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**Role of Exopolysaccharides in the Survival and
Pathogenesis of the Fire Blight Bacterium *Erwinia
amylovora***

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ABSTRACT

Role of exopolysaccharides in the survival and pathogenesis of the fire blight bacterium *Erwinia amylovora*.

Fireblight, a disease of apples and pears, is caused by *Erwinia amylovora*. Mutants of *E. amylovora* that do not produce the extracellular polysaccharide (EPS), amylovoran, are avirulent. A similar EPS, stewartan, is produced by *E. stewartii*, which causes Stewart's wilt of corn, and which has also been implicated in the virulence of this strain. Both stewartan and amylovoran are type 1 capsular polysaccharides, typified by the colanic acid slime produced by *Escherichia coli*. Extracellular polysaccharide slime and capsules are important for the virulence of bacterial pathogens of plants and animals and to enhance their survival and dissemination outside of the host. The goals of this project were to examine the importance of polysaccharide structure on the pathogenicity and survival properties of three pathogenic bacteria: *Erwinia amylovora*, *Erwinia stewartii* and *Escherichia coli*. The project was a collaboration between the laboratories of Dr. Gutnick (PI, *E. coli* genetics and biochemistry), Dr. Coplin (co-PI, *E. stewartii* genetics) and Dr. Geider (unfunded collaborator, *E. amylovora* genetics and EPS analysis). Structural analysis of the EPSs, sequence analysis of the biosynthetic gene clusters and site-directed mutagenesis of individual *cps* and *ams* genes revealed that the three gene clusters shared common features for polysaccharide polymerization, translocation, and precursor synthesis as well as in the modes of transcriptional regulation. Early EPS production resulted in decreased virulence, indicating that EPS, although required for pathogenicity, is not always advantageous and pathogens must regulate its production carefully.

OBJECTIVES

Fireblight, a disease of apples and pears, is caused by *Erwinia amylovora*. Mutants of *E. amylovora* that do not produce the extracellular polysaccharide (EPS), amylovoran are avirulent. A similar EPS, stewartan, is produced by *E. stewartii*, which causes Stewart's wilt of corn, and which has also been implicated in the virulence of this strain. Both stewartan and amylovoran are type 1 capsular polysaccharides, typified by the colanic acid slime produced by *Escherichia coli*.

Recent work in our laboratories, as well as those of others, has shown that the genes encoding the synthesis of these polysaccharides are located in clusters that have similar genetic organization and regulation and share several common alleles. The overall goal of this project was to determine the structure-function relationships between EPS structure and virulence in *E. amylovora* by genetically altering amylovoran structure by transferring *cps* genes for stewartan and colanic acid production into EPS⁻ *ams* mutants of *E. amylovora*. Subsequently strains producing such genetically modified capsules were to be examined for pathogen virulence. Specific objectives were: 1) To construct transgenic *E. amylovora* strains that produce new or altered EPS by introduction of the entire *cps* gene clusters from *E. stewartii* and *E. coli*. 2) To determine the importance of capsule type on the ability of *E. amylovora* to survive adverse environmental conditions and to colonize blossoms. 3) To determine the role of EPS in the infection and colonization of apple tissue. 4) To further characterize the *E. stewartii* and *E. coli* *cps* gene clusters in order to obtain biosynthetic enzymes that will lead to the modification of amylovoran structure and function.

As originally planned, Objectives 2 and 3 were to be carried out with *E. amylovora* *ams* mutants complemented with cosmids containing entire *cps* clusters from *E. stewartii* and *E. coli*. Although we were initially successful in partially restoring EPS production to various *ams* mutants in this manner, we did not produce any strains that produced both an altered amylovoran and normal amounts of that EPS. Consequently, these strains did not show significant differences in blossom colonization (Obj. 2) and were completely avirulent in tree inoculations (Obj. 3). As a result further work along these lines was postponed. During the second year of the project, the complete sequences of the polysaccharide gene clusters for all three bacteria and the corresponding chemical structures of their EPSs became available. The structures of amylovoran and stewartan differed by only three bonds and the *ams* and *cps* gene clusters differed by only three enzymes, thereby accounting for the difference in structure. On the other hand, colanic acid and its gene cluster in *E. coli* were very different in structure and the genes present. We then realized that further efforts to alter EPS synthesis would have to come from introduction of specific *E. stewartii* *cps* genes into *E. amylovora* and that further characterization of the *E. coli*

cps gene cluster would not be directly relevant. At the time we wrote the proposal, we were aware that a simple approach to altering EPS structure might not work. Consequently, we included a complete characterization of the genetics and biochemistry of stewartan synthesis as Objective 4 so that we could take a rationale and defined approach to engineering changes in amylovoran if an empirical one did not work. This objective became the major focus of the second half of the project. We have accomplished most of Objective 4 and even expanded it to include some aspects of the regulation of colanic acid and stewartan synthesis.

