels and allowed to dry at room temperature.

When the nitrocellulose filter was dry, it was placed between 3MM filter paper and baked at 80 C for 2 two hours in a vacuum oven equipped with an ethanol-dry ice water trap. At the end of two hours, the filter was removed from the oven, allowed to cool, and wrapped in aluminum foil until use, at which time the foil was removed and the filter rebaked for 30 minutes.

α-^{32}P Labeling of DNA by Nick Translation. Purified plasmid DNA was labeled with ^{32}P by nick translation using E. coli DNA polymerase I (93, 123). Prior to use, deoxyribonuclease I (DNAse, Sigma) was made as a stock solution of 1 ug/ul in 50 mM Tris-Hcl, pH 7.5, 1 mM $MgSO_4$ 1 mM dithiothreitol (DTT), 50% glycerol, and stored at -20 C. For use, the DNAse stock was diluted 1:200 twice with DNAse dilution buffer (50 mM Tris-HCl, pH 7.5, 10 mM

$MgSO_4$, 1 mM DTT, and 50 ug/ml bovine serum albumin (BSA) (Sigma, Fraction V). When a single ^{32}P-labeled deoxynucleosidetriphosphate was to be incorporated into the DNA, either 2'-deoxycytidine 5'-[α-^{32}P] triphosphate, triethylammonium salt (^{32}P-dCTP) or 2'-deoxyadenosine 5'[α-^{32}P] triphosphate, triethylammonium salt (^{32}P-dATP) (Amersham-Searle) were used. When a double label of deoxynucleosidetriphosphates were to be incorporated into the DNA, both ^{32}P-dCTP and ^{32}P-dATP were used. α-^{32}P-deoxynucleosidetriphosphates were used as either a 50% aqueous ethanol or aqueous solution. Deoxynucleoside triphosphates (dNTP's) (PL Biochemicals, Inc.), 2'-deoxyadenosine 5'-triphosphate, disodium salt (dATP), 2'-deoxycytidine 5'-triphosphate, sodium (dCTP), 2'-deoxyguanosine 5'-triphosphate, sodium (dGTP), and 2'-deoxythymidine 5'-triphosphate, sodium (dGTP), were dissolved in sterile water and stored at -20 C as 500 uM stocks. From the dNTP stocks, a 2 X dNTP mix was prepared for use in the nick translation reaction. The 2 X dNTP mix consisted of 10 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5, 50 uM dXTP (where X = the unlabeled deoxynucleoside triphosphates), and 10 uM dYTP (where Y = the ^{32}P-deoxynucleoside triphosphates). The 2 X dNTP reaction mixture was stored at -20 C.

For the nick translation reaction, 100 uCi of either ^{32}P-dATP or ^{32}P-dCTP, or both, was placed on ice and 25 ul of the 2 X dNTP mix, 0.5 ug of purified plasmid DNA, and

sterile water was added to it to bring the volume up to 49 ul. The DNA-^{32}P-dNTP mixture was then incubated for 15 minutes on ice. If the ^{32}P-dNTP was a 50 % aqueous ethanol solution, the liquid was removed prior to using the label. To dry down the label, 100 uCi of ^{32}P - dNTP was added to a sterile microfuge tube which had been previously washed with ethanol and dried. The tube was then covered with Parafilm and the Parafilm was punctured 3-4 times with a needle. The covered microfuge tube was then placed in a 13 X 100 mm test tube rack and the labeled dNTP dried down under vacuum at room temperature. If the ^{32}P-dNTP was supplied as an aqueous solution, it was not necessary to dry down the label prior to use in the nick translation reaction.

Before addition to the reaction mixture, the DNAse stock was diluted 1 ul to 200 ul twice with the DNAse dilution buffer. One ul of the diluted DNAse was then added to the DNA-^{32}P-dNTP mixture along with 0.4 ul of E. coli DNA Polymerase I (Bethesda Research Laboratories, Inc.), and the reaction mixture was incubated for 3 hours at 14 C. At the end of the 3 hour incubation, the reaction mixture was heated for 10 minutes at 65 C to stop the reaction.

Recovery of Labeled DNA and Test for the Incorporation of ^{32}P into DNA. DNA which had incorporated ^{32}P-dNTP was separated from the unincorporated ^{32}P-dNTP by chromatography through a Bio-Gel P60 column. Bio-Gel P60, 100-200 mesh, (Bio-Rad Laboratories) was swelled by either boiling or autoclaving in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5. After

swelling, the gel was allowed to settle and the fines were poured off. The gel was then resuspended in a equal volume of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.4% SDS. The gel was then poured up to the "crimp" of a sterile 5 3/4 inches Pasteur pipette which had sterile glass wool inserted into the tip.

One ul of the nick translation mix was removed and added to 9 ul of water for determination of the percent ^{32}P incorporation into the DNA. The remainder of the nick translation mix was carefully loaded onto the top of the gel and the drops were collected in sterile 1.5 ml microfuge tubes. The ^{32}P-labeled DNA was eluted from the column with 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 in 200 ul fractions collected from the column in sterile microfuge tubes. The radioactivity of each fraction was monitored with a hand held Geiger counter and fractions were collected until two radioactive peaks could be detected. The three tubes that comprised the first peak were pooled and saved as the labeled DNA fraction. The radioactive material in the second peak was the unincorporated ^{32}P-dNTP. To test for the percent incorporation of ^{32}P-dNTP into the DNA, 2.5 ul from the 1:10 dilution of the nick translation reaction mix was spotted onto each of two Whatman DE81 2.4 cm diameter filters. One filter was placed in a beaker and washed 5 times in 5-10 ml of 5% Na_2HPO_4, 5 minutes per wash. After the sodium phosphate dibasic washes, the filter was allowed to dry on a paper towel, and both the washed and unwashed

filters were placed in scintillation vials with 10-15 ml of water, and the amount of radioactivity was determined in a scintillation counter. The percent ^{32}P-incorporation was determined by comparing the counts-per-minute (cpm) on the washed filter to the cpm on the unwashed filter. The total cpm of the labeled DNA was determined by similarly spotting 2.5 ul of the pooled first peak material onto each of 2 DE81 filters, determining the cpm after washing, and calculating the total cpm after accounting for the total volume of the pooled first peak material.

DNA Hybridization. DNA hybridizations were performed under aqueous conditions by the method of Southern (5, 129). The nitrocellulose filters containing the DNA fragments to be identified by hybridization to ^{32}P-labeled DNA were placed in boilable, sealable food storage bags (Sears, Seal-N-Save Boilable Cooking Pouch Material) and doubly heat sealed (Sears, Seal-N-Save Heat Sealer) so that the bag was 0.5-1.0 cm larger on three sides and about 2 cm larger on the fourth side. The filters were then prehybridized for 1 hour in a 65 C water bath with 100 ul of prehybridization solution per cm^2 of filter. The prehybridization solution was added to the hybridization bag by removing a corner from the end of the hybridization bag containing the extra space between the filter and the bag, and adding the prehybridization solution with a sterile Pasteur pipet using a 1 ml sterile disposable micropipet tip as a funnel. Air bubbles were then removed from the hybridization bag by

squeezing them out through the opening in the bag, and the hybridization bag was doubly sealed as before.

The prehybridization solution contained 5 X Denhardt's reagent (34), (0.02% Ficoll Type 400 (Pharmacia), 0.02% polyvinylpyrollidone-40,000 (Sigma), 0.02% bovine serum albumin (BSA) (Sigma, Fraction V)), 5 X SSPE, (0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA, pH 7.0) and 150 ug/ml heterologous denatured DNA. Denhardt's reagent was stored filtered sterilized as either a 20 X or 50 X stock. SSPE was autoclaved before storing as a 20 X stock. To make 20 X SSPE, 210 g of NaCl, 27.6 g of NaH_2PO_4, and 7.4 g of Na_2-EDTA were added per liter and the pH was adjusted to 7.0 with 6 N NaOH (93). The heterologous DNA, either calf thymus (Sigma, Type I) or salmon sperm (Sigma, Type III), was prepared as a 1 mg/ml solution by allowing the DNA to dissolve overnight in sterile distilled water. The following day the DNA was sheared by passing the solution through an 18-20 guage sterile hypodermic needle until the solution was homogeneous. The heterologous DNA solution was denatured before storage at 4 C by placing in a boiling water bath for 10 minutes, followed by quick cooling on ice. Just prior to use, the heterologous DNA solution was re-denatured by placing in a boiling water bath for 5 minutes and quick cooling on ice (93).

At the end of the prehybridization period, the hybridization bag containing the hybridization filter was removed from the 65 C water bath and the prehybridization

solution was squeezed out of the hybridization bag through a hole by removing the second corner from the long end of the hybridization bag. The hybridization solution, containing 5 X SSPE, 2 X Denhardt's, and 100 ug/ml heterologous DNA per cm^2 of filter, was then added in the same fashion as the prehybridization solution. The hybridization probe, containing 2 X 10^6 cpm prepared by nick translation, was added last and the hybridization bag was again doubly sealed as before. The hybridization continued for 18-72 hours in a 65 C water bath.

At the end of the hybridization period, the filter was cut from the hybridization bag with a razor blade and washed under conditions of "moderate" stringency (5). This consisted of 2 washes of 15 minutes each with 1 ml/cm^2 of 2 X SSPE, 0.1% sodium dodecyl sulfate (SDS), followed by 2 washes of 15 minutes each with 1 ml/cm^2 of 0.1 X SSPE, 0.1% SDS. The SSPE was used from a 20-50 X stock and the SDS was used from a solution of 20% SDS. After washing, the filters were allow to dry on paper towels at room temperature. The dry filters were then mounted on glass with cellophane tape and then both glass plate and filter were wrapped in Saran Wrap® prior to autoradiography.

Autoradiography. The glass plate containing the hybridized filter was placed in a Kodak X-ray Exposure holder with the filter side of the glass plate facing up. In a dark room under red or yellow safe lights, one piece of X-ray film (Kodak XAR-5) was placed on top of the glass plate.

