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Characterization of the *ams*I gene product as a low molecular weight acid phosphatase controlling exopolysaccharide synthesis of *Erwinia amylovora*

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Abstract The *ams* region, responsible for amylovoran synthesis of the fireblight pathogen *Erwinia amylovora*, contains the gene *ams*I encoding a 144 amino acid protein with homology to mammalian low molecular weight acid phosphatases [Bugert and Geider (1995) Mol. Microbiol. 15, 917–933]. A DNA fragment with *ams*I was cloned under the control of the *lac* promoter on a high copy number plasmid. The gene product of *ams*I is about 17 kDa in a protein expression system and had the enzymatic activity of an acid phosphatase. This is the first report about a low molecular weight acid phosphatase activity in prokaryotes. As part of the large *ams* transcript, expression of *ams*I was affected by the activator proteins RcsA and RcsB. Overexpression of *ams*I in *E. amylovora* caused a strong increase of acid phosphatase activity, but additionally a strong reduction in EPS synthesis, phenotypically similar to a mutation in the gene. The gene product may participate in changes of phosphorylation required for the biosynthesis of EPS such as recycling the lipid carrier diphosphate to the monophosphate form.

Key words: EPS capsule; Amylovoran synthesis; Lipid carrier; Phytopathogen; Gene expression

1. Introduction

Erwinia amylovora, the causative agent of fireblight on apple and pear trees and other rosaceous plants, synthesizes the capsular exopolysaccharide (EPS) amylovoran. The EPS is strictly necessary for pathogenicity of *E. amylovora* [1,2]. It is synthesized from a repeating unit, which consists of four differently linked galactose molecules and a glucuronic acid residue [3]. Most likely, the repeating unit is assembled at undecaprenyl phosphate, a phosphorylated lipid carrier, transported to the cell surface and polymerized to high molecular weight EPS. The lipid carrier is recycled from the diphosphate to the monophosphate form. The step requires the action of a phosphatase. Phosphorylation may be necessary during transport of the repeating unit. The amino acid sequence of an open reading frame (ORF) adjacent to *ams*I has significant homology to ABC transporters [4]. Subsequent removal of phosphate groups in this process could also require a phosphatase function.

Phosphorylation steps are ubiquitous in all cells [5]. Regulatory proteins are also adjusted for their activity by phosphorylation. The osmosensor EnvZ in *Escherichia coli* is autophosphorylated and transfers phosphoryl groups to OmpR. Porin expression is apparently regulated by phosphorylation. For the plant tumor inducing bacterium *Agrobacterium tumefaciens*, the sensor/activator proteins VirA/VirG have been shown to act in this manner [6]. Here, we will show the enzymatic function of AmsI as an acid phosphatase and the effect of the gene on amylovoran synthesis.

2. Materials and methods

2.1. Bacterial strains and plasmids

These are listed in Table 1.

2.2. In vivo protein expression

The cloning vector pBluescript KS(+) contains the T7 promoter in the direction opposite to the *lac* promoter near the multicloning site. The plasmids pEA129 and pEA133 with *ams* genes, cloned in the direction of the T7 promoter and pBluescript without an insert, were introduced into the *E. coli* strain K38(pGP1-2) for expression of the encoded proteins. According to the protocol published by Tabor and Richardson [7], overnight cultures of the strains were diluted into fresh LB medium and were grown at 30°C to an A_{590} of about 0.4. The cells were pelleted and resuspended in M9 minimal medium supplemented with 0.02% of 18 amino acids (without cysteine and methionine). After 2 h growth at 30°C, the cultures were incubated at 42°C to induce T7 RNA polymerase expression. The host RNA polymerases were then blocked by the addition of rifampicin (200 μg/ml final concentration) and after 10 min the culture was further incubated at 30°C. A 0.5 ml aliquot of cells was pulsed with 10 μCi of [^{35}S]methionine for 5 min. The cells were spun down, resuspended in 100 μl cracking buffer (60 mM Tris-HCl, pH 6.8; 6 mM 2-mercaptoethanol; 10% glycerol, 0.01% bromophenol blue) and heated to 95°C for 3 min. 40 μl aliquots were loaded on an SDS polyacrylamide gel. The gel was then treated with an enhancer (Amplify; Amersham), dried and exposed to an X-ray film for 1–3 days.