This was originally a three-year project, but due to personnel problems and technical difficulties we requested and received a no-cost extension for an additional year to complete as many of our objectives as possible within our budget.

RELEVANT DATA, METHODOLOGY, RESULTS AND DISCUSSION

RELEVANT WORK FROM THE LABORATORIES OF OUR COLLABORATORS.

EPS structures. After this project started, our colleagues Andrew Mort and Manfred Nimtz reexamined the structures of stewartan and amylovoran using purified stewartan (provided by Dr. Coplin's lab). The two polysaccharides can now be seen to be very similar (Fig. 1). The only differences are: 1) amylovoran has a galactose in the backbone where stewartan has a glucose, and 2) the major side-chain of amylovoran terminates with a pyruvate rather than a glucose moiety. The single glucose, linked to the branched galactose of stewartan is also found in amylovoran, but is present on only 10% of the residues. Apparently *E. amylovora* has the transferase to add this glucose side-chain, but it is not very active. Thus, only three enzyme differences are needed to account for the different structures of the amylovoran and stewartan subunits. As indicated below, these are likely to be CpsD, G, and F and AmsC, D, and E, respectively. Dr. Gutnick studied the structure of colanic acid produced in *E. coli* K-12 (strain JT4000) with Dr. S. Carmeli (Tel-Aviv University) and they have confirmed the basic subunit structure of colanic acid.

Comparison of the *cps* and *ams* gene clusters from *E. stewartii* and *E. amylovora*.

Amylovoran and stewartan synthesis are the functions of the *ams* and *cps* gene clusters, respectively. The nucleotide sequence of 18.5 kb from the *E. stewartii* *cps* cluster was determined (see Obj. 4 below) in Dr. Coplin's laboratory and the nucleotide sequence of the *E. amylovora* *ams* cluster was later completed in Dr. Klaus Geider's laboratory in Germany. Comparison of these clusters involved a collaboration between Drs. Coplin and Geider. In *E. stewartii*, 13 ORFs (Fig. 2) are arranged in the order *cpsA, B, I, C, D, E, F, G, H, J, K, L* and *M*. The predicted amino acid sequences of the CpsA, B, I, C, H, J, K, L and M proteins are over 59% identical (>80% similar) to the products of *E. amylovora* *ams* genes mapping at the same position. Data base searches suggested functions for several gene products. CpsA/AmsG have strong homology to RfbP and may therefore add galactose to the lipid carrier. CpsB/AmsH and CpsC/AmsA are membrane proteins and CpsI/AmsI have homologies to acid phosphatases. The latter three proteins appear to be a common feature of EPS gene clusters in other enteric bacteria, including *E. coli* and *Klebsiella pneumoniae*. They probably encode a transmembrane structure for the export and polymerization of the repeating oligosaccharide unit. The CpsD, E, F, G and K and AmsB, C, D, E and K proteins have homology to a number of different glycosyl-transferases involved in LPS, LOS, and Group II capsule synthesis. These proteins exhibit only 16-37% identity, except for CpsE, which is 74% identical to AmsB, and CpsK, which is 71% identical to AmsK. Furthermore, the first four transferase genes are arranged differently in the central region of the gene cluster. CpsH/AmsF and CpsJ/AmsJ also have similarity to hypothetical proteins found in other polysaccharide gene clusters and may likewise function in subunit assembly. CpsL and AmsL are membrane proteins that may be part of the export apparatus. CpsM and AmsM have homology to GalU and may function in synthesis of UDP-glucose. These results indicate that the *ams* and *cps* gene clusters share a common arrangement and consist of genes for initiating subunit synthesis on the lipid carrier (*cpsA*), exporting the subunit (*cpsB, I, C*), and nucleotide sugar synthesis (*cpsM* and *galE*) that flank a variable region for assembly of the subunit (*cpsD, E, F, G, H, J, K*).

