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# The *Xanthomonas oryzae* pv*. oryzae raxP* and *raxQ* genes encode an ATP sulphurylase and adenosine-5′-phosphosulphate kinase that are required for AvrXa21 avirulence activity

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## Summary

***Xanthomonas oryzae* pv. *oryzae* (*Xoo*) Philippine race 6 (PR6) is unable to cause bacterial blight disease on rice lines containing the rice resistance gene *Xa21* but is virulent on non-*Xa21* rice lines, indicating that PR6 carries avirulence (*avrXa21*) determinants required for recognition by XA21. Here we show that two *Xoo* genes, *raxP* and *raxQ,* are required for AvrXa21 activity. *raxP* and *raxQ,* which reside in a genomic cluster of sulphur assimilation genes, encode an ATP sulphurylase and APS (adenosine-5′-phosphosulphate) kinase. These enzymes function together to produce activated forms of sulphate, APS and PAPS (3′-phosphoadenosine-5′-phosphosulphate). *Xoo* PR6 strains carrying disruptions in either gene, PR6Δ*raxP* or PR6Δ*raxQ*, are unable to produce APS and PAPS and are virulent on *Xa21*-containing rice lines. RaxP and RaxQ are similar to the bacterial symbiont *Sinorhizobium meliloti* host specificity proteins, NodP and NodQ and the *Escherichia coli* cysteine synthesis proteins CysD, CysN and CysC. The APS and PAPS produced by RaxP and RaxQ are used for both cysteine synthesis and sulphation of other molecules. Mutation in *Xoo xcysI*, a homologue of *Escherichia coli cysI* that is required for cysteine synthesis, blocked APS- or PAPS-dependent cysteine synthesis but did not affect AvrXa21 activity, suggesting that AvrXa21 activity is related to sulphation rather than cysteine synthesis. Taken together, these results demonstrate that APS and PAPS production plays a critical role in determining avirulence of a phytopathogen and reveal a commonality between symbiotic and phytopathogenic bacteria.**

## Introduction

Both pathogenic and symbiotic bacteria are able to invade plant hosts in a highly specific manner. A given bacterium can infect and multiply in only a limited number of hosts. In plant pathogenic bacteria, the host specificity is mediated by avirulence (*avr*) gene-encoded effector molecules that can trigger a race-specific defense response in hosts carrying a corresponding resistance (*R*) gene (Staskawicz *et al*., 2001). Bacterial *avr* gene products are quite divergent in structures (Leach *et al*., 2001). In some cases, structural differences, such as repetitive motifs, of the products of an *avr* gene family can affect host specificity (Herbers *et al*., 1992). Post-translational modifications such as acylation can provide additional structural complexity and enhance function of phytopathogenic effectors (Nimchuk *et al*., 2000).

Symbiotic bacteria of leguminous plants use Nod factor, a lipo-chitooligosaccharide (LCO), to elicit the morphogenesis of nitrogen-fixing nodules on the plant roots. The length of the fatty acid moiety and other modifications to the Nod factor determine host specificity (Kamst *et al*., 1998). For example, sulphation of the *Sinorhizobium meliloti* Nod factor determines its symbiotic relation with alfalfa. *S. meliloti* strains carrying mutations in the *nodP, nodQ* or *nodH* genes produce Nod factor that lacks the sulphate group and are severely impaired in their ability to nodulate their normal host alfalfa, but gain the ability to nodulate vetch (Roche *et al*., 1991). Although it has long been hypothesized that the molecular mechanisms governing the interactions of symbiotic bacteria with their hosts share similarities with phytopathogenic bacteria (Roche *et al*., 1991), the role of sulphation in controlling the specificity of phytopathogen–host interactions has not yet been demonstrated.

Sulphation requires the presence of the activated sulphate form PAPS, an important intermediate of the sulphate assimilation pathway in all organisms (Leyh, 1993). PAPS is produced in two steps. First, ATP sulphurylase (ATP:sulphate adenylytransferase, EC 2.7.7.4) transfers

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the adenosine-5′-phosphoryl moiety of ATP to sulphate to form APS (adenosine-5′-phosphosulphate). Secondly, APS kinase (ATP:adenylysulphate-3′-phosphotransferase, EC 2.7.1.25) phosphorylates APS to form PAPS (3′-phosphoadenosine-5′-phosphosulphate). APS and PAPS are primarily used for cysteine synthesis, which may be reciprocally converted to and from methionine. In *E. coli,* CysD, CysN and CysC function together to produce PAPS, which is then reduced successively by the *cysH*-encoded PAPS reductase and the *cysJ* and *cysI* encoded sulphite reductase for cysteine synthesis (Leyh *et al*., 1992). In *S. meliloti,* two genes, *cysD* and *cysN,* encode an ATP sulphurylase. This ATP sulphurylase produces APS, which is preferentially reduced by a *cysH*-encoded APS reductase for cysteine synthesis (Pia Abola *et al*., 1999). *S. meliloti* devotes two other genes, *nodP* and *nodQ*, which are clustered together with other Nod factor synthesis genes, to encode an ATP sulphurylase and APS kinase that function together to produce PAPS. PAPS is then used by a sulphotransferase encoded by *nodH* for Nod factor sulphation (Schwedock *et al*., 1994; Ehrhardt *et al*., 1995).

The rice disease resistance gene *Xa21* confers race specific resistance to the pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal agent of rice bacterial blight disease. *Xa21* encodes a presumed receptor-like kinase with leucine-rich repeats (LRR) in the predicted extracellular domain (Song *et al*., 1995). LRR-containing proteins are involved in protein–protein interactions and regulate signal transduction and cell adhesion as well as other functions (Kobe and Deisenhofer, 1994). The XA21 LRR domain has been shown to be required for race-specific recognition through analysis of truncated forms of the receptor (Wang *et al*., 1998). Based on these results, we have hypothesized that XA21 recognizes an extracellular effector molecule produced by *Xoo* (Song *et al*., 1995). Although the gene encoding this *Xoo* effector remains unknown, a good candidate is the product of the corresponding *avr* gene *avrXa21*. However, *avrXa21 has* not yet been isolated and it is unclear as to how AvrXa21 activity is accomplished.

In an attempt to isolate gene(s) that determine AvrXa21 activity, we carried out Tn*5* mutagenesis on *Xoo* Philippine race 6 (PR6), which is avirulent on *Xa21* rice plants. Two genes, *raxP* and *raxQ,* required for AvrXa21 activity were identified. RaxP and RaxQ show sequence and functional similarity to *nodP* and *nodQ.* RaxP and RaxQ carry ATP sulphurylase and APS kinase activities which function together to synthesize the activated sulphate forms APS and PAPS. These results provide evidence that the plant pathogen *Xoo* and the plant symbiont *S. meliloti* share genes determining host specificity and demonstrate a requirement for sulphation in determining avirulence activity of a bacterial pathogen.

## Results

### *Identification of a virulent* Xoo *PR6 Tn*5 *mutant*

To identify gene(s) that control avirulence of *Xoo* PR6 on *Xa21* plants, we constructed an *Xoo* PR6 mutant library using the Tn*5* transposon. The library was screened for mutants that are virulent on rice line IRBB21 carrying the *Xa21* gene. One mutant, PT1948, was identified from a screen of 2958 Tn*5* mutants. Unlike the parental strain PR6, PT1948 was virulent on IRBB21 and the Taipei309 transgenic line 106-17-3-37 carrying the *Xa21* gene (Fig. 1). PT1984 is also fully virulent on both IR24 and Taipei309, which lack *Xa21*. The mutant produced lesion lengths similar to *Xoo* Korean race 1 (KR1) (strain DY87031), which is fully virulent on all four rice lines. These results indicate that PT1948 has become virulent on *Xa21*-containing lines and retained virulence on IR24 and Taipei309 (Fig. 1).