2.3. Phosphatase assays

For the extraction of total proteins, cells from a 2 ml overnight culture in LB were resuspended in 200 μl 0.1 M sodium citrate buffer (pH 5.6) containing 1% Tween 20. The cells were then frozen in liquid nitrogen for 1 min and thawed in a 37°C waterbath for 5 min. Freezing and thawing was repeated twice followed by 10 min centrifugation. An aliquot of the supernatant was used to determine the total protein content in a Lowry assay. To assay acid phosphatases, up to 100 μl of the supernatant was added to 1 ml substrate solution (1 mg/ml 4-methyl-umbelliferyl-phosphate or 30 mM 4-nitrophenyl phosphate in 0.1 M sodium citrate buffer, pH 5.6) and incubated for 30 min at room temperature. Excitation/emission at 365/455 nm was measured in a spectral photofluorometer (Aminco-Bowman). The absorbance of 4-nitrophenol was determined at 405 nm. For assays of alkaline phosphatase and for cell lysis, the citrate buffer was replaced by 0.1 M glycine buffer (pH 10.4), 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, and after 60 min incubation at 28°C, the reaction was stopped by the addition of 2 ml 0.5 M sodium hydroxide solution.

2.4. CPC turbidity assays

CPC turbidity assays and other techniques were described previously [4].

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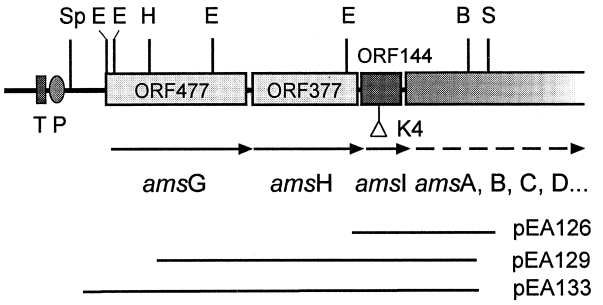

Fig. 1. Map of the early *ams* region. The genes *ams*G (ORF477), *ams*H (ORF377), and *ams*I (ORF144) and various subclones from that region are indicated. T, terminator for transcription; P, promoter of the *ams* operon. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Sp, *Spe*I; K4, insertion site of pfdA2 cassette in mutant Ea7/74-K4.

...SILVVCIGNICRSPTGERLL... *E. amylovora* -AmsI
...NILVVCVGNICRSPTAERLL... *E. coli* -Wzb
...TILVVCIGNICRSPMAQALL... *P. solanacearum*-EpsP
...SVLFVCLGNICRSPIAEAVF... ACP1-Bf (P24666), -Bs (P24667), and BLACP-PTPase (P11064)
* ** *******

Fig. 2. Alignment of amino acids at the N-terminus of AmsI with mammalian acid phosphatases and deduced sequences from bacterial genes. The region shown is supposed to carry the active site for the enzymatic function [10]. Identical amino acids (*) are marked. The complete sequences can be found in references [4] (AmsI), [8] (EpsP), [9] (Wzb) or in the SWISS-PROT Protein Sequence Database (the accession numbers are given in parentheses).

3. Results

3.1. Map of the left end of the ams region and amino acid homologies of AmsI

The *ams* operon of *E. amylovora* encodes for synthesis of the complex exopolysaccharide amylovoran [4]. Its nucleotide sequence revealed 12 ORFs, which are transcribed as a large mRNA. Homology searches in nucleotide sequence data libraries have suggested various functions for individual genes. A physical map of the *ams* start is shown in Fig. 1. The first gene, *ams*G, may encode a galactosyl transferase, and *ams*H, the second gene, may encode a product located in the membrane. AmsI had a striking homology to low molecular weight acid phosphatases with a highly conserved domain at the N-terminus (Fig. 2). These activities and the corresponding genes were first described for mammalian cells, and nucleotide sequence data suggest that similar genes may also occur in many plant-associated bacteria. The three early genes of the *ams* operon of *E. amylovora* were expressed in a protein expression system, and protein extracts were assayed for acid phosphatase activities.