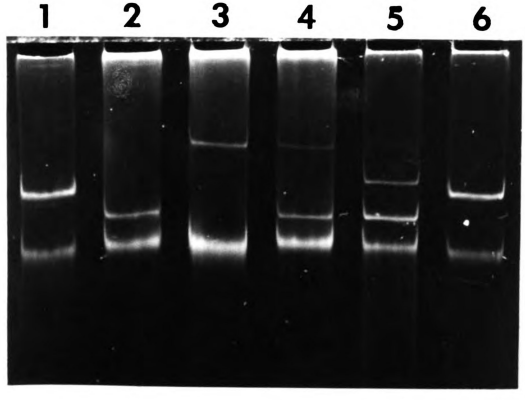
On top of the X-ray film was then placed one intensifying screen (Dupont, Cronex-Xtra Life, Lightning-Plus) such that the side with the taped margins faced up. The X-ray holder was then closed and sealed, wrapped in aluminum foil and clamped firmly shut with a large binder clip on each side of the cassette. The foil wrapped cassette was then placed in a -70 C freezer for 3 hours to 1 week for exposure of the X-ray film. After exposure, the X-ray film was immediately removed from the cassette in the dark room and developed in Kodak D-19 developer for 1-2 minutes, transferred to Kodak Rapid-Fix for 2-5 minutes and then thoroughly washed with tap water before being hung up to dry.

Results:

Previous experiments (see Chapter 1) had shown that when Pseudomonas syringae PSSD211 (pRD1) was recovered from storage or was maintained under the appropriate growth conditions, three different phenotypes could be recovered. The first of these phenotypes appeared to contain a intact plasmid pRD1 in that bacteria of this phenotype were resistant to kanamycin and were phenotypically histidine prototrophic (Kan^r His^+). The second phenotype appeared to be cured of plasmid pRD1 in that they were sensitive to kanamycin and required histidine supplementation for growth on a minimal medium (Kan^s His^-). The third recovered phenotype were resistant to kanamycin but required histidine supplementation for growth on a minimal medium (Kan^r His^-).

When agarose gel electrophoresis of cleared lysates from cultures of the three recovered phenotypes was performed, the following results were obtained (Figure 1). In cultures of the first phenotype, His^+ Kan^r, a plasmid corresponding in size to pRD1 could be detected along with the resident plasmid of PSSD211. In the apparently pRD1-cured cultures of the second phenotype, no plasmid other than the resident plasmid of PSSD211 could be detected. In cultures of the third phenotype, His^- Kan^r, two plasmids could be detected. One plasmid that could be detected was the resident plasmid of PSSD211, while the second plasmid did not correspond in size to either the resident plasmid in PSSD211 or to pRD1. Based on its relative mobility through

Figure 1. Determination of pRD1 in Pseudomonas syringae PSSD211 by agarose gel electrophoresis. Lane 1 and 6, P. aeruginosa PAO8 (R68.45); lane 2, P. syringae PSSD211; lane 3, E. coli JC5466 (pRD1); lane 4, P. syringae PSSD211 (pRD1); lane 5, P. syringae PSSD211 (His-deleted pRD1). Cultures of PSSD211 were phenotypically His^- Kan^S, cultures of PSSD211 (pRD1) were phenotypically His^+ Kan^r, and cultures of PSSD211 (His-deleted pRD1) were phenotypically His^- Kan^r.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0.7% agarose, this new plasmid was a little larger than the 58.2 kb IncP-1 antibiotic resistance plasmid R68.45 (88, 122, 147). It appeared that this new plasmid resulted from pRD1 by deletion of at least the histidine region found on pRD1.

Plasmid pRD1 was transferred from E. coli JC5466 (pRD1) to P. syringae PSSD220, a naturally occurring plasmidless variant of PSSD211. When PSSD220 (pRD1) clones were successively subcultured on a medium that selected for both the pRD1-determined Kan^r and His^+, pRD1 was gradually lost as an extrachromosomal element as detected by agarose gel electrophoresis of cleared lysates of these cultures, even though both kanamycin resistance and histidine prototrophy were still expressed. After three successive transfers on a medium that selected for pRD1-determined Km^r and His^+, pRD1 could only be detected in half of the PSSD220 (pRD1) cultures tested (Figure 2, lanes 2, 5, 6, and 7). In the others, pRD1 could not be detected even though the pRD1-determined Km^r and His^+ were still expressed (Figure 2, lanes 1, 2, 4, and 8). After four successive transfers on a medium that selected for the pRD1-determined Km^r His^+, pRD1 could not be detected by agarose gel electrophoresis in any of the PSSD220 (pRD1) cultures tested (Figure 3).

To determine whether or not the inabililty to detect pRD1 as an extrachromosomal element in these PSSD220 (pRD1) cultures was a result of the integration of pRD1 into the chromosome of PSSD220, PSSD220 (pRD1) clones that expressed

Figure 2. Agarose gel electrophoresis of Pseudomonas syringae PSSD220 (pRD1) transconjugants after 3 successive transfers on a medium that selected for the pRD1-determined kanamycin resistance and histidine prototrophy. Plasmids were detected by the cleared lysate method of Kado and Liu (72). The PSSD220 (pRD1) transconjugants, after mating with E. coli JC5466 (pRD1) as the donor and P. syringae PSSD220 as the recipient, were successively transferred onto a minimal medium plus methionine plus kanamycin before screening for plasmid content. Lanes 1-8, P. syringae PSSD220 (pRD1); lane 9, E. coli JC5466 (pRD1); lane 10, P. syringae PSSD220.

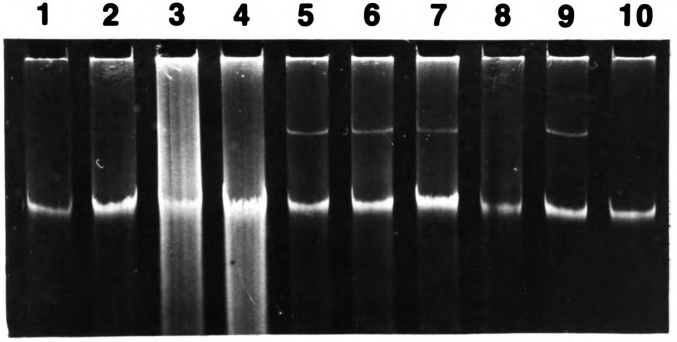
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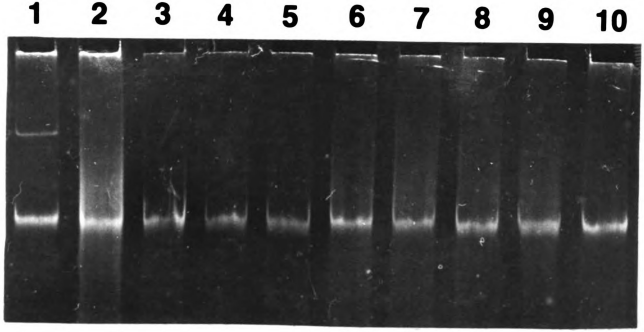
Figure 3. Agarose gel electrophoresis of Pseudomonas syringae PSSD220 (pRD1) transconjugants after 4 successive transfers on a medium that selected for the pRD1-determined kanamycin resistance and histidine prototrophy. Plasmids were detected by the cleared lysate method of Kado and Liu (72). The PSSD220 (pRD1) transconjugants, after mating with E. coli JC5466 (pRD1) as the donor and P. syringae PSSD220 as the recipient, were successively transferred onto a minimal medium plus methionine plus kanamycin before screening for plasmid content. Lanes 1-8, P. syringae PSSD220 (pRD1); lane 9, E. coli JC5466 (pRD1); lane 10, P. syringae PSSD220.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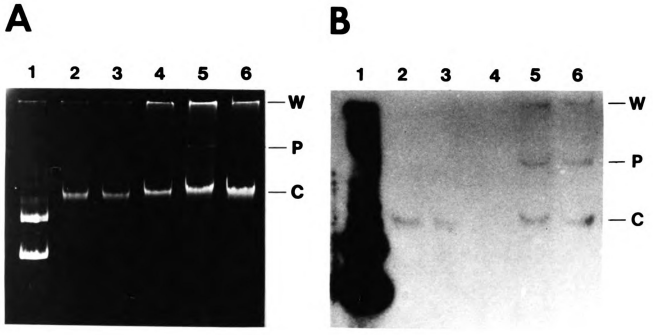
the pRD1-determined Km^r His^+ were first screened for plasmid content by agarose gel electrophoresis. The DNA in the gels was then transferred to nitrocellulose and hybridized to ^{32}P-labeled pSA30 DNA (Figure 4). Plasmid pSA30 is a recombinant plasmid that was constructed from an *E. coli* miniplasmid (22) to contain the *K. pneumoniae* *nif*KDH genes, the structural genes for nitrogenase (19). As a hybridization probe, pSA30 was expected to contain homologous sequences unique to pRD1 and not contain sequences that would be homologous to PSSD220 chromosomal DNA. Plasmid pRD1 was not chosen as the hybridization probe for the reason that it contained the *K. pneumoniae* histidine operon which functioned in PSSD220 to complement histidine mutations. As such, it would not have been unexpected to find sequence homology between the pRD1 histidine genes and the histidine genes found on the PSSD220 chromosome.

The DNA from PSSD220 (pRD1) cultures in which pRD1 was detected extrachromosomally by agarose gel electrophoresis hybridized ^{32}P-labeled pSA30 DNA to both the plasmid and chromosomal bands (Figure 4B, lanes 4 and 5). The DNA from PSSD220 (pRD1) cultures in which pRD1 could not be detected extrachromosomally but still expressed the pRD1-determined Km^r and His^+ hybridized ^{32}P-labeled pSA30 DNA only to the chromosomal band (Figure 4B, lanes 1 and 2). ^{32}P-labeled pSA30 DNA did not hybridize to PSSD220 DNA under these conditions (Figure 4B, lane 3), indicating that there is no homology between pSA30 and *P. syringae* chromosomal DNA.