### *Isolation of an* Xoo *sulphur assimilation gene cluster*

A 15.5 kb plasmid called pPK1 was isolated from PT1984 by plasmid rescue using positive selection for the kanamycin resistance carried on the Tn*5* transposon (Fig. 2A). A 5 kb *Xho*I subclone, pYSX-1, was generated that contains the junction region of the Tn*5* plasmid and *Xoo* genomic sequences (Fig. 2B). Sequence analysis of pYSX-1 revealed that the mutation was due not to the insertion of Tn*5* transposon alone, but rather to the integration of the pSUP102:*Tn*5-B20 plasmid sequence into the *Xoo* chromosome. The integration was apparently mediated by a homologous recombination of a 9 bp sequence, ATGTCTAAC, present in both the Tn*5* plasmid

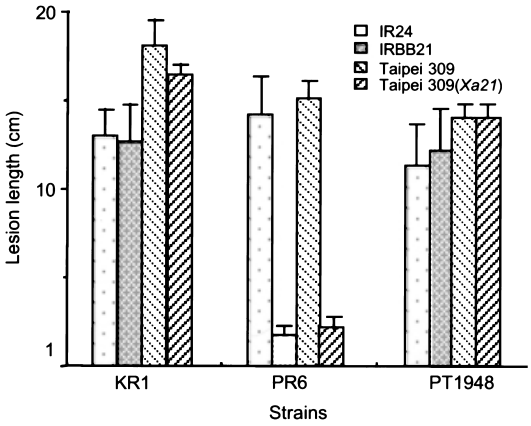


**Fig. 1.** Lesion lengths on IR24, IRBB21, TP309 and the TP309 transgenic line 106-17-3-37 carrying the *Xa21* gene inoculated with *Xoo* KR1, PR6 and the Tn*5* mutant PT1948. Six-week-old-plants were inoculated and scored 14 days after inoculation. Lesion lengths are means of five or six leaves from one plant. Bars represent standard deviation.

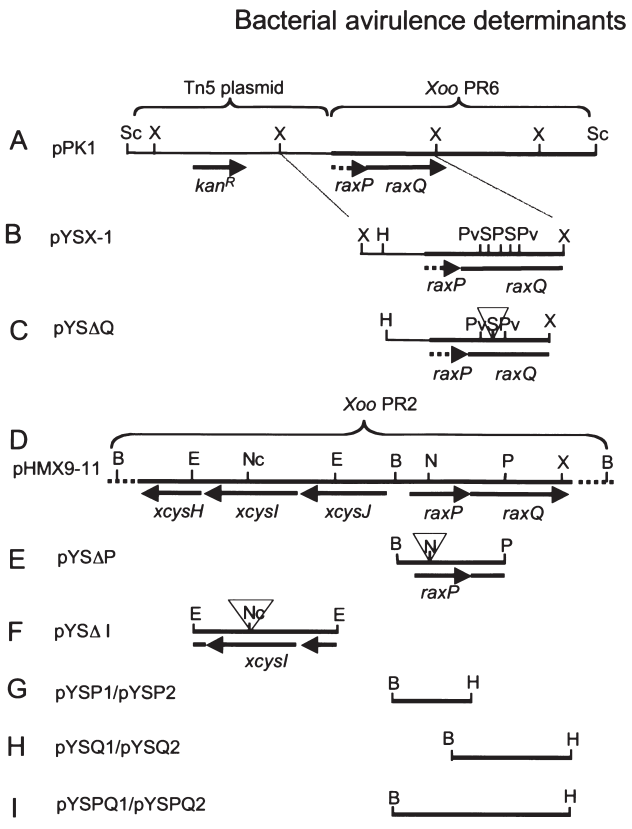


**Fig. 2.** The Tn5 disrupted DNA region and construction of marker exchange mutants and plasmids for complementation.
A. The rescued plasmid pPK1 containing the Tn5 plasmid sequence and the flanking *Xoo* PR6 genomic region carrying the ORFS *raxP* and *raxQ*.
B. A 5-kb *Xho*I DNA fragment subclone from pPK1, called pYSX-1.
C. *raxQ* knockout construct pYSΔQ carrying a 705 bp deletion and a kanamycin resistance gene insertion at the *Sma* I site.
D. Partial map of cosmid clone pHMX9-11 showing the 11 kb and 8 kb *Bam*HI fragments used for sequencing. Dotted lines indicate the rest of the PR2 genomic DNA present in the cosmid clone.
E. *raxP* knockout construct pYSΔP carrying a kanamycin resistance gene insertion at the *Not*I site;.
F. *xcysI* knockout construct pYSΔI carrying a kanamycin resistance gene inserted at the *Nco* I site.
G, H, I. Plasmids used for complementation of *E. coli cys*$^{-}$ mutants (pYSP1, pYSQ1 and pYSPQ1) and for overexpression of RaxP and RaxQ in *Xoo (*pYSP2, pYSQ2 and pYSPQ2). DNA inserts were amplified and the *Bam*HI and *Hin*dIII restriction enzyme sites engineered as described in text. Plasmids pYSΔQ, pYSΔP, pYSΔI, pYSP1, pYSQ1 and pYSPQ1 were constructed in plasmid pUC18. Plasmid pYSX-1 was constructed with plasmid pBluescript SK as a vector. Plasmids pYSP2, pYSQ2 and pYSPQ2 were constructed with pUFR027.
Thin lines in A, B and C indicate the DNA sequence derived from the Tn*5* plasmid pSUP102:Tn5-B20. Horizontal arrows indicate direction of transcription with the dashed arrows denoting the truncated *raxP* gene. Inverted triangles indicate the insertion sites for the kanamycin resistance gene used for marker exchange mutagenesis. B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; Nc, *Nco*I; N, *Not*I; P, *Pst*I; Pv, *Pvu*II; S, *Sma*I; Sc,*Sac*I; X,. *Xho* I. Restriction maps are partial. Size and position of genes are approximate.

and an *Xoo* open reading frame (ORF) designated *raxP (*required for AvrXa21 activity) (data not shown). *raxP* shares similarity to the host specificity gene *nodP* from *S. meliloti* and the cysteine synthesis gene *cysD* from *E. coli*. The ORF immediately downstream of *raxP* shows similarity to *S. meliloti nodQ* as well as *E. coli cysN and cysC*, which are in the same operon as *nodP* and *cysD* respectively. *S. meliloti nodP* and *nodQ* and *E. coli cysD*, *cysN* and *cysC* encode ATP sulphurylase and APS kinase that produce activated sulphate forms, APS and PAPS (Leyh *et al*., 1992; Schwedock *et al*., 1994).

In order to obtain the full-length *raxP* for further characterization, we isolated nine cosmid clones from an *Xoo* PR2 cosmid library using a 1 kb *Pvu*II fragment intragenic to *raxQ* as a probe (Fig. 2B). One of the cosmid clones, pHMX9-11 (Fig. 2D), was digested with *Bam*HI and the fragments were subcloned into pBluescript. One subclone containing *raxP* and *raxQ* and another subclone upstream of the *raxPQ* genes were sequenced. Upstream of *raxP* and *raxQ*, three ORFs in the opposite orientation were identified (Fig. 2D). These ORFs are similar to *E. coli cysJ, cysI* and *cysH* with deduced protein identities of 42%, 49% and 62%, respectively, and were accordingly named *xcysJ*, *xcysI* and *xcysH (x* indicates that they are homologues from *Xoo*) (Ostrowski *et al*., 1989). The *E. coli cysJIH* operon is upstream of and in opposite orientation to the *cysDNC* operon. *cysJ* and *cysI* encode, respectively, the flavoprotein and haemoprotein of a sulphite reductase (NADPH), whereas *cysH* encodes a PAPS reductase. Both the *cysDNC* and the *cysJIH* operons are required for cysteine synthesis (Leyh, 1993). The similarities to the *E. coli* genes suggest that the *Xoo raxP, raxQ* and *xcysJ, xcysI* and *xcysH* also encode components of a sulphur assimilation pathway. The sequence data for the *raxPQ* and *xcysJIH* operons have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AF380010 and AY056057 respectively).