3.2. Products of early ams genes visualized on protein gels

A T7 RNA polymerase expression system [7] was used to visualize the proteins encoded by *ams*G, H and I, which are early genes in the *ams* operon. The *E. coli* strain K38(pGP1-2) carries the plasmid for expression of T7 RNA polymerase under control of the heat inducible λ*p*L promoter. The plasmid pEA129 with the cloned *E. amylovora ams*H and *ams*I genes, and the plasmid pEA133 with the cloned *ams*G gene in the direction of the T7 promoter in pBluescript KS(+) were introduced into the strain K38(pGP1-2). As a negative control, strain K38 was transformed with the cloning vector pBluescript. After induction of the T7 RNA polymerase, the bacterial RNA polymerases were blocked by the addition of rifampicin. To label the proteins encoded by the cloned DNA fragment, the culture medium was then supplemented with [^{35}S]methionine. Total proteins were extracted and aliquots were loaded onto a polyacrylamide gel. Even after 3 days of exposure no signal was seen in the control lane with K38(pGP1-2, pBluescript) (Fig. 3, lane 1). For clone pEA133, one band was detected corresponding to a molecular weight of about 57 kDa (Fig. 3, lane 2). The calculated molecular weight of the encoded *ams*G gene is 55.1, so the protein band represented the *ams*G gene product. The *ams*H and *ams*I genes on plasmid pEA129 expressed three distinct bands around 42, 39 and 17 kDa (Fig. 3, lane 3). DNA sequence analysis [4] suggested that AmsH contains an N-terminal secretory signalling sequence, which is typical for lipoproteins recognized by the signal peptidase II. The cytoplasmic AmsH precursor protein has a calculated molecular weight of 41.4 kDa (377 amino acids), and the exported and processed form

Table 1
Bacterial strains and plasmids used in the experiments

Strain	Properties	Source or reference
E. coli		
E. coli K38	with pGP-2 for T7 expression system	[7]
E. amylovora		
Ea1/79	wild type strain	[19]
Ea1/79-MG	Tn5 in *rcs*A, Km	[20]
Ea7/74-K4	insertion mutant in *ams*I	[4]
Plasmids		
pBluescript KS(+)	*lac*Z′ linker, ori colE1, Ap	Stratagene
pBGS18	*lac*Z′ linker, ori colE1, Km	[21]
pBGS18-126	insertion fragment from pEA126 recloned in pBGS18, Km	This work
pfdC1Z′	ori fd, Km	[22]
pfdC1Z′-*rcs*B2	Ea-*rcs*B on multicopy plasmid, Km	[23]
pEA101	*rcs*A in pSUP106, Cm, Tc	[20]
pEA126	1.5 kb *Eco*RI-*Sal*I subclone from pEA109 with the left hand end of the *ams* region in from pEA126, Ap	[4]
pEA129	3.5 kb *Hin*dIII subclone from pEA109-del100 with *ams*H and *ams*I in pBluescript KS(+), Ap	[4]
pEA133	2.2 kb *Spe*I/*Pst*I subclone from pEA131 with *ams*G in pBluescript KS, Ap	This work and [4]

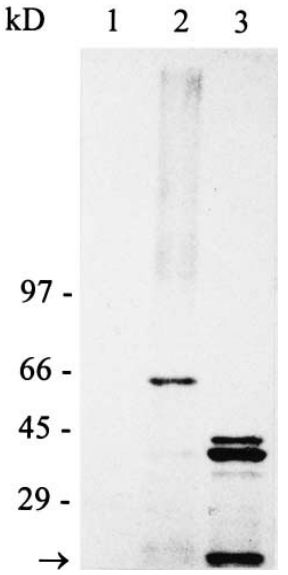

Fig. 3. PAGE of proteins encoded by *E. amylovora amsG*, H and I genes. The proteins were labeled with [^{35}S]methionine in a T7 RNA polymerase expression system. The cloning vector pBluescript KS(+) was used as a negative control (lane 1). The plasmid clone pEA133 (lane 2) carries the *amsG* gene, and pEA129 (lane 3) the *amsH* and I genes on pBluescript in the direction of the T7 promoter. The location of size marker protein bands is indicated on the left hand side, the position of AmsI is marked by an arrow.

with 357 amino acids has an estimated size of 39.2 kDa. The two bands at around 40 kDa visualized on the protein gel might represent the precursor and the processed AmsH protein. The smaller protein band at 17 kDa represented the *amsI* gene product with a calculated molecular weight of 15.7 kDa.

