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Mutations in *ams* genes of *Erwinia amylovora* affect the interactions with host plants

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Abstract

A large region for exopolysaccharide (EPS) production that is involved in the pathogenicity of *Erwinia amylovora* strain CFBP1430, cloned previously, was further characterized in this study. An 8.4 Kb-long sub-cloned fragment from that *ams* (*am*ylovoran *s*ynthesis) region was mutagenized *in vitro* by Tn3Gus insertion. Several new chromosomal mutants were obtained by marker exchange. The phenotype of those mutants was determined with regard to pathogenicity, EPS production, and growth *in planta*. It appeared that alteration in EPS synthesis was correlated with altered pathogenicity and poor (or no) growth *in planta*. Altogether these data argue for a direct role of EPS in bacterial multiplication *in planta*, and/or for an indirect role by protecting the bacteria against host defence reactions.

Abbreviations: cfu – colony forming unit; EPS – exopolysaccharide.

Introduction

Genetics and molecular biology approaches have greatly contributed to the study of plant-microbe interactions, especially the interactions between plants and bacteria (Kerr, 1987; Daniels et al., 1988). This is particularly true for *Erwinia amylovora*, the causative agent of fire blight (Roberts and Coleman, 1991; Vanneste, 1995). The two main classes of avirulent mutants of *E. amylovora* correspond either to acapsular mutants altered in exopolysaccharide synthesis (see below) or to capsulated mutants, most of the latter being mutated in the *hrp* gene cluster (Barny et al., 1990; Beer et al., 1991; Roberts and Coleman, 1991).

The production of a capsule made of exopolysaccharides surrounding *E. amylovora* cells has for a long time been correlated to the bacterium virulence (Bennett and Billing, 1978; Ayers et al., 1979). *E. amylovora* is able to produce at least three exopolysaccharides: levan, a low molecular weight glucan, and an acidic exopolysaccharide called amylovoran. Levan and glucan are probably not essential for pathogenicity (Gross et al., 1992; Geier and Geider, 1993; Smith et al., 1995). Amylovoran is an acidic heteropolymer consisting of a 6-sugar branched repeating unit with galactose and glucuronic acid in a 4 to 1 ratio, the major portion of which is substituted with pyruvate and variously modified with acetate groups. Additional heterogeneity is due to glucose residues present in a minor portion of the repeating units (Nimtz et al., 1996a). Although glucuronic acid and pyruvic acid are common constituents of acidic bacterial EPS, the structure of amylovoran is similar only to that of stewartan, the capsular EPS from the closely related corn pathogen *Pantoea stewartii* (formerly named *Erwinia stewartii*) (Nimtz et al., 1996b). Insertional mutants of *E. amylovora* that are altered in genes involved in EPS biosynthesis produce either no EPS or reduced levels of EPS. They are respectively avirulent (Steinberger and Beer, 1988;

Table 1. Strains, phages and plasmids

Strain, phage or plasmid	Description (phenotype or genotype)	Source or Reference
E. coli		
DH5α	DH5 derivative, highly efficient for transformation	Bethesda Research Labs
S17-1	del*recA*,::RP4-2-Tc::Mu-Km::Tn7, *hsdR, thi, pro*	Simon et al. (1983]
C2110	polA$^-$, Nal^R	
E. amylovora		
CFBP1430	Wild type, isolated from *Crataegus*	Paulin and Samson (1973)
PMV6089	Pat$^-$, EPS$^-$,::MudIIPR13, LacZ$^+$, Cm^R	Tharaud et al. (1994)
PMV6191	Km^{R}Tc^S clones resulting from marker exchange between	
to	Tn3Gus- mutagenized pPMV188 plasmid and CFBP1430 chromosome;	This work
PMV6223	see text and Table 2 for other phenotypes.	
Phages		
4LM		Billing and Garrett (1980)
JV1		Vanneste and Paulin (pers. comm.)
PEa1(h)		Hartung et al. (1988)
Plasmids		
pLA2917	oriT(RK2), cos(l), Km^R, Tc^R	Allen and Hanson (1984)
pRK767	Mobilizable vector, Tc^R	Gill and Warren (1988)
pSShe	Helper plasmid encoding transposase, PolA-dependent, Cm^R	Stachel et al. (1985)
pTn3Gus	PolA-dependent plasmid with transposon containing *uidA* gene, Ap^R, Km^R	B. Staskawicz (unpubl.)
pPMV152	Wild type DNA of CFBP1430 in pLA2917	Tharaud et al. (1994]
pPMV180	Deletion of the 2.3 Kb and 10.2 Kb *Bgl*II fragments in pPMV152	This work
pPMV188	8.4 Kb *Hin*dIII fragment cloned from pPMV180 in plasmid pRK767	This work
pPMV189	idem but in the opposite orientation	This work

Bellemann and Geider, 1992) or partially virulent on immature pear fruit (Bernhard et al., 1990).