Figure 4. Detection of pRD1 in Pseudomonas syringae PSSD220 clones by agarose gel electrophoresis and hybridization to pSA30. (A) Plasmid detection by agarose gel electrophoresis was by the method of Kado and Liu (72). Lane 1, pSA30; lanes 2 and 3, PSSD220 (pRD1) transconjugants after four successive transfers on a medium that selected for the pRD1-determined kanamycin resistance (Km^r and histidine prototrophy (His^+); lane 4, PSSD220; lane 5, PSSD220 (pRD1) transconjugant after three successive transfers on a medium that selected for the pRD1-determined Km^r His^+; lane 6, PSSD220 (pRD1) transconjugant after one transfer on a non-selective medium. A plasmid band corresponding to pRD1 was only detected in lanes 5 and 6. (B) Corresponding autoradiogram of the gel in (A) after hybridization to ^{32}P-labeled pSA30 DNA. pSA30 DNA hybridized to the plasmid band in lanes 5 and 6, and also hybridized to the chromosomal band in lanes 2, 3, 5, and 6. pSA30 DNA did not hybridize to the PSSD220 DNA in lane 4. Abbreviations: P, plasmid band; C, chromosomal band; and W, wells in which the samples were loaded.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1 2 3 4 5 6
W
P
C

These results indicate that the nifKDH genes on pSA30 can be used to locate the pRD1 nifKDH genes within the region of the gel that contain the chromosomal DNA of P. syringae PSSD220. These results gave evidence that pRD1 was no longer detectable as an extrachromosomal element in PSSD220 (pRD1), even though pRD1 determined Km^r His^+ were still expressed, due to the integration of pRD1 into the chromosome of PSSD220. Furthermore, the hybridization of pSA30 to what corresponds to both the chromosomal region and the plasmid region of the gel indicated that pRD1 had integrated into the chromosome even when it still existed as an extrachromosomal element.

To determine whether the histidine genes on pRD1 hybridized to PSSD220 chromosomal DNA, PSSD220 (pRD1) transconjugants were successively transferred on MA plus kanamycin and methionine to select for the pRD1-determined Km^r His^+ phenotype. After each transfer, the PSSD220 (pRD1) cultures were screened for the presence of pRD1 as an extrachromosomal element by agarose gel electrophoresis of cleared lysates. When pRD1 could not be detected extrachromosomally, the DNA was transferred to nitrocellulose and hybridized to nick translated pRD1 DNA.

The ability to detect pRD1 extrachromosomally in agarose gels by ethidium bromide staining after repeated transfers on a medium that selected for the pRD1-determined Km^r His^+ was marginal at best. Of the eight transconjugants originally transferred, pRD1 could still faintly be detected

in four (Figure 5, lanes 3, 4, 5, and 8), and possibly in two others (Figure 5, lanes 2 and 6). Plasmid pRD1 could not, however, be detected in the remaining two (Figure 5, lanes 7 and 9). After hybridization to ^{32}P-labeled pRD1 DNA, pRD1 could not be detected extrachromosomally in those cultures previously identified as lacking extrachromosomal pRD1 (Figure 6, lanes 7 and 9). Furthermore, ^{32}P-labeled pRD1 DNA did not hybridize to the region where PSSD220 chromosomal DNA was located (Figure 6, lane 10). This result indicated that although the pRD1-borne *K. pneumoniae* histidine genes functioned in PSSD220, they were not sufficiently homologous to PSSD220 histidine genes to detect hybridization under the conditions used.

Since pRD1 is a chimeric plasmid under the replication and transfer functions of the IncP-1 antibiotic resistance plasmid RP4, the ability of a plasmid considered identical to RP4 to integrate into the PSSD220 chromosome was determined. *P. syringae* PSSD220 (RP1), previously isolated after mating *E. coli* HB101 (RP1) with *P. syringae* PSSD220, was transferred five times on CA plus kanamycin to select for the plasmid-determined kanamycin resistance. After each transfer, the PSSD220 (RP1) cultures were screened for extrachromosomal RP1 by agarose gel electrophoresis. At the end of the fifth transfer RP1 was still detected as an extrachromosomal element in PSSD220 (RP1). The DNA was then transferred to nitrocellulose and hybridized to ^{32}P-labeled pRD1 DNA. When ^{32}P-labeled pRD1 DNA was hybridized to the

Figure 5. Detection of plasmid pRD1 in Pseudomonas syringae PSSD220 transconjugants by ethidium bromide staining after agarose gel electrophoresis. (A) Plasmid detection by agarose gel electrophoresis was by the method of Kado and Liu (72). Lane 1, cleared lysate of PSSD220 (pRD1) transconjugant previously identified as containing pRD1 as an extrachromosomal element; lanes 2-9, cleared lysates of PSSD220 (pRD1) transconjugants after repeated transfers on a minimal medium plus methionine plus kanamycin to select for the pRD1-determined Km^r His^+; lane 10, cleared lysate of PSSD220. Plasmid pRD1 can be clearly detected in lane 1, faintly detected in lanes 3, 4, 5, and 8, possibly detected in lanes 2 and 6, and not detected in lanes 7, 9, and 10. Abbreviations: Km^r, plasmid-determined phenotypic resistance to kanamycin; His^+, phenotypic histidine prototrophy.

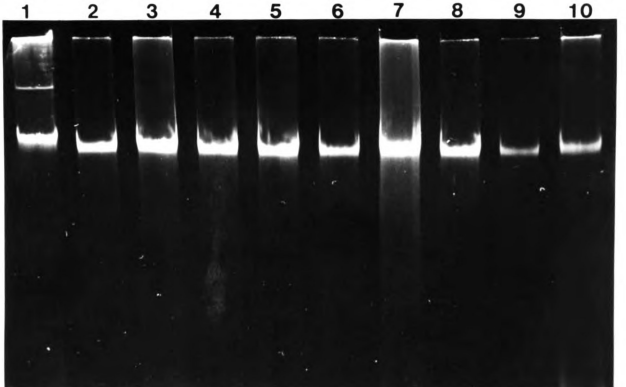
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Figure 6. Detection of plasmid pRD1 in Pseudomonas syringae PSSD220 (pRD1) transconjugants by hybridization to ^{32}P-labeled pRD1 DNA. Corresponding autoradiogram of the gel in Figure 5 after hybridization to ^{32}P-labeled pRD1 DNA. Lane 1, DNA from cleared lysate of PSSD220 (pRD1) transconjugant previously identified as containing pRD1 as an extrachromosomal element; lanes 2-9, DNA from cleared lysates of PSSD220 (pRD1) transconjugants after repeated transfers on a minimal medium plus methionine plus kanamycin to select for the pRD1-determined Km^r His^+; lane 10, DNA from cleared lysate of PSSD220. After hybridization to ^{32}P-labeled pRD1 DNA, pRD1 can clearly be detected as an extrachromosomal element in lanes 1, 3, 4, 5, and 8, faintly detected in lane 6, and possibly detected in lane 2. Plasmid pRD1 cannot be detected as an extrachromosomal element in lanes 7, 9, and 10. Abbreviations: Km^r, plasmid-borne phenotypic resistance to kanamycin; His^+, phenotypic histidine prototrophy.

DNA from cleared lysates from PSSD220 (RP1) cultures, the majority of the hybridizable counts appeared in the extrachromosomal band (Figure 7). In fact, the chromosomal band observed in Figure 7 was only detected after overexposure of the autoradiogram. This result indicated that RP1, unlike pRD1, was unable to integrate into the chromosome of P. syringae PSSD220.

To determine if the deletion plasmids of pRD1 that conferred the His^- Kan^r phenotype in PSSD220 existed both extrachromosomally and integrated into the chromosome, cleared lysates of PSSD220 (deleted-His pRD1) cultures were electrophoresed through 0.7% agarose gels, the DNA transferred to nitrocellulose and hybridized to ^{32}P-labeled pRD1 DNA. The resulting autoradiogram (Figure 8) shows the same pattern of hybridization as when PSSD220 (RP1) cultures were tested for the ability of RP1 to exist integrated into the chromosome of PSSD220. There was strong hybridization of ^{32}P-labeled pRD1 DNA to the plasmid region and only a faintly detectable amount of hybridization to the chromosomal region. This result suggested that like RP1, the deletion plasmids of pRD1 did not exist chromosomally integrated in PSSD220.

Figure 7. Hybridization of ^{32}P-labeled pRD1 DNA to the DNA from cleared lysates from Pseudomonas syringae PSSD220 (RP1). Autoradiogram of the hybridization of ^{32}P-labeled pRD1 DNA to the DNA from cleared lysates of PSSD220 (RP1) cultures after 5 successive transfers on a medium which selected for the RP1-determined kanamycin resistance (CA plus kanamycin). Cleared lysates were prepared by the method of Kado and Liu (101) and the DNA was separated by electrophoresis through vertical 0.7% agarose gels. Lane 1, PSSD220; lane 2-8, PSSD220 (RP1).