3.3. Enzymatic activity of the amsI gene product

To study the enzymatic activities of AmsI, the 1.6 kb *Eco*RI/*Sal*I fragment with the complete *amsI* gene in pEA126 was inserted into vector pBGS18 and gave plasmid pBGS18-126. After transformation into *E. amylovora* strain Ea1/79, plasmid pBGS18-126 expressed the *amsI* gene constitutively via the *lac* promoter. The phosphatase assays were performed under acidic (sodium citrate buffer, pH 5.6) and alkaline (glycine buffer, pH 10.4) conditions with the fluorescent substrate 4-methyl-umbelliferryl-phosphate. The use of *o*-nitrophenyl phosphate substrate produced comparable results and was used to determine the linearity of the enzyme assays (Fig. 4). Strain Ea1/79(pBGS18-126) overexpressing *amsI* exceeded the activity of the wild type strain Ea1/79 considerably at pH 5.6, and cleavage of the substrate was not found at pH 10.4. Thus, AmsI had enzymatic properties of an acid phosphatase based on low phosphatase activities under alkaline conditions in extracts of Ea1/79 and Ea1/79(pBGS18-126).

3.4. Phosphatase activity of an amsI mutant and of rcs-regulatory mutants

4-Methyl-umbelliferyl-phosphate was used as substrate to assay extracts of wild type strains and mutants of *E. amylovora*. A small decrease of phosphatase activity was observed for the *amsI* mutant Ea7/74-K4 compared to the wild type strain Ea1/79 (Fig. 5). These low activities seem to be close to the background of other phosphatase activities of *E. amylovora*. As a part of the *ams* operon, the *amsI* gene is assumed to be regulated by genes connected to expression of amylovoran synthesis. Kinetics for acid phosphatase were performed for the strains Ea1/79, the *rcsA* mutant Ea1/79-MG and Ea1/79 with overexpression of RcsA or RcsB, which are in-

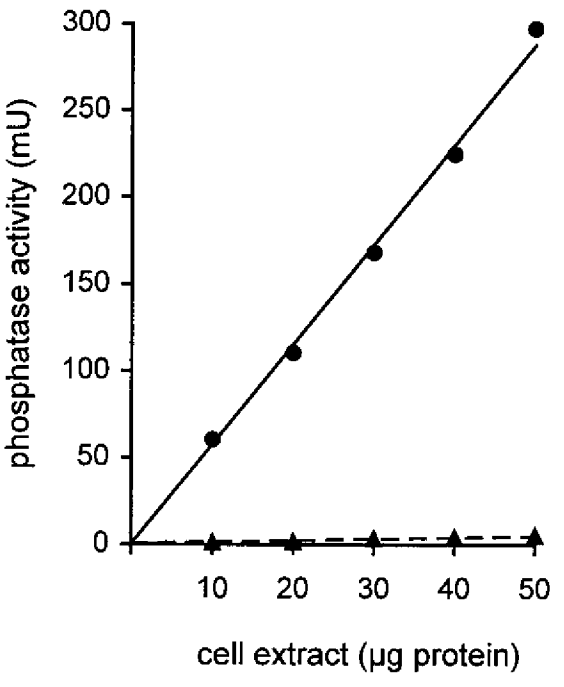

Fig. 4. Acid phosphatase activity of cell extracts from an *E. amylovora* wild type strain and from a strain with overexpression of *amsI*. Cells of the wild type strain Ea1/79 (▲) and Ea1/79(pBGS18-126) (●) were grown overnight in LB medium. Acid phosphatase assays were performed after extraction of total proteins. The color of 4-nitrophenol released was measured in a photometer at 405 nm, and units of activity were calculated according to Bergmeyer [24].