In Gram-negative bacteria, genes for biosynthesis of cell-surface polysaccharides are generally chromosomal and are usually arranged in clusters of one or more transcriptional units (Whitfield and Valvano, 1993). A cluster of 12 amylovoran encoding genes (from left to right: *amsG, amsH, amsI, amsA* to *amsF*, and *amsJ, amsK*, and *amsL*) from *E. amylovora* was described by K. Geider's group (Bernhard et al., 1993; Bugert and Geider, 1995). This cluster proved to be expressed as a 16 Kb long single transcriptional unit (Bugert and Geider, 1995). The *amsI* gene product proved to be a low molecular weight acid phosphatase controlling the EPS biosynthesis of *E. amylovora* (Bugert and Geider, 1997). Putative functions have been assigned to several *ams* genes products by Geider's group either on the basis of functional complementation (Bernhard et al., 1993) or on the basis of sequence similarities (Bugert and Geider, 1995).

In a previous report our group described an overlapping region covering genes *amsG* to *amsD* (from upstream to downstream) (Tharaud et al., 1994). To assess further the role of the genes clustered in that region a set of mutants distributed over this sub-cloned fragment was obtained. We focused on the phenotypes of those mutants with regard to pathogenicity, EPS production and growth *in planta*. Parts of this paper were presented in the proceedings of the sixth workshop on fire blight (Menggad et al., 1993; Laurent et al., 1993).

Materials and methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Either *E. amylovora* or *E. coli* cells were routinely grown in Luria broth (Sambrook et al., 1989). Minimal medium M9 (Miller, 1972) or low phosphate minimal medium (LP-M9) were supplemented with glucose (0.2%) as a carbon source; nicotinic acid (0,02% W/V) was added for growth of *E. amylovora*. When required, these media were solidified by addition of Difco agar (15 g/l) or/and supplemented with antibiotics at the following concentra-

tions: 10 μg/ml of chloramphenicol (Cm), 20 μg/ml of kanamycin (Km), 10 μg/ml of tetracycline (Tc), 50 μg/ml nalidixic acid (Nal), 100 μg/ml of streptomycin (Sm).

DNA manipulation and transformation of bacterial cells

Total DNA was isolated from *E. amylovora* cells by the method of Klötz and Zimm (1972) and purified by CsCl EtBr density gradient centrifugation. Plasmid DNA preparation, analyses and manipulations were performed by using standard techniques (Sambrook et al., 1989). Southern blot and colony hybridizations were performed as described by Barny et al. (1990).

Escherichia coli strains S17-1 and DH5α were transformed by electroporation as described in Sambrook et al. (1989) except that sterile water was used for washing the cells instead of low-salt buffer. Aliquots (150 μl) of cells were mixed with 200-500 ng of cold DNA. The settings for applying the electric pulse were 2.5 kV/cm, 25 μF, and 200 ohms.

Mutagenizing the ams *genes cluster*

To mutagenize the plasmid pPMV188 (see Results, Table 1 and Figure 1), *E. coli* S17-1 cells were electroporated by both plasmids pSShe and pTn3Gus (Table 1) selecting for Cm^R Km^Rcolonies. The resulting strain S17-1(pSShe, pTn3Gus) was used as the recipient strain in a mating with S17-1(pPMV188) selecting for resistance to chloramphenicol, kanamycin and tetracycline. The resulting S17-1 derivative contained the three plasmids pSShe, pTn3Gus and pPMV188. Transposition could occur in any cell of this population. Clones containing any mutagenized pPMV188-derivative plasmid were then selected as Km^R Nal^R after plasmid mobilization into the $polA^-$ *E. coli* strain C2110, in which the polA-dependent plasmids pSShe and pTn3Gus could not replicate. In that way a library of Tn3Gus insertions in pPMV188 was constructed.