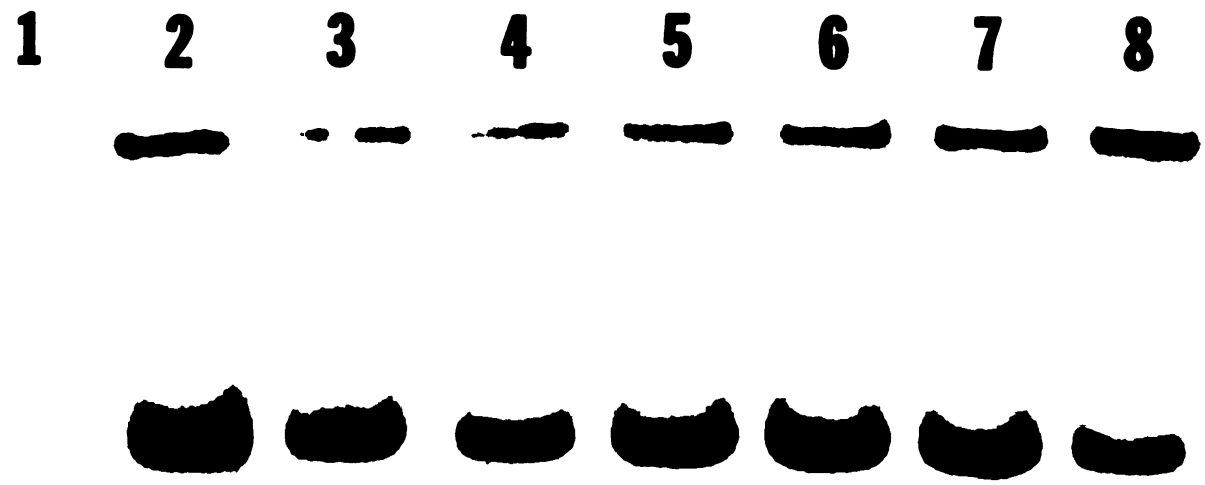
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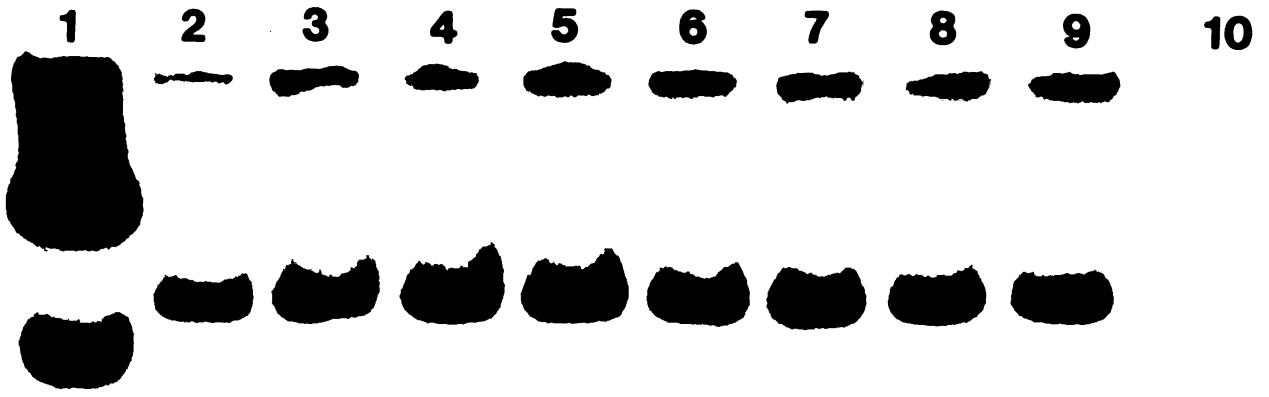
Figure 8. Hybridization of ^{32}P-labeled pRD1 DNA to the DNA from cleared lysates of Pseudomonas syringae PSSD220 cultures containing deletion plasmids of pRD1. Autoradiogram after hybridization of ^{32}P-labeled DNA to the DNA from cleared lysates from cultures of PSSD220 containing deletion plasmids of pRD1. Cleared lysates were prepared by the method of Kado and Liu (72) and the DNA was separated by electrophoresis through 0.7% vertical agarose gels. PSSD220 cultures containing deletion plasmids of pRD1 were identified from His^+ Kan^r PSSD220 (pRD1) colonies after transfers onto CA by their inability to grow on minimal medium without histidine supplementation while still being resistant to kanamycin. Lane 1, PSSD220 (pRD1); lanes 2-9, PSSD220 containing His^- Kan^r deletion plasmids of pRD1; lane 10, PSSD220.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Discussion. When P. syringae PSSD220 (pRD1) was continuously subcultured on a medium that selected for the pRD1-determined Km^r His^+, pRD1 could no longer be detected as an extrachromosomal element by agarose gel electrophoresis, even though the pRD1-determined kanamycin resistance and histidine prototrophy were still expressed. A similar result was observed with E. cloacae MF10 (pRD1), in which the pRD1-determined antibiotic resistances and nitrogen fixation genes were expressed but pRD1 was not detected by agarose electrophoresis (72).

To increase the sensitivity for the detection of extrachromosomal DNA, the DNA from cleared lysates of PSSD220 (pRD1) cultures was hybridized to ^{32}P-labeled DNA after electrophoresis through agarose gels. In these experiments there was hybridization consistently to the chromosomal region whether the hybridization probe was pSA30 or pRD1. This hybridization occurred even when pRD1 was detectable as an extrachromosomal element. These results indicated that pRD1 had integrated into the chromosome of the PSSD220 host.

Plasmid pSA30 was initially chosen for the hybridization probe since it contained the K. pneumoniae structural genes for nitrogenase, nifKDH, and as such was not expected to hybridize to the DNA from the non-nitrogen fixing P. syringae. In experiments where pSA30 was the hybridization probe, there was no hybridization to P. syringae chromosomal DNA. Subsequent hybridization

experiments used pRD1 as the hybridization probe. Since the histidine mutations in P. syringae PSSD220 were complemented by the K. pneumoniae histidine genes contained on pRD1, it was expected that the K. pneumoniae histidine genes would be sufficiently homologous to the P. syringae histidine genes to hybridize pRD1 DNA to P. syringae chromosomal DNA. It was therefore unexpected that no hybridization between pRD1 and P. syringae chromosomal DNA was detected.

It was previously shown that derivatives of the IncP-1 plasmid RP4 which are temperature sensitive for maintenance can mobilize the E. coli K12 chromosome for high frequency gene transfer, presumably by Tn 1 transposition to the chromosome followed by homologous recombination across Tn 1 (53). The role of DNA homology between plasmid and chromosome in IncP-1 plasmid-mediated gene transfer has previously been established with RP4-prime plasmids (6, 9, 46, 47, 48, 52, 71, 143). If DNA homology between plasmid and chromosome alone were sufficient to permit plasmid integration, then it would not be surprising that the histidine genes on pRD1 would supply that homology. In addition, plasmid pRD1 contains the transposable element Tn 1 which has been shown to be capable of mediating RP4 insertion into the E. coli chromosome (31). Plasmid pRD1 therefore carries within it the genetic potential to mediate its own chromosomal integration. That hybridization between pRD1 and the P. syringae chromosome

was not detected does not mean that homology between plasmid and chromosome did not exist, it merely means that any such homology that may exist could not be detected by those experimental conditions.

The chromosomal integration of the E. coli fertility plasmid F, a the model for plasmid integration and subsequent Hfr formation, differs from the integration of IncP-1 antibiotic resistance plasmids in at least one important characteristic. When F has integrated into the chromosome, no extrachromosomal copy of F can exist (91, 92, 126). As a result, it is rare that sex factor F is transferred from Hfr donors to F^- recipients. The IncP-1 plasmid RP4, however, can exist both integrated into the chromosome and extrachromosomally in the same cell (47, 48, 143). The results obtained by hybridization experiments in this study indicated that pRD1 could also exist both integrated and extrachromosomally in P. syringae PSSD220.

Using the criterion of strong hybridization to the chromosomal region of DNA obtained from cleared lysates, PSSD220 (RP1) was screened for the ability of RP1 to integrate into the PSSD220 chromosome. When ^{32}P-labeled pRD1 DNA was used as the hybridization probe in the above experiments, the pattern of hybridization was opposite that of PSSD220 (pRD1) cleared lysates. The majority of the hybridizable counts appeared in the plasmid region of the gel. Therefore, even though pRD1 was formed from the IncP-1 plasmid RP4, it appears that the capability to integrate

into the chromosome of PSSD220 does not extend to plasmids identical to RP4. This suggests that either the K. pneumoniae DNA that was inserted into RP4 is responsible for the ability to integrate, or, less likely, that the region of RP4 where the insertion of the K. pneumoniae DNA occurred contained a function that upon insertional inactivation would now allow the RP4 replicon to integrate.

When deletion plasmids of pRD1 were screened for their ability to integrate into the P. syringae chromosome by hybridization of ^{32}P-labeled pRD1 to the DNA from cleared lysates, the pattern of hybridization was similar to PSSD220 (RP1). This result suggested that like RP1, the deletion plasmids of pRD1 did not exist chromosomally integrated in PSSD220. It appears then that the DNA deleted from pRD1 was responsible for pRD1 integration into the PSSD220 chromosome. Either that or as a result of the deletion, the continuity of RP4 previously disrupted by insertion of the K. pneumoniae DNA was restored and the gene function previously inactivated was also restored.

Although pRD1 could be detected within the chromosomal DNA of PSSD220 (pRD1) cleared lysates whenever the pRD1-determined kanamycin resistance and phenotypic histidine prototrophy were expressed, no pRD1 mediated chromosomal gene transfer was detected. Without genetic evidence for pRD1 integration that would have been provided by linkage between plasmid and chromosomal markers, the evidence for its integration must rely on indirect physical evidence. The

large size of plasmid pRD1, about 150 kb, makes it a very real possibility that during the course of the preparation of cleared lysates of PSSD220 (pRD1), the plasmid was sheared. Once sheared, pRD1 DNA would migrate similarly to chromosomal DNA, which is invariably sheared. The other possible explanation for the appearance of pRD1 DNA among chromosomal DNA would be the formation of plasmid-folded chromosome complexes, which are speculated to form during chromosome replication (101). In this situation, covalently closed circular plasmid DNA comigrates with the chromosomal DNA and, by the detection methods used in this study, would appear to have integrated into the chromosome. The strong hybridization to the chromosomal region from cleared lysates from PSSD220 (pRD1) cultures as compared to the very weak hybridization to the chromosomal region from cleared lysates from PSSD220 (RP1) and PSSD220 (deleted-his pRD1) cultures do not appear to be explained by the above hypotheses. Both RP1 and the deletion plasmids of pRD1 are large plasmids of about 60 kb, and as such would be expected to shear or form plasmid-chromosome complexes similarly to pRD1. That there was little hybridization to the chromosomal DNA from PSSD220 cultures containing these plasmids indicates that pRD1 integrated into the PSSD220 chromosome while RP1 and the deletion plasmids of pRD1 did not. Furthermore, this suggests that since RP1 and the deletion plasmids of pRD1 are not integrated, the capability of pRD1 to integrate is a result of the insertion of *K. pneumoniae* DNA into RP4.

CHAPTER III

The Identification of Plasmid pRD1 Within The Chromosome of *Pseudomonas syringae* PSSD220.

CHAPTER III

The identification of plasmid pRD1 within the chromosome of *Pseudomonas syringae* PSSD220.