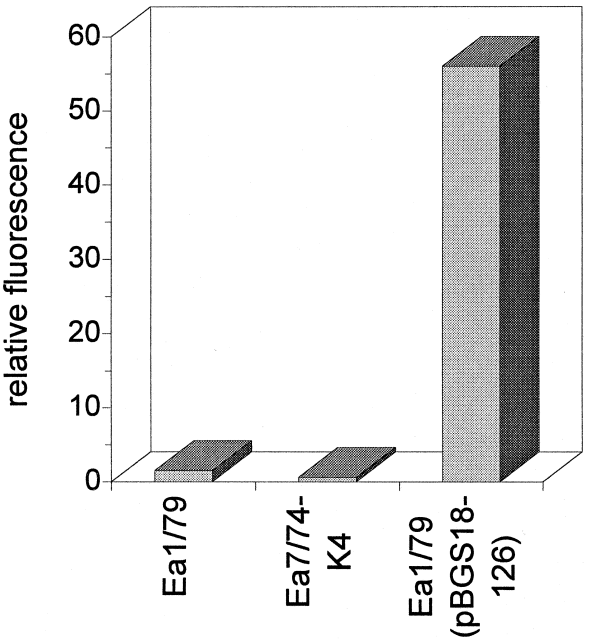

Fig. 5. Acid phosphatase activity of various *E. amylovora* strains. Ea1/79, wild type; Ea7/74-K4, mutated in *amsI*; Ea1/79(pEA126) with overexpression of *amsI*. The assays were done with 4-methyl-umbelliferyl-phosphate as substrate.

volved in the activation of exopolysaccharide synthesis (Fig. 6). A significant increase of phosphatase activity was found after overexpression of the two regulators. In these experiments, the acid phosphatase activity was slightly higher for overexpression of RcsA than for RcsB.

3.5. *Interference of amsI overexpression with EPS synthesis of E. amylovora*

The effect of *ams*I overexpression on EPS synthesis was analyzed by determination of amylovoran in CPC turbidity assays. The wild type strain with the cloning vector pBGS18 showed a slight reduction in EPS synthesis, which is common for an *E. amylovora* strain harboring a high copy number plasmid. The EPS production of the strain Ea1/79(pBGS18-126) was much lower than in the wild type strain (Fig. 7) indicating a considerable inhibition of EPS synthesis by overexpression of *ams*I. On the other hand, the *ams*I-negative mutant Ea7/74-K4 was also deficient in EPS synthesis [4]. Obviously, a balanced amount of AmsI is required for high synthesis of amylovoran.

4. Discussion

The *ams*I gene was previously characterized as the third ORF in the *ams* operon of *E. amylovora*, and the deduced amino acid sequence showed significant homology to low molecular weight acid phosphatases from mammalian cells [4]. Genes related to low molecular weight mammalian phosphatases have also been described for the *eps* cluster of *Pseudomonas solanacearum* [8] and a chromosomal region encoding colanic acid synthesis of *E. coli* [9]. The proteins from mammalian cells share a conserved domain in the N-terminus that is required for the enzymatic activity [10]. Deduced by nucleotide sequence analysis, the predicted AmsI protein has a molecular weight of 15.7 kDa [4], and expression of the gene in a T7 RNA polymerase promoter system revealed a 17 kDa protein. Another closely related gene with 89% similarity in the corresponding amino acid sequence was found in the *cps* cluster of *E. stewartii* [11], and its expression on cosmid pRP449 could complement the *E. amylovora ams*I mutant Ea7/74-K4 for amylovoran synthesis (K. Geider and D. Coplin, unpublished). Here, we describe for the first time the enzymatic function of a bacterial low molecular weight acid phosphatase and its involvement in EPS production. Both a chromosomal mutation in *ams*I [4] and the overexpression of *ams*I in *E. amylovora* diminished amylovoran synthesis drastically. Its role in EPS synthesis could be the dephosphorylation of lipid carrier diphosphates, which have to be recycled to the monophosphate form, when the repeating unit has been polymerized into amylovoran. Only acid but not alkaline phosphatases are active on polyprenols longer than C35 [12,13]. The initial step in biosynthesis of various bacterial polysaccharides is the formation of an undecaprenyl (C55)-linked sugar-1-diphosphate [14]. EPS synthesis may be blocked in the *ams*I mutant due to an accumulation of lipid carrier diphosphates. AmsI could also dephosphorylate lipid carrier monophosphates when the enzyme is present in high amounts. This would explain that *E. amylovora* strains with *ams*I overexpression produce little EPS, because dephosphorylated, inactive lipid carrier molecules may accumulate in the cytoplasmic membrane. In this model, a low level of AmsI keeps the lipid carrier in the monophosphate form and thus regulates EPS synthesis in the initial step of polysaccharide subunit synthesis.