The mutagenized insertions in the plasmids were subsequently marker-exchanged into the chromosome of the *E. amylovora* wild-type strain CFBP1430. First, each mutagenized plasmid was mobilized from S17-1 cells into the *E. amylovora* strain CFBP1430, selecting on minimal medium supplemented with nicotinic acid, kanamycin and tetracycline. Then, the resulting CFBP1430 transconjugants containing the different mutagenized pPMV188 derivative plasmids were grown for eight to eleven passages in liquid low phosphate medium. Plating on kanamycin supplemented plates, and replica-plating on tetracycline agar medium allowed to identify tetracycline sensitive clones. These Km^R Tc^S clones should be marker-exchanged in the *ams* region.

Other methods

Pathogenicity of *E. amylovora* strains was assessed in greenhouse conditions on seedlings of open pollinated cv. 'Passe Crassane' or 'Kirschensaller' for pear and cv. 'Golden delicious' for apple. Inoculation of plants and monitoring of symptoms were performed as described previously (Laurent et al., 1987; Barny et al., 1990). For each assay 10 seedlings were inoculated, usually 2 repeats were done with each mutant.

Bacterial growth *in planta* was performed on pear seedlings of 'Kirschensaller' as described in Tharaud et al. (1994), except for the time periods when the samples were taken, which were 0, 6, 24, 72, and 144 hours after inoculation.

The sensitivity to bacteriophages was determined by spot assays, as described by Expert and Toussaint (1985).

Results/Discussion

Subcloning and mutagenesis of a genomic ams *region*

We previously described cosmids functionally complementing the altered phenotype of the avirulent insertional EPS^- mutant PMV6089 (Table 1) which proved to harbour an insert from the *ams* gene cluster (Tharaud et al., 1994). Screening the genomic library for complementation of PMV6089, it eventually appeared that pPMV152 (Table 1, Figure 1A) was the shortest complementing cosmid extending towards an hitherto unexplored region. On the one hand, providing K. Geider's group with this plasmid stimulated the sequencing of the entire *ams* cluster (Bugert and Geider, 1995). On the other hand, in order to analyse the biology and physiology of the interactions with the plant we decided to obtain more insertional mutants in this region. The first step was to reduce the length of the DNA sequence complementing the mutation as shown in Figure 1A. For that purpose, DNA from pPMV152 was first deleted from internal *Bgl*II fragments. We checked that the resulting cosmid, named pPMV180 (Figure 1A), was able to complement the mutant phe-