Functions of Ams proteins. Dr. Geider's group investigated the functions of the *ams* genes in parallel with similar studies in our laboratories on the functions of the *cps* genes. Their results became available to us subsequent to the termination of this

project . They have proposed the following model (Table 1) based on the incorporation of labeled nucleotide sugars into lipid-linked oligosaccharides by various *ams* mutants. AmsG adds Gal to the lipid carrier. AmsD and AmsC add the next two Gal residues to the backbone to form lip-P-P-Gal-Gal and lip-P-P-Gal-Gal-Gal, respectively. Next, AmsB and AmsK add GlcA and another Gal to form lip-P-P-Gal-Gal-Gal-GlcA-Gal, but the order in which they act is not known. AmsE probably adds a pyruvate to the end of the major sidechain to give P-P-Gal-Gal-Gal-GlcA-Gal-pyr and AmsJ may add a branched Glc to the third Gal on a small fraction of the subunits. Recently, AmsA was shown to encode a protein tyrosine kinase homologous to similar conserved proteins functioning in other EPS biosynthetic operons (I. Rosenshine, personal communication to Dr. Gutnick). Similarly, we have very recently shown by Western analysis using an anti-phosphotyrosine antibody, that

The *cps* cluster from *E. coli*. The sequence of the *E. coli cps* gene cluster was completed in Dr. Peter Reeves' laboratory. Preliminary analysis of the *cps* gene cluster from *E. coli* indicates many interesting features shared by the clusters from the *Erwinia* sp. described above. For example, the first three ORF's (*wza*, *wzb*, and *wzc*) show very strong homology to the AmsH, AmsI and AmsA membrane and acid phosphatase proteins, suggesting the possibility that a similar membrane export apparatus may be involved in EPS production in gram negative organisms. *wcaA* from the *E. coli* cluster shows homology to the first 100 amino acids of CpsE and CpsF of *E. stewartii*, while *wcaB-E* appear by sequence gazing to correspond to glycosyl, pyruvyl and acetyl transferases. In addition, *wcaJ* showed a common region to a portion of AmsG, and *wcaK* showed partial homology to AmsJ. Several differences were clearly observed between the *E. coli* and *Erwinia* clusters, including the presence of seven ORFs in the *E. coli* sequence that encode a number of genes in the mannose pathway leading to the synthesis and incorporation of L-fucose into colanic acid. Also noteworthy, was the lack of AmsG (RfbP), AmsF, and AmsK homologs. It thus appears that there may be a common export apparatus and a number of different trans-glycosylases.

RESULTS OF THIS PROJECT.

Objective 1. To construct transgenic *E. amylovora* strains that produce new or altered EPS by introduction of the entire *cps* genes clusters from *E. stewartii* and *E. coli*.

Complementation of *E. amylovora* mutants with the *cps* cluster from *E. stewartii* (USA). The early work of Drs. Coplin and Geider demonstrated that EPS production could be restored to many *ams* mutants of *E. amylovora* by introduction of cosmid pES2144, which contains the entire *cps* cluster from *E. stewartii*, and that many of the converted strains produced stewartan and lost virulence. During the first and second years we examined the pES2144 transconjugants of *amsA-I* and *amsJ* strains for EPS production, growth rate and stability. Introduction of pES2144 increased EPS production in all of the mutants except *amsF* and *amsJ*. The *amsJ* transconjugant grew very poorly on media for EPS production and could not be scored. The EPS produced by all of the transconjugants, except those for *amsG*, *amsH* and *amsE*, was judged to be stewartan on the basis of sensitivity to phage K9. Unfortunately, only the *amsD* and *amsG* pES2144 transconjugants produced wild-type levels of EPS; all the rest produced two to three times less EPS than the wild-type. Since decreased EPS production could affect the results of *in planta* survival and colonization studies, we tried to increase production by introduction of an *rcsA*⁺ plasmid and secondary mutations, but these treatments only caused the bacteria to grow poorly.

The sequence and structural analysis suggested that the best way to construct *E. amylovora* strains producing altered EPS will be by transfer of *cpsD*, *cpsF* or *cpsG* from *E. stewartii* into the corresponding *ams* mutants. Furthermore, these constructs may avoid the adverse effects on EPS yield and growth rate owing to the presence of competing biosynthetic pathways. During the latter part of the project, we attempted to complement the same *amsA*, *B*, *C*, *D*, *E*, and *F* mutants using the various *cps* subclones shown in Table 4 without success, except for the complementation of an *amsB* mutant with a subclone containing *cpsCDE*⁺ and *amsD* by a *cpsFG*⁺ subclone. Oddly, the complemented *amsD* mutant produced only a small amount of EPS in minimal media, but it formed considerable necrosis and ooze on pear slices. Dr. Geider collected this ooze and purified the EPS from it. Dr. M. Nimtz (GBF,

Braunschweig, Germany), who solved the structure of stewartan, determined the sugar linkages by methylation analysis. The resulting GC profiles representing the backbone of the repeating unit were identical to stewartan thereby demonstrating that we had exchanged Glu for the second Gal in the backbone of the repeating unit. Although amino acid sequence alignments indicate that AmsC and AmsD are most closely homologous to CpsD and CpsG, respectively, the map order and complementation results suggest that AmsC and AmsD are functionally equivalent to CpsF and CpsG. According to our recent model (above) they might then be responsible for adding the third and second sugars residues to the repeating unit.