### raxP *and* raxQ *are required for AvrXa21 activity*

To confirm the requirement of *raxP* for AvrXa21 activity and to test whether *raxQ* and the *xcysJIH* operon are also required, we carried out marker exchange mutagenesis of *raxP, raxQ* and *xcysI.* The resulting knockout strains PR6Δ*raxP,* PR6Δ*raxQ* and PR6Δ*xcysI* were tested on *Xa21* and non-*Xa21* rice plants for their phenotypes. Both PR6Δ*raxP* and PR6Δ*raxQ* grew to high levels and produced long lesions in both *Xa21* and non-*Xa21* plants, indicating that the knockout strains had lost AvrXa21 activity (Fig. 3A–C). To further confirm the requirement of *raxQ* for avirulence, six out of the nine PR2 cosmid clones isolated with the *Pvu*II fragment intragenic to *raxQ* as a probe were used to complement PR6Δ*raxQ*. Two of them,

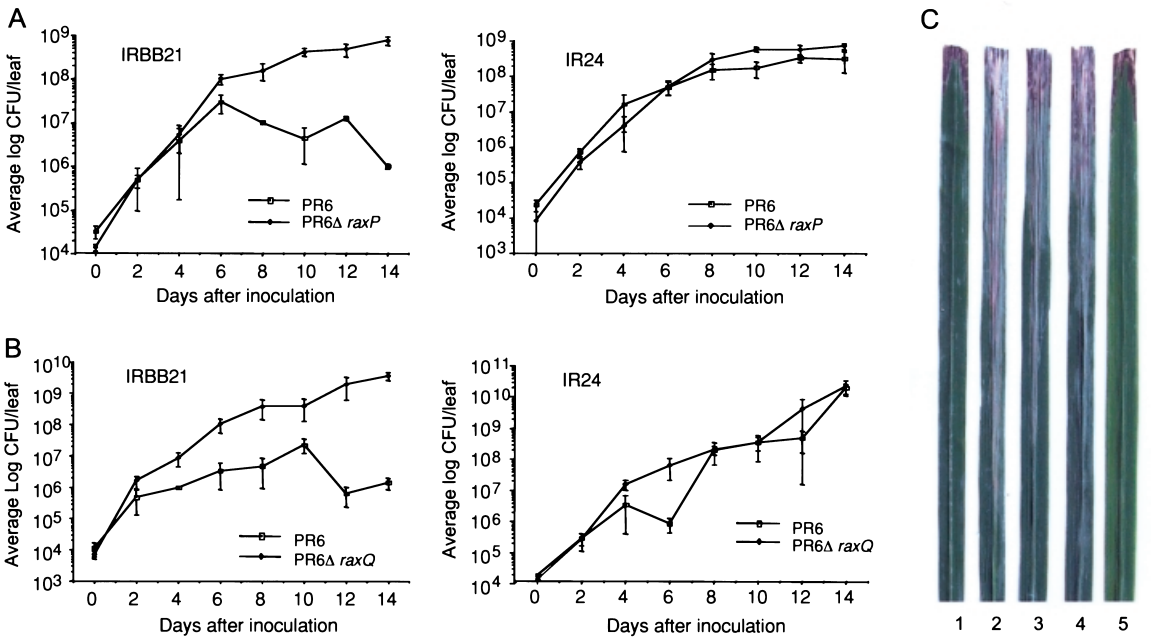


**Fig. 3.** Phenotypic analysis of *Xoo* PR6 *raxP* and *raxQ* mutants.
A and B. Growth curves of *Xoo* PR6 and its *raxP* and *raxQ* mutants, PR6Δ*raxP* and PR6Δ*raxQ*, on IRBB21 and IR24 rice plants.
C. Lesions on IRBB21 plants inoculated with PR6 (1), KR1 (2), PR6Δ*raxP* (3), PR6Δ*raxQ* (4) and PR6Δ*raxQ* complemented with pHMX6-41 (5). Inoculation and scoring were done as described in Fig. 1.

pHMX6-41 and pHMX22-42, were found to restore full AvrXa21 activity to PR6Δ*raxQ.* The *Xa21* plants inoculated with these two complements showed lesion length of 1 cm, whereas those inoculated with PR6Δ*raxQ* displayed lesion length of 14 cm (Fig. 3C and data not shown). Interestingly, the PR6Δ*xcysI* retained AvrXa21 activity, demonstrating that *raxP* and *raxQ,* but not *xcysI,* are required for AvrXa21 activity.

As *raxP* and *raxQ* are required for AvrXa21 activity, we tested for the presence of these DNA sequences in other avirulent and virulent races and for possible complementation of virulence strains by *raxP and raxQ*. A Southern analysis of the *Hin*cII, *Sac*I and *Eco*O109I-digested genomic DNAs from three avirulent strains, including PR6, and six virulent strains, including KR1, was carried out with the *Pvu*II fragment internal to *raxQ* as a probe. No polymorphism was found among the races tested in the *raxP* and *raxQ* region (data not shown), suggesting that *raxP* and *raxQ* may be conserved in both avirulent and virulent races. However, it is possible that small deletion and/or insertion mutations in *raxP* and *raxQ* of the virulent strains would result in loss of AvrXa21 activity and would not be detected. Therefore, a complementation assay of KR1 with *raxPQ* was carried out. *raxPQ* failed to confer AvrXa21 activity on KR1 (data not shown). This result suggests that *raxP* and *raxQ* may not encode effectors *per se,* but rather may be involved in synthesis, secretion or modification of the effector.

### *RaxP and RaxQ are similar to* S. meliloti *NodP and NodQ and* E. coli *CysD, CysN and CysC*

The deduced products of *raxP* and *raxQ*, RaxP and RaxQ, have similarity to CysD, CysN and CysC from *E. coli* as well as NodP and NodQ from *S. meliloti* (Fig. 4). The genes encoding these sets of proteins are found within operons and arranged in tandem in both *E. coli* and *S. meliloti*. Unlike the *S. meliloti nodP* and *nodQ* genes, which are plasmid borne, but similar to the *E. coli cys* genes, both *raxP* and *raxQ* are chromosomally located (data not shown). RaxP is similar to NodP and CysD, which encode the small subunit of ATP sulphurylases (Schwedock and Long, 1989; Leyh *et al*., 1992). The N-terminal domain of RaxQ and NodQ is similar to CysN and the C-terminal domain of these proteins is similar to CysC. CysN and the N-terminal domain of NodQ encode the large subunit of ATP sulphurylase, whereas CysC and the C-terminal domain of NodQ encode an APS kinase. RaxP is 64% identical to NodP and shares 67% identity with CysD (Fig. 4A). RaxQ and NodQ share 59% identity. RaxQ is also similar to CysN and CysC with 49% and 63% identity respectively (Fig. 4B). RaxQ shows highest similarity with NodQ and CysN in the consensus GTP-binding