f1

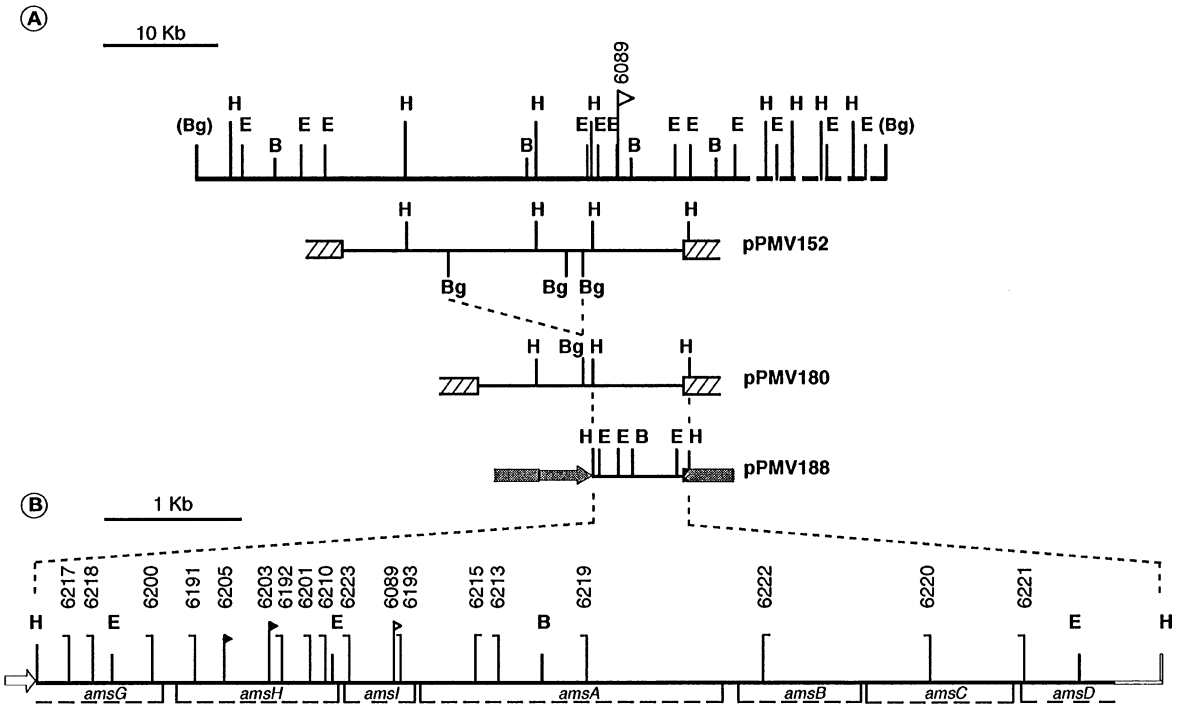

Figure 1. Physical map of an *Erwinia amylovora* chromosomal fragment complementing PMV6089 and mapping of the Tn3Gus mutants in the DNA insert of pPMV188.

A) The top line represents the restriction map of the chromosome of *Erwinia amylovora* in the studied region (▬). Restriction sites for *Bam*HI (B), *Bgl*II/*Sau*3A (Bg), *Eco*RI (E), and *Hin*dIII (H) are indicated. The *Bam*HI sites were not located in the right-hand part (▪▪▪▪). The location of the Mu*d*IIPR13 insertion in PMV6089 is marked (⚑). The flag (⚑) is oriented in the direction of transcription of the *LacZ′* reporter gene. Below the map, is illustrated the strategy for sub-cloning, from pPMV152, a chromosome fragment in pPMV188 which complements PMV6089: restriction sites as above mentioned except that Bg stands for *Bgl*II, *E. amylovora* DNA in the cosmids and plasmids (▬), pLA2917 DNA (▨), pRK767 DNA (▭), *LacZ′* promoter in pRK767 (⇨).

B) Enlargement of the *E. amylovora* chromosomal insert in pPMV188: restriction sites for *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H) are marked. The location of Tn3Gus (⚑, ⌜) or Mu*d*IIPR13 (⚑ for PMV6089) insertions are indicated. The flags (⚑, ⚑) and pointers (⌜) are oriented in the direction of transcription of the reporter gene. Flags indicate insertions which could be used to monitor gene expression *via* ß-galactosidase (⚑) or ß-glucuronidase (⚑) activity. The large arrow (⇨) corresponds to the *LacZ′* promoter from pRK767 in pPMV188. The genes *amsG*, *amsH*, *amsI*, *amsA*, *amsB*, *amsC*, and *amsD*, were located according to sequence data from Bugert and Geider (1995).

notype of PMV6089 and therefore carried the functional gene corresponding to the alteration in PMV6089.

Sub-cloning was further performed by introducing the 8.4 Kb *Hin*dIII fragment in both orientations into the vector pRK767, thus giving the plasmids pPMV188 (Fig. 1A) and pPMV189 (not shown). The cloned sequence was still able to complement the colony morphology and pathogenicity phenotypes of PMV6089 in the transconjugants containing pPMV188 but not in those containing pPMV189. This result indicated that the *E. amylovora* genes cloned in the insert of pPMV188 were expressed from the LacZ′ promoter present on the vector plasmid pRK767 (Table 1) (Gill and Warren, 1988) and that, therefore, the cloned fragment lacked any promoter activity upstream to the mutation in PMV6089. Actually the strong promotor in *amsG* is not present in pPMV188 (Fig. 1B) (Bugert and Geider, 1995). At the same time these data confirmed the direction of transcription of the complementing gene, which is from left to right on Figure 1, and indicated that all the *ams* genes upstream to the PMV6089 insertion were expressed from a single promoter. This was confirmed by LacZ or Gus fusion expression (data not shown) and is coherent with sequence data and transcription assays (Bugert and Geider, 1995).

Transposon-mediated mutagenesis of pPMV188 was performed with Tn3Gus as described in Materials and methods. The insertions from 33 pPMV188 deriv-

ative plasmids with a unique Tn3Gus insertion in the *E. amylovora* genomic insert were marker-exchanged in the chromosome of *E. amylovora* strain CFBP1430. The resulting $Km^R Tc^S$ *Erwinia amylovora* clones were named PMV6191 to PMV6225 (Table 1).