Complementation of *E. amylovora* mutants with *cps* genes from *E. coli*. (Israel)

During the first year we attempted to introduce a series of plasmids encoding various portions of the *cps* gene cluster from *E. coli* into *cps* and *ams* mutants of *E. stewartii* and *E. amylovora*. These transformations did not succeed and we were not able to construct heterologous strains introducing *cps* genes from *E. coli* into the *Erwinia* strains. Moreover, pES2144 was unable to complement any of the non-polar *cps* mutants in *E. coli*. Because of more extensive progress in other objectives of the program as outlined below, this aspect of the project was not continued. One possible explanation for our lack of success is that the fragments from *E. coli* are simply too small to give complete complementation (there is no single cloned fragment from *E. coli* which spans the entire *cps* gene cluster that is analogous to pES2144. However, towards the end of the project we did obtain a cosmid library from *E. coli* but it was unable to complement any of the mutants of *E. stewartii*. It is likely, therefore that while there is some structural similarity between the EPS of the *Erwinia* strains and the colanic acid of *E. coli*, the gene products as well as the substrates are not sufficiently similar to allow replacement of one enzyme by the other.

Objective 2. To determine the importance of capsule type on the ability of *E. amylovora* to survive adverse environmental conditions and to colonize blossoms.

Desiccation and regulation studies (Israel). During the first year, we continued our studies on the environmental signals leading to the induction of capsule synthesis in *E. coli*. We confirmed previous findings showing that strains expressing capsule were at least ten-fold more resistant to desiccation than mutants unable to synthesize

the capsule. In addition, during the second year we showed that a strain carrying a *cps B::lac* fusion in the chromosome was also induced by desiccation. This induction was dependent on the two regulatory proteins RcsA and RcsB and partially dependent on the putative environmental membrane sensing protein RcsC. We also screened other environmental signals for their ability to replace desiccation in the induction of *cps* transcription in the *cpsB::lac* host.

In addition to desiccation, transcription was induced in the presence of cadmium, ethanol, glycerol, and high (5M) NaCl (Table 6). As in the case of desiccation, induction by these chemical agents was also dependent on the regulatory proteins RcsA, B and C. These results were confirmed using the *lux* luminescence system as a reporter cassette. We initially utilized a liquid scintillation counter for luminescence measurements, but subsequently began a collaboration with Prof. Shimshon Belkin formerly of the Arid Zone Research Institute in Sdeh Boker and currently in the School of Environmental Sciences in the Hebrew University in Jerusalem. In this system a multisample luminometer enabled us to measure light emission from 90 ELISA plate wells simultaneously. Of course, desiccation was not an option for these studies, but we did use the alternative signals in liquid culture (see Table 7 and Fig3).

A 0.5-kb *cps* promoter fragment was obtained from Dr. V. Stout of Arizona State Univ. This fragment was initially isolated in our laboratory as a 3-kb fragment capable of driving *lac* transcription in an Rcs-dependent fashion. This fragment, when cloned into a high copy number plasmid, was able to titrate the Rcs regulatory proteins as evidenced by the conversion of a Lon^- *cpsB::lac* strain to Lac^- . The fragment was subsequently subcloned by Dr. Stout, who showed that the introduction of the cloned fragment into a *cps ::lac* fusion strain turned the cells Lac^- , presumably by titrating the Rcs regulatory proteins. We isolated a series of mutations within the promoter region on the basis of their inability to titrate these proteins such that the cells remained Lac^+ in the presence of the plasmid (Fig.3). Such non-binding mutants were sequenced, and shown to be distributed throughout the promoter. The mutant promoters were cloned in front of either the *lac* or the *lux* reporter genes. Preliminary evidence indicates that mutations localized throughout the 500 bp

fragment dramatically reduce transcription in all of the genetic backgrounds we tested (*lon*⁺, *lon*, *rscA*, *rscB*, and *rscC* respectively, and in cells grown either at 30 or 37C).