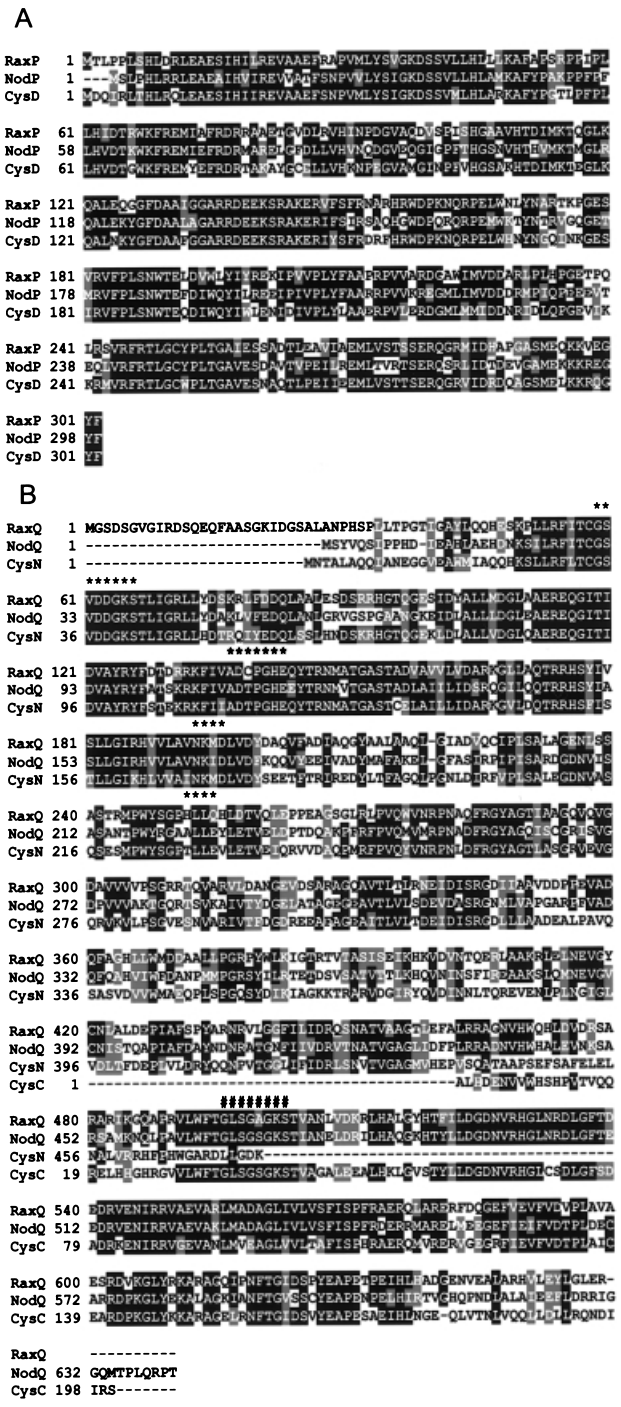

**Fig. 4.** Amino acid sequence comparison of RaxP and RaxQ.
A. Alignment of *Xoo* RaxP, *R. meliloti* NodP and *E. coli* CysD.
B. Alignment of RaxQ, NodQ, CysN and CysC. Amino acids that are identical in all the sequences compared are in black boxes whereas amino acids that are similar are in hatched boxes. Amino acids for presumed GTP binding are indicated by asterisks above the sequence. The region of the ATP-binding consensus domain is indicated with pound signs above the sequence.

sites and with NodQ and CysC at the purine-binding site (Fig. 4B) (Leyh, 1993). Although RaxQ shows highest similarity to NodQ, there are 27 amino acids in the N-terminus that are absent relative to NodQ (Fig. 4B). Like their *E. coli* and *S. meliloti* counterparts, RaxP and RaxQ do not have a recognizable transmembrane domain and signal peptide, suggesting that they are both cytoplasmic proteins. Although RaxQ is similar to *E. coli* CysN and CysC as well as *S. melliloti* NodQ in protein sequences, it is structurally more similar to NodQ. Both RaxQ and NodQ are single proteins whereas CysN and CysC are two separate proteins.

### raxP *and* raxQ *encode ATP sulphurylase and APS kinase*

Because the *S. meliloti* and *E. coli* proteins have ATP sulphurylase and APS kinase activities, we reasoned that *raxP* and *raxQ* probably possess ATP sulphurylase and APS kinase activities as well. To test this hypothesis, we used *raxP* and *raxQ* to complement *E. coli cysD*$^-$, *cysC*$^-$ and *cysN*$^-$*C*$^-$ mutants. Previous experiments have shown that these *E. coli* cysteine auxotrophic mutants could be complemented by the *S. meliloti nodP* and *nodQ* genes (Schwedock and Long, 1990; Schwedock *et al*., 1994). The *E. coli* cysteine auxotrophic mutants were transformed with plasmids carrying the corresponding *Xoo* genes. The resulting transformants were cultured in M9 media in the absence of cysteine. We found that *raxP (*pYSP1) (Fig. 2G) alone could not complement the *cysD*$^-$ mutant (TSL3), whereas *raxPQ (*pYSPQ1) (Fig. 2I) was able to complement this mutant. Moreover, *raxQ (*pYSQ1) (Fig. 2H) complemented the *cysC*$^-$ mutant (JM81A) and *raxPQ* complemented both the *cysC*$^-$ mutant and the *cysN*$^-$*C*$^-$ double mutant (DM62) (Table 1). Thus, genetic complementation of the *E. coli* mutants suggests that RaxP and RaxQ together confer ATP sulphurylase and APS kinase activities.

Based on the similarity of RaxP and RaxQ with NodP and NodQ, we used anti-NodP and anti-NodQ antibodies to assess protein levels in the *E. coli* cysteine auxotrophic mutants and *Xoo* PR6. The NodP antibody did cross-react with RaxP; however, the NodQ antibody did not cross-react with RaxQ (data not shown). In *E. coli* cysteine auxotrophic mutants, RaxP was very weakly expressed in TSL3 *cysD*$^-$ cells containing pYSP1 (*raxP*), but was very strongly expressed in DM62 *cysN*$^-$*C*$^-$ with pYSPQ1 (*raxPQ*) independent of the presence of isopropyl-β-D-thiogalactoside (IPTG) in culture medium (Fig. 5). These results suggest that RaxP expressed alone may not be stable and explain why *raxP* alone could not complement *cysD*$^-$ mutant. Interestingly, a similar result was obtained with *Xoo* PR6 containing plasmid-borne *raxP*. The NodP

**Table 1.** Complementation of *E. coli* cysteine auxotrophs by *raxP* and *raxQ*.

| Plasmid | Plasmid-borne gene(s) | Cell density ($OD_{600}$) | | |
|---|---|---|---|---|
| | | TSL3 (*cysD*$^-$) | DM62 (*cysN*$^-$, *cysC*$^-$) | JM18A (*cysC*$^-$) |
| – | – | 0.033 ± 0.002 (1.451 ± 0.043) | 0.124 ± 0.005 (1.197 ± 0.068) | 0.482 ± 0.003 (1.385 ± 0.056) |
| PYSP1 | *raxP* | 0.050 ± 0.007 | NT | NT |
| PYSQ1 | *raxQ* | NT | 0.135 ± 0.003 | 1.379 ± 0.005 |
| PYSPQ1 | *raxP, raxQ* | 0.917 ± 0.003 | 1.104 ± 0.027 | 1.417 ± 0.078 |

All *E. coli* mutants and their complements were cultured overnight in M9 minimal medium supplemented with thiamine and proline. Data are the means of three replicates ± SD. Data in brackets reflect the cell density when the cells are cultured in M9 medium supplemented with cysteine. NT, not tested.

antibody detected the plasmid-borne copy (pYSP2) but was not sensitive enough to detect the chromosomally encoded RaxP protein. Overexpression of RaxP was detected only in *Xoo* cells harbouring the plasmid pYSPQ2 with both *raxP* and *raxQ* but not in cells containing the plasmid (pYSP2) with *raxP* alone (data not shown).