Abstract

Total DNA was isolated from *Pseudomonas syringae* pv. *syringae* PSSD220 (pRD1) after determining that pRD1 could not be detected extrachromosomally and that ^{32}P-labeled pRD1 hybridized to *P. syringae* PSSD220 (pRD1) chromosomal DNA. DNA restriction analysis of *P. syringae* PSSD220 (pRD1) total DNA and subsequent hybridization indicated that pRD1 integrated into the chromosome. Integration of pRD1 into the PSSD220 chromosome resulted in a deletion of pRD1 DNA which showed homology to plasmid RP4. The deletion included a region where RP4 Tra functions had previously been mapped.

Deletion plasmids of pRD1, identified in *P. syringae* PSSD220 by their inability to complement the histidine mutation of PSSD220, were isolated. Restriction endonuclease analysis of these deletion plasmids indicated that these plasmids had not formed as a result of excision of integrated pRD1 from the PSSD220 chromosome.

Introduction. The integrated state of the Escherichia coli fertility plasmid F was discovered by its ability to promote a frequency of recombination higher than that achieved by F^+ donors (14, 54, 85). E. coli cells previously infected with the coliphage Lambda, which were immune to superinfection by Lambda, show that Lambda was closely linked to the genes for galactose utilization (86, 150). In both of these cases, non-chromosomal covalently-closed-circular (CCC) DNA integrated into the chromosome of E. coli. It has been suggested that the integration of F is the result of recA dependent homologous recombination between insertion sequences (IS) found on both F and the chromosome (64, 65, 107, 108, 109). In the case of Lambda integration, a specific site for integration (att) is located on both Lambda and the chromosome, and the integration is not controlled by the bacterial homologous recombination system. The ability of Lambda to integrate into the chromosome is specified by the bacteriophage encoded int genes (43, 44, 128).

A temperature-sensitive replication mutant of the IncP-1 R plasmid RP4 has also been shown to integrate into the E. coli chromosome by recA-dependent homologous recombination in cells containing the chromosomally inserted transposable element Tn 1 (31). This transposable element is also located on RP4 and encodes for ampicillin resistance normally associated with RP4. When RP4 had integrated into the chromosome of E. coli, the RP4-containing donor strain

was able to transfer chromosomal DNA at a much higher frequency than when the donor strain did not contain an integrated RP4 (53, 143).

When plasmids capable of mediating chromosomal gene transfer have integrated into the chromosome of its host, these strains may be identified by their ability to transfer plasmid and chromosomal markers as one linkage group. However, the identification of chromosomally integrated plasmids when there is no linkage data available requires a different approach than that used to identify F and Lambda integrates. The Campbell model for lysogenic Lambda formation (18) has been the basis for the identification of excision plasmids in P. syringae pv. phaseolicola which contain P. syringae pv. phaseolicola chromosomal DNA (134).

The identification of chromosomally inserted pRD1 DNA within P. syringae pv. syringae PSSD220 genomic DNA was achieved by restriction endonuclease analysis and DNA hybridizations. Deletion plasmids of pRD1 were also analyzed to determined whether or not they had formed as a result of excision from the P. syringae pv syringae PSSD220 chromosome.

Materials and Methods:

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this study are listed in Table 1, Chapter 1.

Media. The media used to grow bacterial cultures was previously described in Chapter 1.

Plate mating and Selection. Plate matings and selection for transconjugants was as previously described in Chapter 1.

Identification of Potential pRD1 Integrates in P. syringae PSSD220. The method of identification of potential pRD1 integrates in P. syringae PSSD220 was described in Chapter 2.

Plasmid Isolation. A variation of the plasmid isolation method of Currier and Nester (30) which was used to isolate pRD1 and deletion plasmids of pRD1 was described in Chapter 2.

DNA Hybridization. The method for DNA transfer to nitrocellulose and hybridization was described in Chapter 2.

Restriction Endonuclease Digestion. Restriction endonucleases were purchased from Bethesda Research Laboratories and used in the manner prescribed by the manufacturer. All restriction endonucleases were titrated before use against the DNA on which they were to be used in order to determine the units of enzyme per ug of DNA which would ensure complete digestion in 1 hour at the prescribed temperature.

The bovine serum albumin (BSA) used in the restriction endonuclease digests was prepared as nuclease free BSA by the following method. A 50 mg/ml solution of BSA (Sigma Fraction V) in 100 mM Trizma-Base (Sigma) was stirred at 4 C until the BSA was dissolved. The BSA solution was placed in a boiling water bath for 15 minutes and then allowed to cool at room temperature. The alkalified BSA solution was then adjusted to pH 7.4 by the drop-by-drop addition of concentrated HCl and stored at 4 C until use. For use, the BSA solution was considered to be 50 mg/ml.

Isolation of P. syringae PSSD220 Genomic DNA. Genomic DNA was isolated by the method of Marmur (94). PSSD220 (pRD1) was sub-cultured as described in Chapter 2 until pRD1 could no longer be detected as an extrachromosomal element. The absence of pRD1 as an extrachromosomal element was then confirmed by hybridization of the DNA from cleared lysates of PSSD220 (pRD1) to ^{32}P-labeled pRD1 DNA. For genomic DNA isolation, PSSD220 (pRD1) in which pRD1 was not detected extrachromosomally was grown in 100 ml of minimal broth supplemented with methionine and kanamycin until a dense bacterial suspension was obtained (2-3 days). For the genomic isolation of PSSD220, the bacteria were grown overnight in 100 ml of complete broth. Bacterial cells for genomic DNA isolation were pelleted by centrifugation and resuspended in 5 ml of 50 mM Tris, 50 mM EDTA, pH 8.0. A 5 mg/ml solution of lysozyme (Sigma) in 0.5 ml of 50 mM Tris, 50 mM EDTA, pH 8.0 was predigested for 30 minutes in a 37 C

water and then added to the bacterial cell suspension to produce a final lysozyme concentration of 1 mg/ml. The bacterial cell suspension-lysozyme mixture was incubated on ice for 30 minutes followed by the addition of 1 ml of 0.5% sodium dodecyl sulfate (SDS), 50 mM Tris, 0.4 M EDTA, pH 7.4, 1 mg/ml predigested Pronase (see Chapter 2), and then heated for 15 minutes in a 50 C water bath. Six ml of distilled phenol saturated with 50 mM Tris-Hcl, pH 7.4 was then added and the subsequent solution was mixed by inversion until emulsified. After phenol extraction, the upper aqueous phase was separated by centrifugation and removed from the lower phenol phase. To the aqueous phase was added 0.6 ml of sterile 3 M sodium acetate and the solution was gently mixed by inversion. Exactly 2 volumes of cold 95% ethanol was then layered on top and the nucleic acids were collected from the interface by spooling on a sterile glass rod. Excess ethanol was removed from the nucleic acids by gentle squeezing. The nucleic acids were then dissolved in 5 ml of 50 mM Tris, 1 mM EDTA, pH7.5, 50 ug/ml ribonuclease (Sigma Type I-A) and incubated for 30 minutes in a 37 C water bath. The nucleic acid solution was then allowed to cool at room temperature and 5 ml of chloroform was added. The solution was mixed by inversion until emulsified and the upper layer was removed after centrifugation. The DNA was then recollected by spooling after the addition of 2 volumes of cold ethanol and stored in sterile 50 mM Tris, 1 mM EDTA, pH 7.0 at 4 C until use.

Agarose Gel Electrophoresis. Agarose gel electrophoresis of restriction endonuclease digested plasmid and genomic DNA was performed through 20-25 cm 0.5-0.7% horizontal agarose gels submerged in electrophoresis buffer (see Chapter 2) at a constant current of 40 mA for 16-24 hours. The agarose gels were stained and photographed as previously described (Chapter 2). Lambda HindIII restriction fragments (Bethesda Research Laboratories) were used as molecular weight standards. For size determination of plasmid pRD1 DNA restriction fragments, HindIII, SalI, and KpnI digests of purified RP4 DNA were used as molecular weight standards in addition to the Lambda HindIII standards. The sizes of the large pRD1 fragments were determined after electrophoresis through a 25 cm 0.5% agarose gel, while the sizes of the smaller pRD1 fragments were determined after electrophoresis through a 25 cm 1.5% agarose gel. Restriction fragments of total DNA from P. syringae PSSD220 (pRD1) were electrophoresed to achieve size separation and the determination of chromosomally integrated pRD1 was accomplished by hybridization of these fragments to ^{32}P-labeled pRD1 DNA. Purified pRD1 DNA, in an amount calculated to coincide with 1 copy of pRD1 per chromosome, was also electrophoresed to determine the number of copies of integrated pRD1 per chromosome. For these calculations, the P. syringae PSSD220 genome size was considered to be identical to that of E. coli, 4 X 10^3 kb.

Isolation of pRD1 deletion derivatives in P. syringae

PSSD220. The identification of P. syringae PSSD220 isolates containing deletion derivatives of plasmid pRD1 was by selection for those strains were no longer able to grow on a minimal medium without histidine supplementation. These isolates could also be identified by selection for kanamycin resistance on complete agar after mating with JC5466 (pRD1).