In order to determine if the Tn3Gus insertions had been transferred to the right place, Southern blot hybridization of *Hin*dIII digested genomic DNA from each km^R Tc^S clone was performed using pTn3Gus DNA as a probe. Twenty-two strains presented the hybridization patterns expected with a single Tn3Gus insertion (data not shown). Southern blot hybridization of *Eco*RI digested genomic DNA from some of these mutants (PMV6191, PMV6192, PMV6193, and PMV6196) was performed probing with pPMV188 DNA. The expected hybridization profiles were obtained (not shown) confirming that homologous recombination had occurred in the *ams* cluster. The locations of the mutational insertions were defined using restriction mapping and alignment with published sequences of specific *ams* genes (Bugert and Geider, 1995). However, given the loose accuracy of agarose gels, some insertions mapping close to a gene limit were yet assigned to this gene on the basis of phenotype and complementation data (Table 2, Figure 1B).

EPS and pathogenicity phenotypes of the Tn3Gus insertional mutants in the ams *cluster*

The Tn3Gus insertional mutants which proved to map in the targeted DNA fragment showed a non-mucoid colony aspect on agar plates. They were also resistant to infection by the phages 4LM, PEa1(h), and JV1. When assayed for pathogenicity on pear and apple seedlings, most of them were unable to induce any fire blight symptom. Mutants in *amsG*, PMV6200, PMV6217 and PMV6218, were able to induce local symptoms (vein- or petiole-necrosis) on most of the inoculated pear seedlings and on some apple seedlings. Although weakly virulent they appeared non invasive because stem necrosis was never observed. Weak necrosis on pear slices was also observed by Bugert and Geider (1995) with a non-mucoid $AmsG^-$ mutant. These authors also showed that, when grown on an asparagin-sorbitol medium, $AmsG^-$ mutants were able to produce a low but detectable amount of EPS as measured by cetylpyridinium chloride turbidity assay (Belleman et al., 1994; Bugert and Geider, 1995). Thus, when the *amsG* gene is mutated, the synthesis of EPS would be null or very weak in non-inducing media and slowed down even in inducing environment, for instance *in planta* (see below).

Functional complementation of Tn3Gus insertional mutations

Functional complementation of Tn3Gus insertional mutants by the plasmid pPMV188 confirmed the assignment of these mutants to the portion of the *ams* cluster inserted in pPMV188. For most mutants mucoidy of colonies as well as phage sensitivity and pathogenicity were restored in presence of pPMV188 (Table 2). To interpret such a result one has to remember that there was no complementation of the Ams^- mutant PMV6089 by pPMV189, holding the same *E. amylovora* genomic insert as the complementing plasmid pPMV188 but in the reverse direction. This indicates that the weak promotors which might exist upstream to individual genes (Bugert and Geider, 1995) either are not functional or, more probably, are not efficient enough. Therefore when complementation is observed, which is the case for several mutants in *amsG*, *amsH*, *amsI*, and *amsA*, it indicates that, even if there is a polar effect of Tn3Gus insertion, a multigene functional unit is transcribed and translated from the plasmid insert.

The mutants in *amsB*, *amsC*, and *amsD* were not complemented. The $AmsD^-$ mutant was not complemented because this gene is not entirely present in pPMV188. However, the failure of pPMV188 to complement $AmsB^-$ and $AmsC^-$ mutants suggests that Tn3Gus insertions in these genes are polar on downstream genes, when not included in pPMV188.

It is worth mentioning the surprising complementation profile of $AmsG^-$ mutants: although mucoidy was not restored in the presence of pPMV188, phage sensitivity and pathogenicity were fully and partially restored respectively. This indicates that the merodiploids may produce at least a low level of EPS such as some phage receptors are present. Similarly other Tn3Gus insertional mutants, although not fully complemented for pathogenicity, were complemented at least for mucoidy and phage sensitivity. This was also observed by Bugert and Geider (1995). Remembering that *amsG*, the most upstream gene in pPMV188, lacks at least its promoter region and that the *E. amylovora* genes cloned in the insert of pPMV188 are expressed from the LacZ′ promoter present on the vector, gene for gene complementation would be unlikely as far as *amsG* is concerned. The slight complementation of $AmsG^-$ mutants that was