To verify the biochemical function of RaxP and RaxQ as ATP sulphurylase and APS kinase, we carried out an *in vivo* assay with PR6, PR6Δ*raxP* and PR6Δ*raxQ* cells cultured in M9 medium in the presence of [$^{35}$S]-$Na_2SO_4$. Thin-layer chromatographic (TLC) analysis of the supernatants of permeabilized cells showed that wild-type PR6 cells synthesized a substantial amount of APS but produced only trace amount of PAPS. In contrast, the *raxP* and *raxQ* mutants generated neither APS nor PAPS (Fig. 6). This result confirms that RaxP and RaxQ carry ATP sulphurylase and APS kinase activities. Furthermore, it also suggests that RaxP and RaxQ may be the only enzymes activating sulphate into APS and PAPS.

### *The APS and PAPS produced by RaxP and RaxQ are used for both cysteine synthesis and sulphation of other molecules*

As RaxP and RaxQ produce activated sulphate forms, APS and PAPS, we wanted to determine the fate of these molecules. To do so, we carried out a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) assay of total cell proteins from PR6 and its three knockout mutants, PR6Δ*raxP*, PR6Δ*raxQ* and PR6Δ*xcysI,* cultured to stationary phase in the presence of [$^{35}$S]-$Na_2SO_4$. The results showed that, in PR6, many proteins incorporated the $^{35}$S label, whereas proteins extracted from PR6Δ*raxP* and PR6Δ*raxQ* were not labelled (Fig. 7), indicating that the $^{35}$S-labelled PR6 proteins were derived from the APS and PAPS produced by RaxP and RaxQ. In a separate experiment using cells cultured in the presence of supplemented cysteine, incoporation of the $^{35}$S label into the proteins was basically abolished, indicating that $^{35}$S label was incorporated via cysteine synthesis (data not shown). Furthermore, the *xcysI* knockout strain also showed a drastic reduction in the number of labelled proteins, proving that *xcysI* is required for cysteine synthesis. Thus, because the *xcysI* mutant has no APS- or PAPS-dependent cysteine synthesis but does retain AvrXa21 activity, it appears that AvrXa21 activity is not related to cysteine synthesis. Interestingly, one strongly labelled protein band of about 100 kDa and some other weakly labelled bands were observed in the *xcysI* knock-

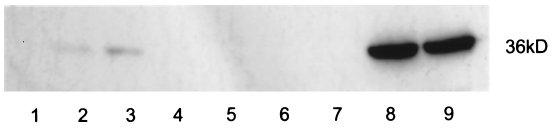


**Fig. 5.** Immunodetection of RaxP overexpressed in *E. coli* with anti-NodP antibody. *E. coli* cells were cultured overnight in M9 medium without cysteine supplement and with or without IPTG induction. Samples are *E. coli* TSL (*cysD*$^-$) *(*1), TSL (*cysD*$^-$)/pYSP1 without and with induction (2, 3); JM18 (*cysC*$^-$) *(*4), JM18A (*cysC*$^-$)/pYSQ1 without and with induction (5, 6); DM62 (*cysN*$^-$*C*$^-$) *(*7), DM62 (*cysN*$^-$*C*$^-$)/pYSPQ1 without and with induction (8, 9).

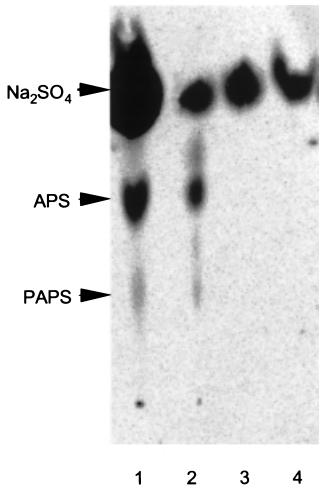


**Fig. 6.** ATP sulphurylase and APS kinase assay of the cell extracts of *Xoo* PR6 and its *raxP* and *raxQ* mutants, PR6Δ*raxP* and PR6Δ*raxQ*. Lane 1, *in vitro* reaction of yeast ATP sulphurylase and *R. meliloti* NodQ used for APS and PAPS standards; Lanes 2–4, extracts of PR6, PR6Δ*raxP* and PR6Δ*raxQ* cultured to $OD_{600}$ of 0.5 in M9 medium supplemented with [$^{35}$S]-$Na_2SO_4$.

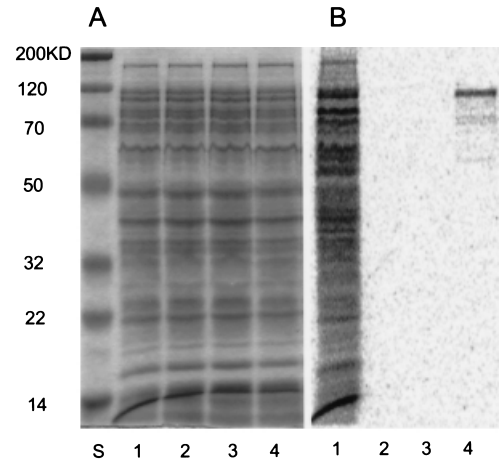


**Fig. 7.** Analysis of the use of activated sulphate forms produced by RaxP and RaxQ. A. Coommassie blue-stained SDS-PAGE of total cell proteins from PR6 (1), PR6Δ*raxP* (2), PR6Δ*raxQ* (3) and PR6Δ*xcysI* (4) along with prestained protein standards (S). *Xoo* cells were cultured to stationary phase in M9 medium in the presence of [$^{35}$S-]$Na_2SO_4$ without cysteine supplement. Sizes of the standards are marked on the left side. B. Phosphoimage of the SDS-PAGE.

out, suggesting that the labelled sulphuryl group in PAPS can also be directly transferred to other molecules (Fig. 7) (Leyh, 1993).

## Discussion

Sulphation of proteins and carbohydrates plays a key role in controlling specificity of a diverse range of extracellular recognition events (Bowman and Bertozzi, 1999; Kehoe and Bertozzi, 2000). In humans, for instance, the N-terminal LRR domain on the human glycoprotein chorionogonadotropin (hCG) receptor binds the C-terminal region of the alpha subunit of the hCG ligand with high affinity (Bhowmick *et al*., 1996). The hCG alpha subunit is sulphated at a tyrosine residue on the C-terminus, suggesting that sulphation is required for the ligand–receptor recognition (Bielinska, 1987). In another example, the recruitment of leucocytes to the sites of inflammation is mediated by the interaction of the receptor P-selectin on activated endothelial cells with the P-selectin glycoprotein ligand (PSGL)-1 present on cognate leucocytes (Kansas, 1996). Sulphation of three tyrosine residues near the N-terminus of PSGL-1 is essential for P-selectin binding (De Luca *et al*., 1995; Pouyajni and Seed, 1995; Wilkins *et al*., 1995). More recently, sulphation of the chemokine co-receptor CCR5 has been found to be required for attachment of the human immunodeficiency virus (HIV) and its subsequent invasion. Mutation of the four sulphated tyrosine residues in the N-terminal region of CCR5 inhibits HIV infection by 50–70% in cultured cells (Farzan *et al*., 1999). Finally, in bacteria, sulphation of the *S. meliloti* Nod factor is required for specific recognition by its host alfalfa (Roche *et al*., 1991).