In addition to the regulation studies outlined above, we observed that when the *cpsB* from *E. coli* was cloned into any of a variety of *Lon*⁺ strains, the hosts turned mucoid in spite of the fact that at least genotypically they could be shown to be *lon*⁺. A series of investigations showed that the cells became phenotypically *Lon*⁻ as characterized by: i) sensitivity to MMS and UV irradiation, ii) constitutive high expression of LacZ in a *cps::lac* fusion strain, iii) dependence on RcsA and RcsB for mucoidy to occur. The simplest explanation for this effect is that CpsB from *E. coli* may be a substrate for Lon and when highly expressed, titrates Lon. We have very recently obtained some preliminary evidence to show that CpsB does in fact turn-over more rapidly in a *Lon*⁺ background. However, after about 90 min, CpsB appears to start to degrade in a *Lon*⁻ cell. This relative sensitivity of a biosynthesis gene to proteolysis may involve an additional level of control on the entire system, so that cells entering a transient stress condition can still avoid a permanent commitment to capsule biosynthesis, a huge energy drain on the cell, as long as possible.

***Cps* transcription during *E. coli* colonial development.** Previous results from our laboratory has demonstrated that the capsule in a variety of organisms plays a key role in protecting colonies from desiccation. During the last few months of the research, we developed a new system for following the expression of *cps* genes and their regulation. The availability of a multiplate system for monitoring *lux* expression led to the development of a system for following *cps* transcription during colonial development. ELISA plates were filled with supplemented agar (nutrients, antibiotics and environmental signals) and inoculated with spot inocula of various strains. The strains all carried the promoterless *lux* operon cloned behind either the intact 0.5 kb promoter fragment, or three mutant promoters (mutants 20, 21 and 51 respectively) in the *lux* plasmid pUDC615 which carries a kanamycin resistance gene as well. As shown in Figs. 4-7, only the *Lon*⁻ *Rcs*⁺ strains emitted significant quantities of light, once the cells had grown for over 3.5 days. In sharp contrast, however, *Lon*⁺ strains exhibited lower, but very significant levels of luminescence in the presence of stimuli

such as ethanol, NaCl, polyethylene glycol, procaine, or Cd. As shown in other systems, this induction of luminescence required both RcsA and RcsB, and was partially dependent on RcsC. That the luminescence response was due to enhanced transcription of the *cps* genes was shown by the fact that none of the mutant promoter *lux* transcriptional fusions were expressed under any conditions.

Blossom colonization and pathogenicity studies (Israel). In order to evaluate the pathogenicity of the mutant strains of *E. amylovora* before and after complementation with the plasmid carrying the entire *cps* region from *E. stewartii*, three sets of experiments were conducted in collaboration with Dr. Shula Manulis of the Volcani Agricultural Research Institute in Israel. In one set of experiments, flowering branches from Spadona pear trees from orchards in the Northern Galilee region infected with various complemented and virulent strains of *E. amylovora*. Infection was either by spraying with 10^9 cells/ml or by direct application of a 20 ml drop of the culture containing 10^4 cells. A second set of experiments was performed on similar preparations from potted pear trees of Spadona and Costia varieties cultured in netted growth chambers (Table 2). At the end of the experiment (typically several days incubation) the tissue was scored for necrosis, which was taken as a measure of pathogenicity. In addition, at the end of the experiment tissue was macerated and plated for viable cell counts. It can be seen that the only complemented strain to show any level of pathogenicity in the flower assay was strain *E. amylovora* D41 *amsE* (pES2144). None of the other strains showed any pathogenic effect. Nevertheless, all of the cells grew as judged from the increase in viable counts from 10^7 to 10^9 cells/ml in treatment. A possible explanation for the lack of pathogenicity in these assays may be the production of somewhat lower amounts of the heterologous polysaccharide. Another approach to studying pathogenicity was developed by Dr. Geider. The test is based on the direct application of bacteria to immature pear slices, which results in necrosis and the accumulation of bacterial cells and EPS (termed "ooze") on the surface of the slice. There may be some question as to precise relationship between ooze production and pathogenicity. As illustrated in Table 3, D41 *amsE* (pES2144), K4 *amsI* (pES2144) and K15 *amsG* (pES2144) were found to produce ooze when applied to pear slices. Of these, only the complemented *amsI* strain appeared to generate wild-type levels of ooze. The results obtained thus far

suggest that pathogenicity on pear slices and pear shoots may not be perfectly correlated. Nevertheless, wild-type virulent strains are all positive in both assays, while avirulent mutants (or Cps⁻ mutants) are always negative. It is likely that ooze composition varies under particular conditions and probably represents a suite of biopolymers of various sizes and viscosities. It is also possible that certain Cps⁻ mutants may be defective in capsule synthesis under standard laboratory conditions, but produce ooze in-planta. This material may not contain the necessary components required for efficient pathogenicity.