Table 2. Phenotypes of the *ams* insertion mutants carrying plasmid pPMV188

Mutant strain[a]	*ams* locus[b]	Phenotype after complementation			
		Colony[c] Morphology	Phage[d] Sensivity	Symptoms[e] on Apple	Pear
PMV6217[f]	*amsG*	-	+	- ($5^{\#}$)	$\pm$ (2^{*} $5^{\#}$)
PMV6218[f]	*amsG*	-	+	$\pm$ (2^{*} $8^{\#}$)	$\pm$ (4^{*} $6^{\#}$)
PMV6200[f]	*amsG*	-	+	- ($2^{\#}$)	- ($6^{\#}$)
PMV6191[f]	*amsH*	+	+	$\pm$ (1^{*} $8^{\#}$)	$\pm$ (1^{*} $8^{\#}$)
PMV6205	*amsH*	+	+	$\pm$	$\pm$
PMV6203[f]	*amsH*	+	+	- ($7^{\#}$)	$\pm$ (7^{*} $3^{\#}$)
PMV6206	*amsH*	+	+	$\pm$	$\pm$
PMV6192	*amsH*	+	+	+	+
PMV6201[f]	*amsH*	+	+	$\pm$ (4^{*} $6^{\#}$)	$\pm$ (3^{*} $7^{\#}$)
PMV6210	*amsH*	+	+	+	+
PMV6223	*amsI*	-	$\pm$	$\pm$	$\pm$
PMV6193	*amsI*	+	$\pm$	ND	+
PMV6215	*amsA*	+	+	+	+
PMV6213[f]	*amsA*	+	+	- ($7^{\#}$)	+ (10^{*})
PMV6219	*amsA*	-	-	-	-
PMV6222	*amsB*	-	$\pm$	-	-
PMV6220	*amsC*	-	-	-	-
PMV6221	*amsD*	-	-	-	-

[a]: all mutants formed rough colonies on agar, were insensitive to the four EPS-specific phages (4LM, JV1, and PEa1(h)), and did not induce symptoms on apple and peer seedlings, except local symptoms in the case of the AmsG^{-} mutants and two AmsH^{-} mutants (PMV6191 and PMV6201).

[b]: from top to bottom the genes are ordered as they are from left to right on the map (Figure 1B). Similarly the mutants are ordered as are the insertions on the map.

[c]: morphology of colonies on agar plates: mucoid (+), non mucoid (-), intermediate ($\pm$).

[d]: clear plaques (+ = sensitivity), no plaque (- = resistance) or turbid plaques ($\pm$) when infected by any of the four EPS-specific phages.

[e]: pathogenicity assay on apple and pear young seedlings: (+) more than 8 plants out of 10 showed stem necrosis 10 days after inoculation; ($\pm$) between 2 and 7 plants presented stem necrosis 10 days after inoculation of 10 plants; (-) the 10 inoculated plants showed no stem symptom 10 days after inoculation; for some mutants with intermediate virulence (($\pm$) detailed data are given: (*) number of plants with stem necrosis at 10 days after inoculation of 10 plants; (#) number of plants with local symptoms at 10 days after inoculation of 10 plants (local symptoms: vein- or vein and petiole-necrosis on the inoculated leaf); ND: not determined.

[f]: these mutant strains were further studied for bacterial growth *in planta*. Moreover, for the derivatives of these strains supposed to contain pPMV188, the presence of the cosmid *in planta* was checked by isolating Km^{R} Tc^{R} colonies from the plants, 10 days after inoculation.

observed was thus phenotypical and apparently due to the presence of the plasmid. We cannot explain how this might result in a shift of EPS synthesis, perhaps via another sugar transferase coding gene, such as the EPS production would be sufficient to confer phage sensitivity but not enough for mucoidy nor for a proper interaction *in planta*.