In the present study, we isolated the *Xoo xcysJ, xcysI, xcysH*, *raxP* and *raxQ* genes. Like the *E. coli cys* gene cluster (59′), the *raxPQ* and *xcysJIH* operons are also tandemly arranged in the opposite orientations (Fig. 2) (Leyh, 1993). *raxP* and *raxQ* were shown to be required for AvrXa21 activity and recognition of *Xoo* PR6 by the presumed rice receptor kinase XA21 (Fig. 3). It was also demonstrated that the *raxP*- and *raxQ*-encoded proteins possess ATP sulphurylase and APS kinase activities, and catalyse the production of APS and PAPS (Table 1 and Fig. 6). Mutations in *raxP* and *raxQ* abolish incorporation of radioactive sulphate into proteins, indicating that these genes are required for cysteine synthesis. A mutation in *xcysI* also blocks APS- or PAPS-dependent cysteine synthesis (Fig. 7). These experiments indicate that sulphur-containing amino acids incorporate a large share of the APS or PAPS produced by RaxP and RaxQ. Clearly, then, the *raxP*- and *raxQ*-encoded proteins are the major components of a sulphate assimilation pathway in *Xoo*. Further experimentation showed that, in addition to its use for cysteine synthesis, the RaxP- and RaxQ-produced PAPS is also used for sulphation of other molecules (Fig. 7). Because disruption of the cysteine synthesis gene *xcysI* does not affect AvrXa21 activity, it is likely that the main role for *raxP* and *raxQ* in specifying AvrXa21 activity is in production of APS and PAPS for sulphuryl group transfer to another molecule. Interestingly, we have recently isolated a gene encoding a protein that shares similarity to both tyrosyl protein and oligosaccharide sulphutransferases. This gene is also required for *Xoo* PR6 avirulence activity (F. G. da Silva, Y. Shen, C. Dardick, P. Sharma *et al*., unpublished data), supporting the hypothesis that sulphation controls AvrXa21 activity. It is not clear if sulphation controls other R–Avr interactions.

Phytopathogenic bacteria and plant symbiont bacteria display common mechanisms for successful colonization of a particular host. The most notable mechanisms include quorum sensing and two-component regulatory systems, which allow adaptation to constantly changing environmental conditions (Hentschel *et al*., 2000). However, in terms of the mechanism for determining host specificity, little common ground has been noted. Although the *y4lO* gene product of *Rhizobium* sp. NGR234 shares similarity to *avrRxv* gene product from *Xanthomonas campestris* pv. *vesicatoria*, the biological function of the *y4lO* gene product is not yet known (Ciesiolka *et al*., 1999). Here we show that genes encoding ATP sulphurylase and APS kinase activities that are required for host specificity are conserved in both plant pathogenic bacteria and symbiotic bacteria.

It is interesting to note that RaxQ and *S. meliloti* NodQ are single proteins possessing both ATP sulphurylase and

APS kinase activities, whereas these activities are encoded by two separate genes, *cysN* and *cysC,* in *E. coli*. Such a genomic arrangement is unusual for sulphate assimilation genes in bacteria. The only other case in a survey of bacterial genome databases is in the plant pathogen *Xylella fastidiosa* (Simpson *et al*., 2000). The significance of the similarity of such a gene arrangement and structure in plant pathogens and symbionts is not yet known.

In mammals, ATP sulphurylase and APS kinase exist as a single bifunctional protein in which the ATP sulphurylase site produces pyrophosphate ($PP_i$) and the intermediate APS. The APS is not subsequently released but is rather channelled to the APS kinase (Schwartz *et al*., 1998). In contrast, the ATP sulphurylase and APS kinase encoded by *nodP* and *nodQ* are thought to form an enzymatic complex (Schwedock *et al*., 1994). Similarly, RaxP expressed alone in *Xoo* was not detectable on Western blot although it could be weakly detected in *E. coli*, which may be because of the high copy number of the plasmid used for RaxP expression. Only when it was expressed along with RaxQ was it robustly detected in both *Xoo* and *E. coli* (Fig. 5 and data not shown). These results suggest that RaxP may be stable only in the presence of RaxQ and that RaxP and RaxQ may also form a sulphate-activating complex.

*S. meliloti* has two copies each of *nodP (nodP1* and *nodP2*) and *nodQ (nodQ1* and *nodQ2*) that are complementary. A knockout of one copy of a pair only partially affects nodulation (Schwedock and Long, 1989). Interestingly, the *raxQ* probe hybridized weakly to another DNA sequence, suggesting the existence of another gene similar to *raxQ*. However, a mutation in *raxQ* alone abolishes APS and PAPS synthesis (Fig. 6), indicating that a functional *raxQ* is required for this activity. Thus, the role for the *raxQ*-hybridizing sequence is unclear. In a search of the DNA database, we found that *X. fastidiosa* and *Pseudomonas fluorescens* have 'orphan' APS kinase genes in addition to APS kinase genes linked to ATP sulphurylase-coding genes (Simpson *et al*., 2000) (http://www.jgi.doe.gov/JGI_microbial/html/pseudomonas/pseudo_homepage.html). These 'orphan' APS kinases may use ATP sulphurylase produced APS to generate PAPS for a specific purpose, such as sulphation, rather than cysteine synthesis. As the probe we used for the Southern analysis covers part of the *raxQ*-encoded APS kinase domain and APS kinases from different organisms are highly similar, it is possible that such an 'orphan' APS kinase gene was detected. This presumed 'orphan' APS kinase may use APS produced by the *raxP* and *raxQ* encoded ATP sulphurylase to synthesize PAPS. Experiments are in progress to isolate and characterize the *raxQ* homologue and to determine if an 'orphan' APS kinase is also involved in AvrXa21 activity.

The involvement of RaxP and RaxQ in AvrXa21 activity and recognition of *Xoo* by the rice receptor kinase Xa21 is similar to the requirement for *S. meliloti* NodP and NodQ in recognition by alfalfa. In the *S. meliloti*–alfalfa interaction, it is clear that a Nod factor perception system exists, but it is unknown how sulphated Nod factors are perceived by the plant (Cullimore *et al*., 2001). Intriguingly, the alfalfa *Nork (*nodulation region-linked receptor kinase) gene encodes a receptor kinase similar to the rice protein XA21 with an LRR in the putative external domain. *nork* was found to be truncated in $Nod^-Myc^-$ alfalfa mutant MN NN1008 (G. B. Kiss, personal communication) that is blocked in the early stage of nodulation and Nod factor signal transduction (Albrecht *et al*., 1999). Thus, the NORK protein is a good candidate for the Nod factor receptor. Based on the results presented here and in line with the role of sulphation in Nod factor activity, we propose that *raxP* and *raxQ* synthesize an activated form of sulphate, PAPS, that is then used for sulphation of the AvrXa21effector, which is yet to be identified. Extracellular secretion of the sulphated avirulence effector would make it available for race-specific interaction with the LRR domain of the rice XA21 receptor kinase. Alternatively, PAPS may be transferred to an unknown molecule that modulates the function of the avirulence effector. For example, the sulphated modulator may affect secretion of the effector or act as an enzyme to activate the effector. Experiments following the fate of the RaxP/Q-synthesized PAPS and identification of the effector should help distinguish these possibilities.

## Experimental procedures

### *Bacterial strains, plasmids and culture media*

Bacterial strains and plasmids used in this study are described in Table 2. *Xoo* cells were routinely grown in liquid or solid PSA media (10 g of peptone, 10 g of sucrose, 1 g of L-glutamic acid (monosodium salt), 16 g of agar per litre), nutrient broth (NB) medium (Difco) or M9 medium supplemented with methionine at 28°C. *E. coli* strain DH10B and plasmids pUC18, pBluescript SK and pUFR027 were used for all cloning experiments. *E. coli* cysteine auxotrophic mutants were generously provided by Dr Sharon Long, Stanford University, CA, USA. *E. coli* cells were cultured in LB or M9 at 37°C. The concentrations of antibiotics used for *E. coli* and *Xoo* are described in Shen *et al*. (2001).