Effect of desiccation on survival of complemented strains (Israel). As illustrated in Table 9, *ams* mutants of *E. amylovora* were highly sensitive to desiccation. When complemented with pES2144 from *E. stewartii*, the mucoid transconjugants apparently synthesized a hybrid capsule which provided significant protection from desiccation.

Objective 3. To determine the role of EPS in the infection and colonization of apple tissue.

(USA) These studies are seasonal in Ohio due to the availability of plant material. Preliminary studies done on M26 apple saplings in growth chambers in August, 1995 verified that our methods for isolating the wild-type strain from cortical tissue works well. However, the *amsD* (pES2144) and *amsE*(pES2144) transconjugants did not produce symptoms and could not be recovered from the inoculation site. This essentially precluded any studies on movement and colonization. We then postponed further studies in the hope that complemented *ams* mutants expressing wild-type levels of amylovoran would become available. Unfortunately, we were not able to construct the *amsB/cpsCDE*⁺ and *amsD/cpsFG*⁺ merodiploid strains soon enough before the termination of the project to test its pathogenicity on trees, but we still intend to do this.

Objective 4. To further characterize the *E. stewartii* and *E. coli cps* gene clusters in order to obtain biosynthetic enzymes that will lead to the modification of amylovoran structure and function.

We have spent the major portion of our time on this objective, because it appeared to be the best approach to constructing the transgenic *E. amylovora* strains that we needed for objectives 1, 2 and 3. The complete sequence of the *E. stewartii cps* cluster enabled us to identify those genes most likely to be responsible for the differences in EPS structure between *E. stewartii* and *E. amylovora* and to plan experiments for making defined changes in EPS structure by genetic manipulation. For reasons explained above, similar work was not done in *E. coli*.

GENETICS OF STEWARTAN BIOSYNTHESIS IN *E. STEWARTII*.

Sequence analysis. The nucleotide sequence of the entire *E. stewartii cps* cluster was completed during the first half of this project. Thirteen ORFs were present in addition to *galE* (Fig. 1). As discussed above, all but three ORFs were also present in the *E. amylovora ams* gene cluster. The homologies are summarized in Fig. 1. An “*rca* box” was present in front of *cpsA* and no other promoters or strong terminators were apparent elsewhere in the cluster. This suggests that, like *E. amylovora*, the entire cluster is transcribed as one operon. Although, the transposon mutants used to define the *ams* cluster do not appear to have polar effects on downstream genes, such was not the case in our earlier analysis of the *cps* cluster, where mutants fell into definite complementation groups, such as *cpsA*, *cpsBIC*, and *cpsDEFG* due to polar effects of transposons. This suggests that the *cps* cluster is transcribed both as a long, *Rcs*-dependent transcript from the *cpsA* promoter and as shorter transcripts, which are not controlled by *RcsA/RcsB* regulation.

Construction of *cps* mutants and subclones. During the second half of the project, Dr. Coplin focussed almost exclusively on i) obtaining subclones that express only one or several functionally-related *cps* genes from a *lac* promoter and ii) constructing non-polar mutations in each of the *cps* ORFs believed to be involved in subunit assembly. Our initial strategy was to use restriction sites for subcloning whenever possible in order to avoid PCR artifacts and eliminate the need for resequencing PCR products. A major difficulty in working with EPS genes has been that *cps* mutations frequently have detrimental effects on growth and viability. Either overproducing a glycosyl-transferase or mutating it can quickly lead to poor growth and the accumulation of secondary suppressing mutations. We reasoned that by using small

subclones to complement the non-polar mutants we could simultaneously verify that the clone was functional and that the mutant did not contain any second-site mutations.

The inserts listed in Table 4 were cloned into pBluescript and pRK415 (Keen *et al.* 1988. Gene 70:191-197). The later vector is mobilizable, has a low copy number, and offers expression of the insert from a P_{lac} promoter. In *E. stewartii*, the presence of Lac repressor means that this promoter is expressed at a low level when present on a plasmid. Such low level expression is probably needed when there is an imbalance of *cps* genes, to avoid depletion of the lipid carrier. We were successful in obtaining clones that contain *cpsI*, *cpsD*, *cpsE*, *cpsFG*, *cpsDEFGH*, *cpsJK*, *cpsL*, *cpsK*, and *cpsKL* (Table 4).