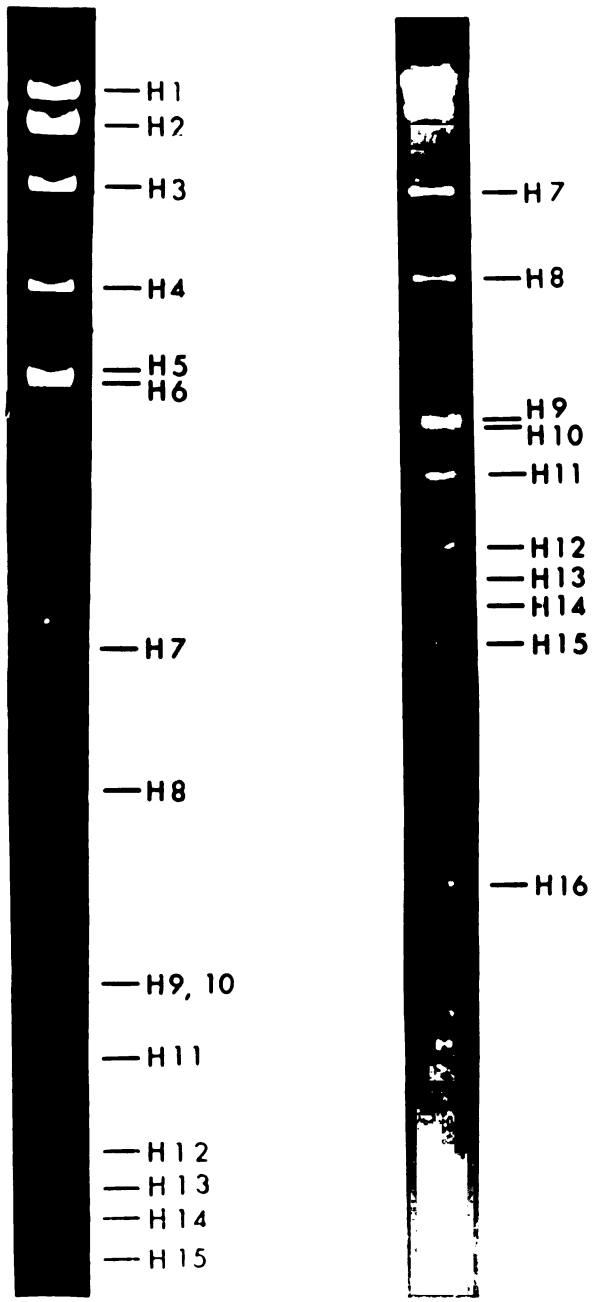
Results:

The emphasis of research concerning plasmid pRD1 had previously focused on the K. pneumoniae genes for nitrogen fixation that are found on pRD1. In fact, little study of pRD1 has been performed since these nitrogen fixation genes were sub-cloned from pRD1 onto multi-copy plasmid vectors in E. coli (118, 119). The results of these studies did, however, produce a restriction map of pRD1 for the restriction endonucleases EcoR1 and HindIII. The restriction pattern of isolated plasmid pRD1 after HindIII digestion was compared to the HindIII restriction pattern established by Puhler et al (119). HindIII digestion of purified plasmid pRD1 DNA produced the same number of restriction fragments, sixteen, that Puhler et al (118, 119) reported, although the size of these fragments differed from those previously reported. Figure 9 shows the result of HindIII digestion of purified pRD1 DNA and gives an estimation of the sizes of the resulting fragments. Size estimates of pRD1 HindIII fragments were obtained by using Lambda HindIII fragments and RP4 SalI, HindIII, and KpnI fragments (82) as molecular size standards. The size of pRD1 was calculated to be about 190 kb, much larger than the 150 kb previously reported (117).

The SalI restriction pattern of PSSD220 genomic DNA was compared to the SalI restriction pattern obtained from total DNA of PSSD220 (pRD1) cultures which expressed pRD1-determined histidine prototrophy and kanamycin resistance

Figure 9. Plasmid pRD1 Size Determination. The sizes of fragments H1, H2, H3, H4, H5, H6, H7, and H8 were determined after HindIII digestion and electrophoresis through 0.5% agarose. The sizes of the remaining HindIII fragments were determined after electrophoresis through 1.5% agarose. Molecular Size standards, in kb, were as follows: Lambda HindIII, 23.5, 9.7, 6.6, 4.3, 2.2, 2.1, 0.56; RP4 HindIII, 60; RP4 SalI, 40.1, 19.9; RP4 KpnI, 31, 14.5, 12.7, 1.8. Kb = kilobase pairs.

Fragment	kb
H1	53.7
H2	36.3
H3	24.8
H4	17.1
H5	12.5
H6	12.3
H7	7.0
H8	5.2
H9	3.6
H10	3.5
H11	3.2
H12	2.6
H13	2.5
H14	2.3
H15	2.1
H16	1.2


H1
H2
H3
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H5
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even though pRD1 could not be detected extrachromosomally (Figure 10). Restriction endonuclease digests of pRD1 by SalI produced fragments 1 and 2 could clearly be detected within the PSSD220 genomic background (Figure 10, PSSD220 (pRD1) SalI fragment 1 and 2). Fragment 3 from the same digest could not, however, be detected even though there were no interferring PSSD220 genomic DNA fragments which migrated to where pRD1 SalI fragment 3 should have been located. Furthermore, a SalI fragment that was not present in PSSD220 genomic DNA was detected in PSSD220 (pRD1) DNA (Figure 10, PSSD220 (pRD1) SalI fragment 5).

The SalI restriction patterns of PSSD220 (pRD1) DNA in which pRD1 had integrated (PSSD220 Ω pRD1) and pRD1 were compared after hybridization to ^{32}P-labeled pRD1 DNA. If the Campbell model for the integration of double stranded DNA into the chromosome was applicable to pRD1 integration into the PSSD220 chromosome, the following results would be expected. The new SalI fragment that resulted after pRD1 integration (Figure 10) would be one of the two border fragments of integrated pRD1 and the second border fragment should have been detectable in PSSD220 (pRD1) DNA after hybridization to ^{32}P-labeled pRD1 DNA. No second border fragment was detected using the above approach (Figure 11). In one isolate of PSSD220 Ω pRD1 no new SalI restriction fragment was detected. Furthermore, in each of the two isolated analyzed, the same two SalI fragments of pRD1 were undetectable when pRD1 had integrated into the PSSD220

Figure 10. Identification of chromosomally integrated pRD1 in Pseudomonas syringae PSSD220 (pRD1). P. syringae PSSD220 (pRD1) in which pRD1 could not be detected extrachromosomally, and P. syringae PSSD220 total DNA was isolated by the method of Marmur (94). Plasmid DNA was isolated by the method of Currier and Nester (30). DNA was digested with SalI and electrophoresed through 0.5% agarose. Lane 1, PSSD220; lane 2, PSSD220 Ω pRD1; lane 3, pRD1. Within the PSSD220 genomic background, pRD1 SalI fragments 1 and 2 can be detected while pRD1 SalI fragment 3 cannot. A new fragment, PSSD220 (pRD1) SalI fragment 5, not found in PSSD220 or pRD1 appears after pRD1 integration into the PSSD220 chromosome. pRD1 SalI fragments 1 and 2, and the new SalI fragment within PSSD220 are indicated by arrows.

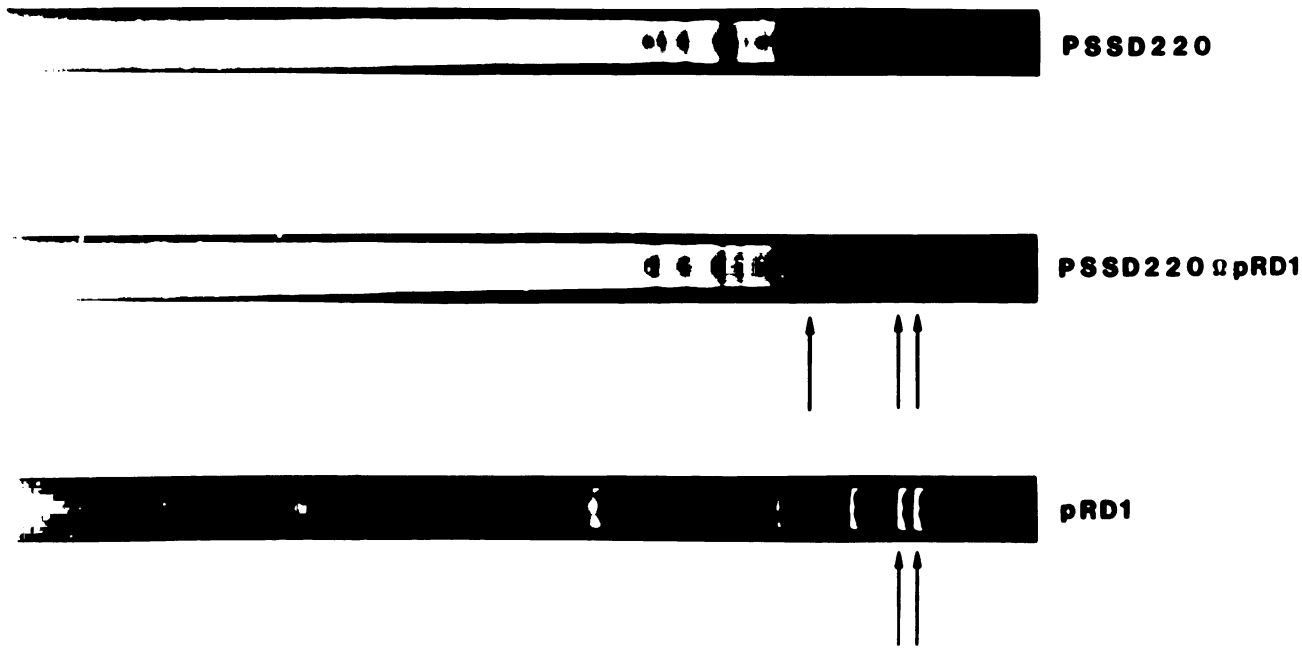
PSSD220
PSSD220ΩpRD1
pRD1

Figure 11. Comparison of integrated pRD1 in Pseudomonas syringae PSSD220 to purified pRD1 after SalI digestion. Total DNA from two isolates of P. syringae PSSD220 in which pRD1 had integrated was isolated by the method of Marmur (94), digested with SalI, electrophoresed alongside SalI digested purified pRD1 DNA (30), and subsequently hybridized to ^{32}P-pRD1. The amounts of PSSD220 (pRD1) and pRD1 DNA were adjusted so that the amount of pRD1 DNA approximated one copy per chromosome. Lanes 1A and 1B, P. syringae PSSD220 Ω pRD1; lanes 2A and 2B, pRD1. In lanes 2A and 2B, the arrows show pRD1 SalI fragments not present when pRD1 has integrated into the P. syringae PSSD220 chromosome. In lane 1B, the arrow shows the new SalI fragment which is present after pRD1 integration in P. syringae PSSD220. No new SalI fragment could be detected in lane 1A.

chromosome (Figure 11). Hybridization of ^{32}P-labeled pRD1 DNA to KpnI digested DNA from one of the cultures used for the SalI analysis also resulted in the loss of two pRD1 restriction fragments upon its integration. Furthermore, only one new fragment could be detected with PSSD220 chromosomal DNA (Figure 12).