### *Molecular techniques*

Standard recombinant DNA techniques were used (Sambrook *et al*., 1989). *Xoo* genomic DNA was prepared according to Wilson (1994). For Southern DNA hybridization, [$^{32}$P]-dCTP (NEN Life Science Products, Boston, MA, USA) was used to label DNA probes with a random labelling kit (Amersham Life Science, Arlington Height IL, USA). DNA sequencing of DNA fragments cloned in pBluescript was performed by the dideoxy chain termination method by using an automated sequencer (Model 400 l; Li-Cor, Lincoln,

**Table 2.** Bacterial strains and plasmids used in this study.

| Strain and plasmid | Relevant genotype or properties | Source or reference |
|---|---|---|
| *Escherichia coli* | | |
| DH10B | F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80 dLacZÆM15 ÆlacX74 deoR recA1 endA1 araD139 Æ (ara, leu) 7697 galU galK λ⁻ rpaL nupG | Gibco BRL, Grand Island, NY, USA |
| TSL | $Cm^R$, *cysD*⁻ | Leyh *et al*. (1988) |
| JM18A | *cysC*⁻ | Leyh *et al*. (1988) |
| DM62 | $Km^R$, *cysN*⁻*C*⁻ | Leyh *et al*. (1988) |
| TSL(pYSP1) | TSL(cysD⁻) containing raxP | This study |
| TSL(pYSPQ1) | TSL(cysD⁻) containing raxP and raxQ | This study |
| JM18A(pYSQ1) | JM18A(cysC⁻) containing raxQ | This study |
| JM18A(pYSPQ1) | JM18A(cysC⁻) containing raxP,and raxQ | This study |
| DM62(pYSQ1) | DM62(cysN⁻C⁻) containing raxQ | This study |
| DM62(pYSPQ1) | DM62(cysN⁻C⁻) containing raxP and raxQ | This study |
| *Xanthomonas oryzae* pv. *oryzae* | | |
| PXO99 | Philippine race 6 (PR6) strain, avirulent on *Xa21* rice plants | Hopkins *et al*. (1992) |
| DY87031 | Korean race (KR1) strain, virulent on *Xa21* rice plants | Wang *et al*. (1996) |
| PT1948 | Tn5 mutant of PXO99, virulent on Xa21 rice plants | This study |
| PR6Æ*raxP* | *raxP*⁻, PXO99 mutant with $Kan^R$ insertion at *Nco*I site of *raxP* | This study |
| PR6Æ*raxQ* | *raxQ*⁻, PXO99 mutant with $Kan^R$ replacement of *Sma*I fragment of *raxQ* | This study |
| PR6Æ*cysI* | *cysI*⁻, PXO99 mutant with $Kan^R$ insertion at *Nco*I site of *cysI* | This study |
| PR6(pYSP2) | PXO99 overexpressing raxP | This study |
| PR6(pYSQ2) | PXO99 overexpressing raxQ | This study |
| PR6(pYSPQ2) | PXO99 overexpressing raxP and raxQ | This study |
| *Plasmid and cosmid* | | |
| pUC18 | OripUC, $Ap^R$ | Biolabs, Beverly, MA, USA |
| pBluescript SK | OriColE1, $Ap^R$ | Stratagene, La Jolla, CA. USA |
| pUC4K | OripBR322, $Ap^R$, Containing $Kan^R$ cassette | Pharmacia, Pisctaway, NJ, USA |
| pUFR027 | IncW, $Nm^R$, Mob +, *mob*(P) | DeFeyter *et al*. (1990) |
| pPK1 | Plasmid rescued from PT1948 containing Tn*5* disrupted region | This study |
| pYSX-1 | pUC18 with a 5 kb *Xho*I fragment from pPK1 containing truncated *raxP* and *raxQ* | This study |
| pHMX9-11 | A cosmid clone of PR2 containing *raxPQ* locus | This study |
| pYSΔQ | pYSX-1 with $Kan^R$ replacement of a *Sma*I fragment in *raxQ* | This study |
| pYSΔP | pUC18 containing a *Bam*HI–*Pst*I fragment with $Kan^R$ inserted in the *Not* I site of *raxP* | This study |
| pYSΔI | pUC18 containing a 2.8 kb *Eco*I fragment from pHMX9-11 with $Kan^R$ inserted in the *Nco* I site of cysI | This study |
| pYSP1 | pUC18 with a 1.1 kb PCR fragment containing *raxP* | This study |
| pYSQ1 | pUC18 with a 2.0 kb PCR fragment containing *raxQ* | This study |
| pYSPQ1 | pUC18 with a 3.0 kb PCR fragment containing *raxP* and *raxQ* | This study |
| pYSP2 | pUFR027 with the 1.1 kb fragment containing *raxP* | This study |
| pYSQ2 | pUFR027 with the 2.0 kb fragment containing *raxQ* | This study |
| pYSPQ2 | pUFR027 with the 3.0 kb fragment containing *raxP* and *raxQ* | This study |

NE, USA) (Sanger *et al*., 1977). To fill in gaps, a primer walking strategy was used with synthesized primers (Operon, Alameda, CA, USA). PCR was performed with Taq polymerase (Qiagen Inc. Valencia, CA, USA) using the Programmable Thermal Controller (MJ Research, Inc. Watertown, MS, USA). PCR products were cloned with the TA cloning kit (Invitrogen Corp. Carlsbad, CA, USA). Bacterial transformations were carried out with a BRL Electroporator (Life Technologies, Inc. Gaithersburg, MD, USA). *Xoo* plasmid profiling was conducted according to Eckhardt (1978). DNA and protein sequence analyses and similarity searches were performed with Sequencher (Gene Codes Corp., Ann Arbor, MI, USA), NCBI BLAST (Altschul *et al*., 1997) and CLUSTALW (Thompson *et al*., 1994).

### *Tn*5 *mutagenesis of the* Xoo *genome and screening for virulent* Xoo *mutants*

Random *Tn5* mutagenesis of *Xoo* PR6 strain PXO99 was carried out according to Simon *et al*. (1989) with the following modifications. PR6 cells were grown on PSA plates for 72 h, suspended in NB medium to $OD_{600}$ 0.25–0.30, and incubated for 5–6 h at 30°C with shaking until the cell density reached $OD_{600}$ 0.5–0.6. Fresh *E. coli* S17-1 cells harboring pSUP102:*Tn*5-B20 (provided by Werner Klipp, University of Bielefeld) cultured on Luria–Bertani (LB plates) were suspended in NB medium and grown for 4–5 h at 37°C with shaking until $OD_{600}$ 0.5–0.6. Cultures of *Xoo* and *E. coli* were mixed in a ratio of 15:1 in an Eppendorf tube and were pelleted. The pellet was resuspended in 200 µl of NB and the suspension was spotted on PSA plates and incubated for 48 h. The mating mixture was resuspended in 200 µl of water and spread on PSA plates containing kanamycin (50 µg ml$^{-1}$) and cephalexin (25 µg ml$^{-1}$). Transconjugants resistant to kanamycin were purified on the same medium and stored in 10% glycerol in microtiter plates at –80°C. The $Kan^R$ mutants were screened for their virulence phenotype on rice plants of IRBB21 containing the *Xa21* gene by scissors clip inocula-

tion (Kauffman *et al*., 1973). Candidate mutants virulent on IRBB21 were further verified for their virulence on IR24, Taipei309 and *Xa21* transgenic line 106-17-3-37 of Taipei309 (Song *et al*., 1995).