To construct non-polar mutants, we used a kanamycin cartridge (Galan *et al.*, 1992. J. Bacteriol. 174:4338-49) that has a strong promoter in front of the *aphT* gene and no terminator. Consequently transcription proceeding out of the cartridge can drive downstream genes, if it is inserted in the correct orientation. Clones containing several *cps* genes were constructed in pBluescript and a unique site was identified within each *cps* gene. These sites were cleaved, the ends filled with Klenow fragment, and the *aphT* cartridge was introduced by blunt-end ligation. Clones were then screened for insertion of the cartridge in the correct orientation. The entire insert was then removed from pBluescript by *XbaI*-*SalI* digestion and recloned into a suicide vector for marker-exchange into *E. stewartii*. In most cases, we used either plasmid pKNG101 (Kaniga *et al.* 1991. Gene 109:137-141) or pGP704 (Miller and Mekalanos 1988. J. Bacteriol. 170:2575-2583), which have an R6K origin of replication. The mutant plasmid was first integrated into the chromosome of wild-type strain DC283 by a single-crossover and selection for Km^R. Subsequent, excision of the plasmid, by a cross-over on the opposite side of the cartridge left the mutant allele in the chromosome. With pKNG101 we could positively select for this event by using the plasmid's *sacB* genes, which are lethal to *Erwinia* in the presence of sucrose, but this did not always work too well. We have been successful in constructing non-polar mutations in *cpsD*, *cpsE*, *cpsF*, *cpsG*, *cpsH*, *cpsJ*, and *cpsK* (Table 5) by this

technique. Each marker-exchange mutagenesis was confirmed by Southern blot hybridizations.

In complementation tests with the non-mucoid mutants we have been able to show that the *cpsD*, *cpsF*, *cpsG* and *cpsK* mutants were complemented by the *cpsD*, *cpsFG*, *cpsJK* and *cpsKL* subclones, respectively. However, several other strains or clones have presented problems. The *cpsE* mutant gives mixed colonies in complementation tests with the *cpsE* subclone, suggesting that recombination or secondary mutation is necessary to restore mucoidy. The *cpsF* mutant was complemented by the *cpsDEFGH* subclone and a cloned PCR fragment containing *cpsFG*, but not several other *cpsFG* clones. This has left the possibility that in some cases either the subclones or the plasmids used to create the nonpolar mutations have acquired secondary mutations. In each case, we complemented several non-polar mutants of each type on the assumption that cross-overs occurring in different locations might separate out possible second site mutations. In addition, the *cpsFG*, *cpsKL*, *cpsJK*, *cpsK* and *cpsE* subclones were completely reconstructed in *E. coli* JM109, which contains the *lacI^q* mutation so that there will be no expression of the insert from the *lac* promoter. In total, we have constructed and characterized over 90 different subclones and 50 site-directed mutants for this project in order to obtain those listed in Tables 4 and 5, which appear to be working correctly.

Preliminary studies on the labeling of oligosaccharide intermediates in stewartan synthesis (below) indicated that we needed to prevent the interconversion of UDP-glucose and UDP-galactose by UDP-galactose-4-epimerase and that we need to channel more of the label into EPS and away from LPS synthesis. To accomplish the former, we introduced *galE* mutations into our *cpsA*, *cpsC*, *cpsI*, *cpsD*, *cpsE*, *cpsF* and *cpsG* mutants. These were verified by complementation analysis and Southern blotting. Then to increase EPS production, we introduced a multicopy plasmid containing the transcriptional activator gene *rcsA*⁺ into each double mutant.

The 434 bp *cpsI* gene has homology to acid-phosphatase genes and may function to recycle the lipid carrier following release of the repeating unit. A 1.1 kb *EcoRV* fragment containing *cpsI* was subjected to PCR mutagenesis to introduce a frameshift

mutation at aa 59 and a new *PmlI* site. An *aphA-3* cartridge was ligated into the *PmlI* site and the resulting mutation was marker-exchanged into the chromosome. The resulting *cpsI* mutant was non-mucoid; it could be complemented by the wild-type subclone, but not by the one with a frameshift mutation. When introduced into a wild-type *E. stewartii* strain, the *cpsI*⁺ clone increased acid-phosphatase activity from 55 to 36,500 units. Control plasmids containing the insert in the opposite orientation to *P_{lac}* or the frameshift mutation did not increase enzyme activity. A similar increase was seen with the *cpsI*⁺ clone in *E. coli*. However, the normal acid-phosphatase activity in *E. stewartii* was so low that we could not detect a significant decrease in the *cpsI::aphA-3* mutant as compared to the wild-type level.