The sizes of the restriction fragments that appeared to be involved in the integration of pRD1 into the PSSD220 chromosome were strikingly similar to restriction fragments of the IncP-1 antibiotic resistance plasmid RP4, one of the two plasmids from which pRD1 was formed (36). One of the two SalI fragments of pRD1 that were no longer present after pRD1 integration corresponded to the 19.9 kb SalI fragment of RP4 (Figure 13). The two KpnI fragments of pRD1 that wee no longer present after pRD1 integration corresponded to the 14.5 kb and 12.7 kb KpnI fragments of RP4 (Figure 13). To determine the extent of involvement of RP4 DNA in the integration of pRD1 into the PSSD220 chromosome, PSSD220 DNA, PSSD220 Ω pRD1 DNA, RP4 DNA, and pRD1 DNA were digested with SalI and hybridized to ^{32}P-labeled RP4 DNA. In each of the two cultures of PSSD220 with integrated pRD1 examined, it was clearly shown that the 19.9 kb SalI of RP4 was altered as a result of pRD1 integration into the PSSD220 chromosome (Figure 14). Similar to the results obtained after hybridization to ^{32}P-labeled pRD1 DNA, one new restriction fragment resulted after pRD1 integration. Using RP4 as the hybridization probe instead of pRD1 resulted in

Figure 12. Comparison of integrated pRD1 in Pseudomonas syringae PSSD220 to purified pRD1 after KpnI digestion. Total DNA from P. syringae PSSD220 in which pRD1 had integrated was isolated by the method of Marmur (94), digested with KpnI, electrophoresed alongside KpnI digested purified pRD1 DNA (30), and subsequently hybridized to ^{32}P-pRD1. The amounts of PSSD220 (pRD1) and pRD1 DNA were adjusted so that the amount of pRD1 DNA approximated one copy per chromosome. Lane 1, P. syringae PSSD220 Ω pRD1; lane 2, pRD1. The two pRD1 KpnI fragments that were not detected within PSSD220 chromosomal DNA are indicated by arrows in lane 2. The new KpnI fragment that resulted from pRD1 integration is indicated by an arrow in lane 1.

Figure 13. Comparison of SalI and KpnI restriction fragments from pRD1 and RP4. Purified plasmid RP4 and pRD1 DNA was isolated from E. coli by the method of Currier and Nester (30),digested with SalI or KpnI and electrophoresed through 0.5% agarose. Lane 1, RP4 DNA digested with KpnI; lane 2, pRD1 DNA digested with KpnI; lane 3, RP4 DNA digested with SalI; lane 4, pRD1 DNA digested with SalI. RP4 restriction fragments that appeared to remain intact in pRD1 are RP4 KpnI fragments 2, 3 and 4, and RP4 SalI fragment 2, and are indicated by arrows.

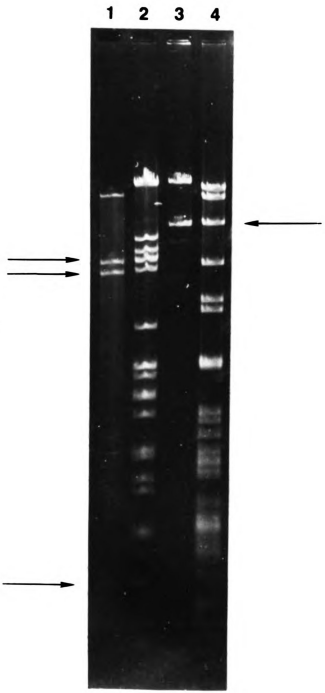
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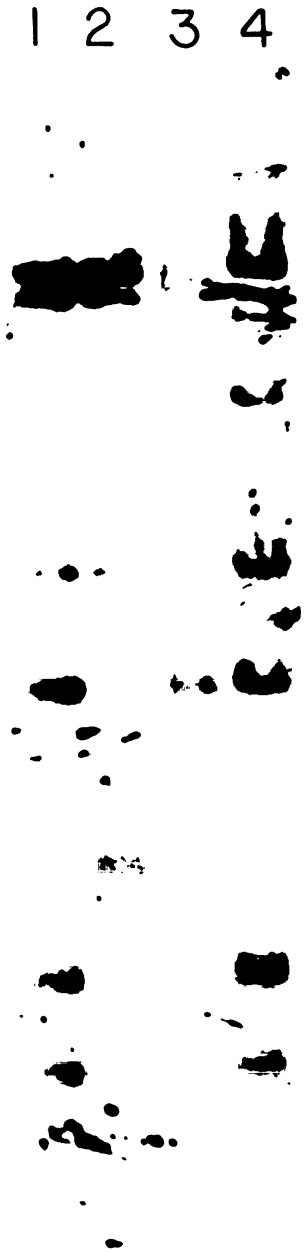
Figure 14. Hybridization of pRD1, RP4 and Pseudomonas syringae PSSD220 (pRD1) SalI digested DNA to ^{32}P-labeled RP4. Total DNA from P. syringae PSSD220 Ω pRD1 was isolated by the method of Marmur (94), digested with SalI, electrophoresed alongside SalI digested purified pRD1 and RP4 DNA (30), and subsequently hybridized to ^{32}P-RP4. The amounts of PSSD220 (pRD1) and pRD1 DNA were adjusted so that the amount of pRD1 DNA approximated one copy per chromosome. RP4 DNA was added in the same amount as pRD1 DNA. Lane 1, P. syringae PSSD220 lane 2, pRD1; lane 3, RP4; lanes 4 and 5, P. syringae PSSD220 Ω pRD1.

the ability to detect a new fragment formed after pRD1 integration in both isolates. Even when RP4 was the hybridization probe, no second pRD1 SalI fragment could be detected after pRD1 integration.

The restriction pattern obtained after hybridization of ^{32}P-labeled RP4 DNA to PstI digested pRD1 and RP4 DNA was compared to the restriction pattern obtained after similar hybridization to PstI digested DNA from two the PSSD220 (pRD1) isolates in which pRD1 had integrated into the chromosome (Figure 15). By comparing the patterns obtained from purified pRD1 and RP4 DNA, it can be clearly seen that RP4 PstI fragment 4 was not present in pRD1 after hybridization to ^{32}P-labeled RP4 DNA (Figure 15, lane 2). Furthermore, pRD1 contained three restriction fragments hybridizable to RP4 DNA that were not present in RP4 itself (Figure 15, lane 1). By comparing the patterns obtained from pRD1 and RP4 DNA to that of the two isolates of PSSD220 Ω pRD1, it can be seen that the second PstI fragment of both pRD1 and RP4 was absent when pRD1 had integrated into the PSSD220 chromosome. As was also the case when the DNA from these same isolates was digested with SalI and KpnI and hybridized to ^{32}P-labeled pRD1 DNA, an additional fragment appeared after pRD1 integration.

Sixteen transconjugants obtained after mating E. coli JC5466 (pRD1) with P. syringae PSSD220 were followed during continuous selection for resistance. These Kan^r transconjugants were also screened for their susceptibility to ampicillin and

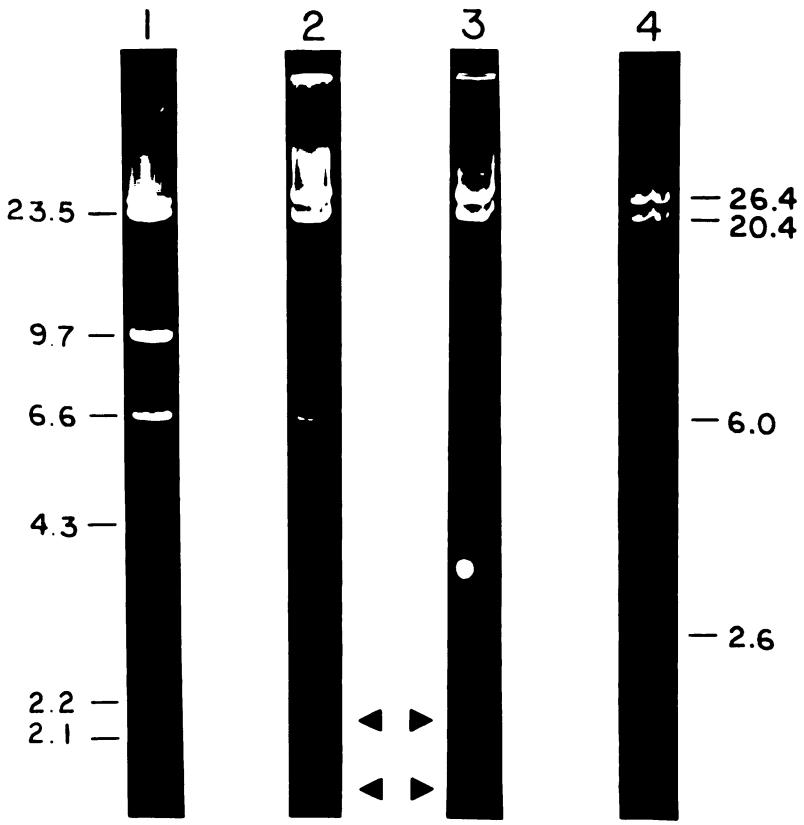
Figure 15. Hybridization of ^{32}P-labeled RP4 to PstI digested RP4, pRD1, and Pseudomonas syringae PSSD220 Ω pRD1. Total DNA from P. syringae PSSD220 in which pRD1 had integrated was isolated by the method of Marmur (94), digested with PstI, electrophoresed alongside PstI digested purified pRD1 and RP4 DNA (30), and subsequently hybridized to ^{32}P-RP4. The amounts of PSSD220 (pRD1) and pRD1 DNA were adjusted so that the amount of pRD1 DNA approximated one copy per chromosome. RP4 DNA was added in the same amount as pRD1 DNA. Lane 1, pRD1; lane 2, RP4; lanes 3 and 4, P. syringae PSSD220 Ω pRD1.