### *Cloning of the Tn5-disrupted Xoo genomic region*

To clone the *Xoo* region disrupted by the Tn*5* transposon, we used tail PCR with primers designed with Tn*5* transposon end sequences. These experiments indicated that not only the Tn*5* transposon but also Tn*5* plasmid pSUP102:Tn*5*-B20 sequences were integrated into the *Xoo* chromosome. Therefore, a plasmid rescue approach was used to isolate the Tn*5* mutated gene region. PT1948 genomic DNA was digested with *Sac*I, which cuts once inside the Tn*5* plasmid (Fig. 2A), and recircularized with T4 DNA ligase. *E. coli* DH10B cells transformed with the ligation mix were selected on kanamycin. The rescued plasmid pPK1 carried the 4.5 kb *Xho*I fragment internal to the Tn*5* transposon as verified by Southern analysis (Fig. 2A and data not shown). To identify the *Xoo* DNA sequence flanking the Tn*5* plasmid sequence, an *Xho*I subclone pYSX-1 was generated and sequenced (Fig. 2B). pYSX-1 contains the junction region of Tn*5* plasmid and the *Xoo* genomic DNA sequence. To obtain the corresponding wild-type gene of the disrupted open reading frame, a 1-kb *Xoo Pvu*II DNA fragment from pYSX-1 was used as probe to screen a cosmid library of *Xoo* Philippine race 2 (PR2) strain PXO86 (provided by Jan Leach, Kansas State University), also avirulent on IRBB21 plants. From one of the isolated cosmid clones, pHMX9-11 (Fig. 2D), two *Bam*HI fragments were subcloned into pBluescript II SK for sequencing.

### *Construction and complementation of marker exchange mutants*

For ORF *raxQ*, a *Hin*d III–*Xho* I fragment from pYSX-1 was subcloned into pUC18 to generate marker exchange construct pYSΔQ (Fig. 2C) carrying a *Sma*I fragment deletion and a kanamycin resistance gene insertion in the *raxQ.* For ORF *raxP*, a *Bam*HI/*Pst*I fragment from pHMX9-11 was subcloned into pUC18, to which the kanamycin resistance gene was inserted at the *Not*I site in *raxP,* resulting in construct pYSΔP (Fig. 2E). For ORF *xcysI*, an *Eco*RI fragment from pHMX9-11 was subcloned into pUC18 and a kanamycin resistance gene was inserted at the *Nco*I site in *xcysI* to form construct pYSΔI (Fig. 2F). The marker exchange mutageneses were carried out according to Shen *et al*. (2001). At least three samples for each marker exchange mutant were verified by Southern analysis and tested for their virulence on IRBB21 plants and *in planta* growth. The resulting knockout strains were named PR6Δ*raxP,* PR6Δ*raxQ* and PR6Δ*xcysI*. The isolated PXO86 cosmid clones were introduced by electroporation (Shen *et al*., 2001) to complement the PR6Δ*raxP* and PR6Δ*raxQ* knockout mutants. The phenotypes of the transformants were determined by plant inoculation and/or growth curves (Wang *et al*., 1998).

### raxP *and* raxQ *plasmid construction for complementation of* E. coli *cysteine auxotrophic mutants and overexpression in* Xoo

For complementation of *E. coli* cysteine auxotrophic mutants, four primers were designed for amplification of *raxP*, *raxQ* and *raxPQ*. All primers were engineered with a *Bam*HI site on the forward primer and a *Hin*dIII site on the reverse primer (shown in bold). They are: PC24, 177 bp upstream of the start of *raxP*, 5′_TTC**GGATCC**AGTGGCTGGAT GAGAA-3′; PC23, immediate downstream of *raxQ*, 5′_TCG**AAGCTT** AGCGTTCCAGACCCA A-3′; PC25, 103 bp upstream of the start of *raxQ*, 5′_GTA**GGATCC**GAAATGCTGGTCAGCAC-3′; and PC26, 21 bp downstream of *raxP*, 5′_ACG**AAGC TT**GACTCCCGAATCACTGCCC-3′. The primer pairs of PC24/PC26, PC24/PC23 and PC25/PC26 were used in the PCR to amplify *raxP*, *raxQ* and *raxPQ* respectively. The PCR products containing *raxP, raxQ* and *raxPQ* were inserted into the *Bam*HI and *Hin*dIII site of pUC18 to form pYSP1, pYSQ1 and pYSPQ1 respectively (see Fig. 2G–I). The constructs were verified by sequencing. LacZ promoter in the pUC18 vector was used for expression of *raxP* and *raxQ* in *E. coli*. For overexpression of *raxP* and *raxQ* in *Xoo*, the same set of the PCR fragments containing *raxP, raxQ* and *raxPQ* were inserted into pUFR027 in the same orientation as the LacZ promoter in the vector by three-fragment ligation to form pYSP2, pYSQ2 and pYSPQ2 respectively (Fig. 2G–I).

### *Thin-layer chromatographic analysis*

*Xoo* PR6, PR6Δ*raxP* and PR6Δ*raxQ* cells were cultured in modified M9 medium. The M9 medium was supplemented with 1.5 mmol $l^{-1}$ methionine and carrier-free [$^{35}$S]-$Na_2SO_4$ (ICN Pharmaceuticals, Costa Mesa, CA, USA) ($3.7 \times 10^5$ Bq per 10 ml). The $MgSO_4$ component in the M9 medium was replaced by $MgCl_2$ in one-tenth of the molar concentration of $MgSO_4$ to eliminate the non-radioactive sulphate. The cells were grown to an $OD_{600}$ of 0.5, collected and washed once with water. The cells were resuspended in 50 μl of 10 mmol $l^{-1}$ TE and subjected to freeze and thaw in liquid nitrogen and warm water five times. The cell lysates were centrifuged for 15 min. The supernatants were filtrated through Microcon MY10 filters to reduce proteins. The filtrated cell lysates were then analysed by thin-layer chromatography (TLC) with PEI-cellulose TLC plates (Selecto Scientific, GA, USA) according to Schwedock *et al*. (1994). The developed TLC plates were visualized with Phosphoimager (Molecular Dynamics, CA, USA). The APS and PAPS standards were synthesized with yeast ATP sulphurylase (Sigma, St. Louis, MO, USA) and *R. meliloti nodQ* encoded APS kinase (provided by Sharon Long, Stanford University) according to Schwedock *et al*. (1994).

### *Protein electrophoresis and Western blotting*

For detection of the expression of *raxP* and *raxQ* in both *E. coli* and *Xoo*, the *E. coli cysD*$^-$, *cysC*$^-$ and *cysN*$^-$*C*$^-$ mutants harbouring pYSP1, pYSQ1 and pYSPQ1, respectively, and cells of *Xoo* PR6 containing pYSP2, pYSQ2 and pYSPQ2 were cultured with or without 0.5 mmol $l^{-1}$ IPTG. Total cell proteins were resolved in SDS-polyacryamide gel and were transferred to ECL™ nitrocellulose membrane (Amersham Pharmacia) by using standard Western blotting technique (Sambrook *et al*., 1989). Rabbit anti-NodP and anti-NodQ antibodies were provided by Sharon Long. Secondary antibody against rabbit IgG was from ICN Pharmaceuticals. The

ECL™ immunodetection kit (Amersham Pharmacia) was used to detect the cross-reactions.

To analyse the use of activated sulphate forms in cysteine synthesis and sulphation, PR6, PR6Δ*raxP* and PR6Δ*raxQ* cells were cultured as for TLC analysis except that the cells were cultured to stationary phase. Total cell proteins were resolved in SDS-PAGE and radioactive signal was detected with phosphoimage.

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