Growth in planta

Altogether EPS and pathogenicity phenotypes indicate that functions involved in EPS synthesis were altered in Ams^{-} mutants and simultaneously affected their pathogenicity. The mutants located in *amsG* and, to a lower extent, PMV6191, an AmsH^{-} mutant, appeared to be able to induce local symptoms on host plants. The growth characteristics *in planta* have been postulated to explain these observations (Tharaud et al., 1994). To try this hypothesis, several mutants in genes *amsA*,

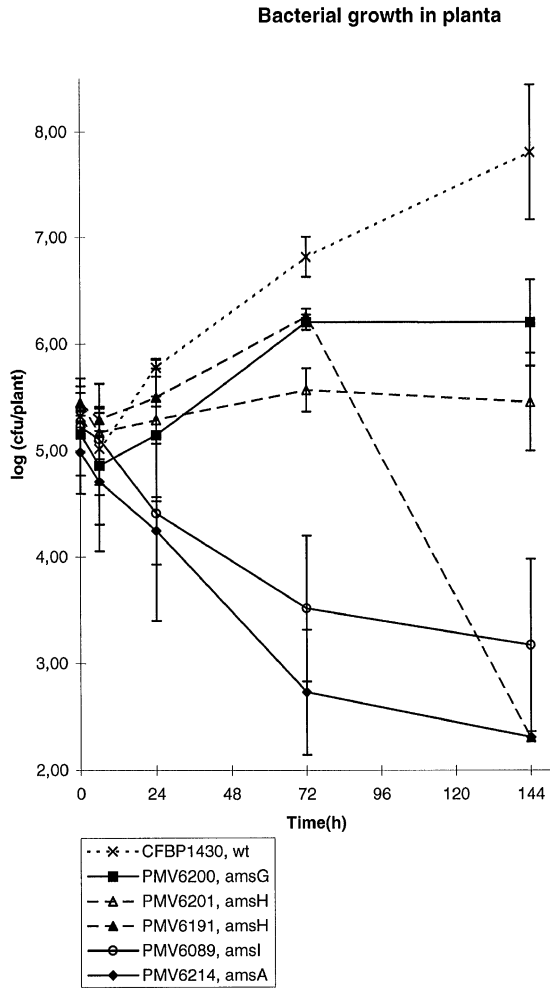

Figure 2. Bacterial growth *in planta* of wild type parental strain CFBP1430 and some *ams* mutants representative of each class of EPS and pathogenicity phenotypes.
Young pear seedlings, cv. 'Kirschensaller', were inoculated with ca 3×10^5 cfu per leaf. At intervals, five seedlings were separately crushed and dilutions were plated on King's B medium (King et al. 1954). Data are the mean determined from four or five replicate samples. Error bars indicate 1 standard deviation calculated from four or five replicate samples. Data points at 2.31 cfu/plant actually correspond to a lower number of cfu/plant, this value being the limit of detection.

amsG, *amsH*, and *amsI*, were assayed for growth on pear seedlings (Figure 2). It is noteworthy that all the studied mutants grew normally *in vitro* on complete and/or minimal medium.

As previously shown the population level of all strains decreased during the first 6 h after inoculation (Tharaud et al., 1994). The wild-type strain CFBP1430 then multiplied and reached about 10^8 colony forming units per plant after six days (144 h). Avirulent mutants showed one of the following growth characteristics (Figure 2): either a continuous decrease of the bacterial population or a stabilization at the inoculum level (around 3×10^5 cfu/plant). The AmsG$^-$ mutants, however, which induced local symptoms and the AmsH$^-$ mutant PMV6191, which gave local symptoms on half the inoculated pear seedlings, were able to reach a bacterial population of ca. 10^6 cfu/plant, at least at 3 days after inoculation (Figure 2). In the case of PMV6191, to explain that there was less than 2×10^2 cfu in each plant at 6 days, one might suppose that there was a sample effect: at 24 h, the population sizes ranged between 10^5 and 10^6 cfu/plant, the bacterial population may have decreased in some seedlings and it may have stabilized around 10^6 cfu/plant in other seedlings.

Together with previous work (Tharaud et al., 1994), these data indicate that there must be a continuous growth from six hours to at least 6 days after inoculation for expression of total virulence. This suggests that a minimal level of EPS production is necessary for plant invasion by the bacteria. This might reflect either a direct role of EPS in the multiplication of the bacteria and/or intercellular movement, or an indirect role by protecting the bacteria against host defence reactions, or both. It is worth mentioning that the local symptoms induced by AmsG$^-$ mutants concern a larger number of attacked plants when the host is a rather sensitive one, like the pear cultivar 'Kirschensaller', than when it is less sensitive, like the apple cultivar 'Golden delicious'. This argues for a role of EPS to help the bacteria overtaking the host defence reactions either by physical protection or by specific interaction.