On the other hand, AmsI might also participate in an adjustment of phosphorylation in transport processes of repeat-

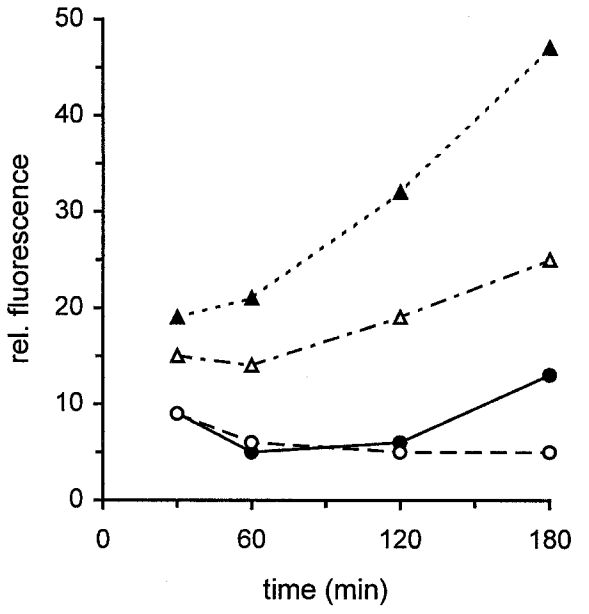

Fig. 6. Time course for acid phosphatase activity of *E. amylovora* strains with different regulation of EPS synthesis. Ea1/79, wild type strain (●); Ea1/79-MG, *rcs*A mutant (○); Ea1/79(pEA101), overexpression of *rcs*A (▲), and Ea1/79(pfdC1-RB2) (△) carrying the *rcs*B gene. The assays were done with 4-methyl-umbelliferyl-phosphate as substrate.

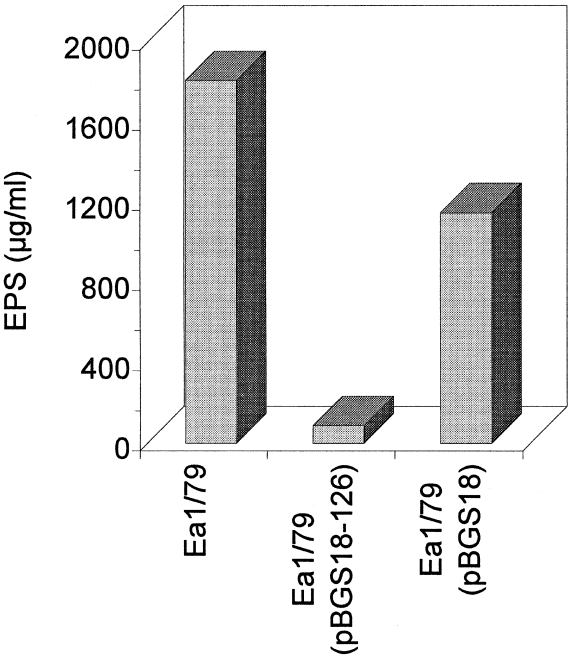

Fig. 7. The effect of *ams*I overexpression on the EPS production of *E. amylovora*. EPS was measured in CPC turbidity assays with supernatants of cell cultures grown for 2 days in MM2. Ea1/79, wild type strain; Ea1/79(pBGS18), wild type with the cloning vector pBGS18; Ea1/79(pBGS18-126), overexpressing *ams*I. The amounts of EPS were calculated from a standard curve.

ing units as suggested for *E. coli* [9]. Transport of molecules across cellular membranes is mediated by proteins, which were classified as ABC transporters [15]. They contain ATP-binding sites and transfer phosphoryl groups during transport processes.

Regulatory proteins could be another important target for a phosphatase function [5]. In analogy to *E. coli* [16], it can be assumed that RcsC acts as the sensor, which activates RcsB by phosphorylation. The control of phosphorylation of RcsB may not be a dominant task of AmsI, since overexpression and mutation of *ams*I should affect amylovoran production diametrically.

Regulation of sugar uptake has been associated with phosphorylation of enzyme IIA of the PTS system [17]. Finally, AmsI may catalyze sugar transphosphorylation as recently described for a phosphotransferase from *Morganella morganii* [18]. At present, there is no evidence that sugars in the repeating unit of amylovoran are transiently phosphorylated. That leaves the ultimate role of AmsI open, until intermediate stages in amylovoran synthesis are characterized for *ams*I-deficient strains.

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