1 2 3 4

and tetracycline. All sixteen transconjugants were resistant to both antibiotics. The above transconjugants were also screened for their ability to grow on a minimal medium without histidine supplementation, a method used previously (Chapter 2) to identify deletion plasmids of pRD1 in PSSD220. All transconjugants selected for resistnce to kanamycin were unable to grow on a minimal medium which lacked histidine supplementation.

Plasmid DNA isolated from two isolates of PSSD220 containing pRD1 deletion plasmids, pJV421 and pJV521, was digested with PstI and compared to the RP4 PstI restriction pattern (Figure 16). Neither pJV421 or pJV521 contained a PstI fragment comparable to the 2.6 kb PstI fragment 4 of RP4. Both pJV421 and pJV521 did, however, contain two PstI fragments of about 2.15 and 1.8 kb each not found in RP4. Furthermore, pJV421 contained an additional PstI fragment of about 4.4 kb.

Figure 16. A comparison of the PstI restriction pattern of RP4 with two pRD1 deletion plasmids, pJV421 and pJV521, isolated from Pseudomonas syringae PSSD220. Plasmid RP4 was isolated from Escherichia coli and plasmids pJV421 and pJV521 were isolated from Pseudomonas syringae PSSD220 by the method of Currier and Nester (30). pJV421 and pJV521 were identified as deletion plasmids of pRD1 by their inability to complement the histidine mutation of P. syringae PSSD220 while still conferring resistance to kanamycin. Lane 1, Lambda HindIII size standards; lane 2, pJV521; lane 3, pJV421; lane 4, RP4. Two PstI fragments of pJV421 and pJV521 which were visible by ethidium bromide fluorescence but photographed poorly are indicated by arrows in lanes 2 and 3.


1
2
3
4
23.5
9.7
6.6
4.3
2.2
2.1
26.4
20.4
6.0
2.6

Discussion. When plasmid pRD1 could not be detected in P. syringae PSSD220 (pRD1) cultures expressing pRD1 markers, either by agarose gel electrophoresis or by hybridization of ^{32}P-labeled pRD1 DNA to the DNA from cleared lysates from these cultures, it was proposed that pRD1 had integrated into the PSSD220 chromosome. When total DNA from these cultures was digested with SalI, some of the pRD1 SalI fragments could be identified within the background of PSSD220 genomic DNA. pRD1 SalI fragment 3 could not, however, be detected either by agarose gel electrophoresis or by subsequent hybridization to ^{32}P-labeled pRD1 DNA. These results indicated that pRD1 had integrated into the PSSD220 chromosome through a crossover within pRD1 SalI fragment 3. Comparing the intensity of hybridization of ^{32}P-labeled pRD1 DNA to total DNA from these cultures with the intensity of hybridization to an amount of purified pRD1 determined to correspond to one copy of pRD1 per chromosome, it appeared that only one copy of integrated pRD1 existed per PSSD220 chromosome. The IncP-1 plasmids have been shown to maintain themselves at four to seven copies per chromosome in other hosts (37). It would be, therefore, reasonable to assume that pRD1 would be maintained at that same copy number in P. syringae PSSD220, unless integrated pRD1 was under chromosomal replication control, in which case one copy of pRD1 per chromosome would be expected.

The inability to detect pRD1 SalI fragment 3 within P. syringae PSSD220 (pRD1) genomic DNA indicated that pRD1 had

indeed integrated into the PSSD220 chromosome and that the Campbell model for Lambda integration might also be applicable to pRD1 integration. This model would predict that the restriction fragment that contained the region through which pRD1 integration occurred would no longer be detected as a fragment of its original size after pRD1 integration. The resulting pRD1 integration would split this restriction fragment into two pieces such that one piece would now be found at each end of the integrated pRD1, at the border between integrated pRD1 DNA and PSSD220 chromosomal DNA. Hybridization of ^{32}P-labeled pRD1 DNA to total DNA from PSSD220 Ω pRD1 isolates identified two pRD1 fragments which were lacking when pRD1 had integrated and the subsequent formation of only one fragment. Clearly pRD1 integration by a single crossover, as expected using the Campbell model, cannot explain these results.

Although these results did not clarify the mechanism by which pRD1 had integrated into the PSSD220 chromosome, they did indicate that as a result of integration, pRD1 suffered a deletion within the region of pRD1 that contained homology to RP4. This region was determined to be at least within the 19.9 kb SalI fragment of RP4. Because the second SalI fragment of pRD1 that disappeared after integration into the PSSD220 chromosome (approximately 4 kb) was not detected after hybridization to ^{32}P-labeled RP4 indicated that a portion of the Klebsiella pneumoniae DNA was also deleted after pRD1 integration.

The data obtained from the SalI digestions are inconsistent with the data obtained after KpnI digestion. The two KpnI fragments lost after pRD1 integration appear to be the contiguous 12.7 and 14.5 kb KpnI fragments of RP4 origin. The 19.9 kb SalI fragment of RP4 lies entirely within the above mentioned two KpnI fragments such that the common KpnI site almost perfectly bisects the SalI fragment. Since SalI cuts RP4 into only two fragments with the larger fragment remaining intact in pRD1, it would appear that the smaller fragment of pRD1 that disappears after pRD1 integration would be found within the RP4 DNA located within the 14.5 kb KpnI fragment.

Digestion of pRD1 with PstI followed by hybridization to ^{32}P-labeled RP4 DNA was used to identify the 2.6 kb PstI fragment of RP4 as the region of insertion of the K. pneumoniae DNA into RP4 during the formation of pRD1. By comparison of the above restriction pattern to the RP4 PstI restriction fragments, the 2.6 kb PstI fragment of RP4 was determined to be the one disrupted by insertion of K. pneumoniae DNA into RP4 to form pRD1. This fragment is located between 6 kb and 8.6 kb on the RP4 map (82) and is outside of the region of RP4 that appears to be involved in the integration of pRD1 into the PSSD220 chromosome. This region also coincides with the region of RP4 known to contain Tn 1, a transposable element that encodes resistance to ampicillin (7, 45).

Deletion formation in IncP-1 plasmids has been

frequently observed in relation to loss of enhanced chromosome mobilizing ability in R68.45 (29, 51). These deletions have extended into the Tra regions of R68.45 and appear to originate from IS21, the insertion sequence on R68 whose spontaneous duplication resulted in the phenotypic property of enhanced chromosome mobilization (136). On RP4, IS21 is located within the larger SalI fragment, along with the origin of transfer. The region containing this DNA appears to be intact in pRD1. Therefore the deletions observed after pRD1 integration in PSSD220 are not IS21 derived. At present, no insertion sequence is known within the region of pRD1 that is deleted after its integration. However, within that region are located the Tra 2 and Tra 3 regions. Previous studies on the conjugation systems of RP1, RP4, and RK2 have shown that deletions in the Tra 2 region results in the loss of self-transmissibility and resistance to the IncP-1 specific bacteriophages PRR1 and PRD1 (7, 8, 38, 144). When PSSD220 (pRD1) was used as the donor in intraspecific matings, no gene or plasmid transfer was detected (Chapter 1). In addition, PSSD220 (pRD1) was not susceptible to bacteriophages PRR1 and PRD1. The deletion of part of the Tra genes on pRD1 would explain bacteriophage resistance and the inability to detect plasmid transfer in those PSSD220 strains where the only copy of pRD1 in the cell had integrated. However, no plasmid or chromosomal gene transfer could be detected even when pRD1 could be detected extrachromosomally in PSSD220 (pRD1).

Clearly the loss of chromosomally-located plasmid Tra genes does not fully explain these results and other factors may be involved. These factors may include inefficient mating pair formation between P. syringae PSSD220 (pRD1) donors and P. syringae recipients. This conclusion seems reasonable since it has recently been shown that mobilization of both the E. coli and Rhizobium meliloti chromosomes required only the chromosomal insertion of the RK2 origin of transfer, oriT, and a functional RK2 replicon to initiate chromosomal transfer from oriT (150). That the RP4 oriT on pRD1 appears to be present in chromosomally integrated pRD1 implies that chromosome mobilization should be possible given the presence of a transfer proficient IncP-1 replicon in P. syringae PSSD220.

Deletion plasmids of pRD1 isolated from P. syringae PSSD220 still maintain the region deleted from pRD1 upon its integration into the chromosome. It is apparent then that these plasmids did not result from imprecise chromosomal excision of pRD1. The spontaneous degradation of pRD1 in recA$^+$ E. coli strains has previously been reported (117). This spontaneous degradation resulted in a 59 kb plasmid which had lost the his and nif genes of pRD1. It was speculated that the boundaries of the his-nif region contained homologous DNA. The region of insertion of the K. pneumoniae DNA in the formation of pRD1 from RP4 was identified in the present work to be within the Tn 1 region of RP4. Among the molecular rearrangements

associated with transposable elements is the duplication of the transposable element during cointegrate formation and fusion (for a review of transposable elements, see Kleckner (74)). The additional PstI fragments observed in pRD1 after hybridization to ^{32}P-labeled RP4 DNA, and the identification of the same fragments in pJV421 and pJV521 allow the following speculations. The additional PstI fragments appear to be the result of the duplication of Tn1 during the replicon fusion of RP4 and FN68 to form pRD1. A duplicated Tn 1 at both ends of the inserted K. pneumoniae DNA would then supply the necessary homology for the recA mediated recombination observed in the deletion of the his-nif region of pRD1. A duplicated Tn 1 would also explain the similarities in the RP4-hybridizable regions of pRD1 to pJV421 and pJV521.

The criterion for the identification of integrated plasmids from the DNA of cleared lysates, based on the relative intensities of hybridization of plasmid probes to plasmid and chromosomal regions, appears to be justified in the case of plasmid pRD1 in P. syringae PSSD220 (see Chapter 2). By this same criterion, RP4 and pRD1 deletion plasmids such as pJV421 and pJV521 had not integrated into the PSSD220 chromosome. The implication from these results is that RP4 DNA is not sufficient for integration in PSSD220 and that the ability of pRD1 to integrate is the result of the K. pneumoniae DNA present on pRD1.

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