Conclusion

The capsular polysaccharides are considered to be determinative in the ability of bacteria to colonize a specific niche. They may protect the bacterial cells against desiccation. EPS has also been suggested to be a requisite for the survival and growth of bacteria in the plant, for instance as a binder of nutrients and cations (Coplin and Cook, 1990; Leigh and Coplin, 1992; Tharaud et al., 1994). EPS$^-$ bacterial cells, however, present the same ability as capsulated cells to induce electrolyte leakage from plant cells suggesting a passive role of EPS at least in the electrolyte leakage process (Brisset and Paulin, 1992). It has been postulated a regulatory function for the capsule of *E. amylovora* cells in the infection process and the disease progress (Hignett and Roberts, 1985; Hignett, 1988).

EPS$^-$ mutants of *E. amylovora* are agglutinated by plant agglutinins isolated from pear, whereas EPS$^+$ strains are not, it has therefore been postulated that EPS may protect bacteria against agglutination inside the host tissue (Bradshaw-Rouse et al., 1981; Romeiro et al., 1981). One aspect of the role of EPS in pathogenesis would be to mask the presence of bacteria in the plant (Newman et al., 1994). More generally, as it is the case for other plant pathogenic bacteria, EPS might play a major structural role in colony development *in planta* and modify the environment to which bacterial cells are exposed within the intercellular space (Brown et al., 1993). Particularly EPS may allow the invading bacteria to prevent plant defence reactions, including specific recognition steps (Leigh and Coplin, 1992; Parniske et al., 1994) possibly leading to host-specificity (Kingsley et al., 1993).

This study focused on the biology and the physiology of the interactions of *Erwinia amylovora* with host plants. The upstream region of the *ams* genes cluster of *E. amylovora*, which is involved in the exopolysaccharide amylovoran synthesis, was characterized with regards to diverse biological and pathogenicity properties of bacterial insertional mutants.

In order to get information about the function and the expression of the genes in the upstream part of the *ams* region, an 8.4 kb-long wild-type *E. amylovora* genomic insert was mutagenized by Tn3Gus insertion. Chromosomal mutants in genes *amsG*, *amsH*, *amsI*, *amsA*, *amsB*, *amsC*, and *amsD* were obtained by marker exchange.

The phenotypes of our Tn3Gus insertional mutants showed a correlation between EPS production as revealed by colony morphology and phage sensitivity, pathogenicity on apple and pear seedlings, and growth *in planta* (pear seedlings). From our data, which involve several mutants in each of the four most upstream genes in the *ams* region, we were able to extend the results of Bugert and Geider (1995), who obtained only one mutant in each gene. Moreover the analysis of the growth *in planta* gives a more complete view of the role of EPS in the disease process.

In brief, all the mutants presented non mucoid, phage resistant colonies and most of them were avirulent, but those mapping in *amsG*, which were not invasive although they repeatedly induced localized symptoms. Particularly AmsG$^-$ mutants were able to multiply *in planta* such as a plateau at ca. 10^6 cfu per plant was reached 3 days post inoculation, whereas mutants in the other *ams* genes failed to multiply so that their population either decreased or stabilised at the initial inoculum level. This indicates that the ability of the bacterium to rapidly reach a minimal population level is essential for the development of fire blight symptoms.

In conclusion, we could make it clear that there is a need for a minimal level of EPS production by *Erwinia amylovora* for sufficient growth *in planta*, which in turn is required for pathogenicity. The different degrees of symptom development exhibited by two hosts with different susceptibility to fire blight suggest that EPS might help *Erwinia amylovora* to overcome the host defence reactions possibly by specific interaction. This is consistent with data from a study of the protection against fire blight by avirulent mutants (Tharaud et al., 1997). Amylovoran might play a crucial role in the recognition process between the bacterial and the plant cells, which appears necessary for induction of the disease (Cooper et al., 1990), the virulent strains being able to invade the host by preventing and/or avoiding its defence reactions. As proposed for the exopolysaccharides from other bacteria interacting with plant, either in symbiotic or in pathogenic interactions (Kingsley et al., 1993; Niehaus et al., 1993; Sundin et al., 1996), amylovoran would be involved in a complex process affecting the plant defence system and necessary for the colonization of the plant by the bacterial cells.

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