Phenotypes of *E. stewartii* *cps* mutants. In our early genetic work on the *cps* gene cluster, we reported that transposon induced *cps* mutants were non-mucoid and unable to cause systemic wilt in corn plants. Except for the mutations in *cpsA*, *cpsB* and *cpsC*, these mutations could not be easily mapped with respect to the ORFs revealed by sequencing. Consequently, previously isolated Tn3HoHoI and Tn5*lac* insertion mutations in *cpsA*, *cpsB*, and *cpsC* were located by sequencing. In addition, a number of previously unmapped insertions were also sequenced. These included two insertions in *cpsF*, three in *cpsH*, one in *cpsK*, and two in *cpsL*. These mutants as well as the non-polar mutants constructed above were examined for mucoidy and virulence on corn seedlings. Mutations in *cpsI*, *cpsD*, *cpsE*, *cpsF*, *cpsG*, and *cpsK* resulted in loss of EPS production and virulence. The non-polar *cpsH* and *cpsJ* mutants produced 4 to 5 fold more EPS/cell than wild-type. Normal stewartan was determined to be ca. 1.5 mDal in size using SEC-DRI/LALLS (A. Huber, Universitt Graz, Austria), whereas that produced by the *cpsH* and *cpsJ* mutants was 4 to 6 times greater in molecular mass. Interestingly, increasing the size and amount of EPS did not affect virulence of the mutants on corn seedlings. Complementation of the *cpsH* and *cpsJ* mutants by subclones resulted in production of normal sized EPS. *cpsL* mutants were intermediate in EPS production and virulence, but the structure of the EPS was unaltered.

The mucoid phenotypes of the non-polar *cpsH* and *cpsJ* mutants and that of the transposon induced *cpsL* mutants differed from that of the corresponding

transposon-induced *ams* mutants of *E. amylovora*, which are all non-mucoid. However, in the case of *cpsH*, our transposon induced mutations are non-mucoid and avirulent. One explanation for this maybe that only a small fragment of CpsH is needed for activity. However, subsequent work with the two *cpsH::Tn3HoHoI* mutations revealed that they cannot be complemented and are dominant to the wild-type allele, which suggests that they make a truncated protein that poisons the pathway. Consequently the true null phenotype may be represented by the non-polar mutation. Dr. Geider has had problems complementing the *amsF* mutant we used, so it may be similar to our transposon mutants. Likewise, the same problems may account for the difference in phenotypes with *cpsJ/amsJ* and *cpsL/amsL* mutants, which to our knowledge have not been complemented by any small *ams* subclones and could possibly contain secondary mutations or have polar effects.

***E. stewartii* cps gene regulation.** In related studies, we have continued to investigate the regulation of the *E. stewartii* *cps* cluster by *rcaA* and *rcaB* and we have collaborated with Dr. Suzanne Beck-von Bodman (now at the University of Connecticut) in demonstrating that EPS production is also dependent on cell-density dependent signalling involving an autoinducer (AI) molecule. As part of this project, we have constructed a mini-Tn5 transposon carrying the *cpsA* promoter fused to *lacZ*. This transposon has been useful in studying the expression of *cps* genes in *E. amylovora* and *E. stewartii*, since it enables us to easily introduce a *cps* reporter gene fusion into the chromosome of any strain without disrupting normal EPS synthesis. Using this system we have shown that the *cpsA* promoter region is specifically regulated by *rcaA* and *rcaB* in both species. Work in Beck-von Bodman's laboratory has shown that *E. stewartii* produces an AI, N-(3-oxohexanoyl)-L-homoserine. Using our reporter gene constructs, we have collaborated with her in showing that AI is needed for full expression of both the *cps* and *wts/hrp* gene clusters. *E. stewartii* contains a *luxI* homologue, *esaI*, responsible for production of the AI, and a *luxR* homologue, *esaR*, that is a transcriptional repressor. This is the first example of a repressor associated with an autoinduction system. *esaI* mutants are non-mucoid and completely avirulent, whereas an *esaR* mutant and a *esaIR* are fully mucoid and intermediate in virulent. The derepressed mutants differ from the wild-type strain in that they make EPS at low cell densities. Together this is an interesting result because it suggests that production of EPS too early in the infection process interferes with

pathogenesis. A similar *esa* regulatory system does not appear to be present in *E. amylovora*. One joint project planned for the future will include modeling the expression of *cps* genes from *E. stewartii* during colonial development according to protocols developed in the Gutnick laboratory and using a variety of defective mutants including those defective in the quorum-sensing apparatus. One might expect that in such mutants, induction of luminescence would begin almost immediately, rather than after a very long lag period.

CAPSULE BIOSYNTHESIS IN *E. COLI* (ISRAEL).

During year 2 of the project we began our development of a method for studying the biosynthesis of capsule *in vitro*. The procedure is based on one described by Reuber and Walker for the cell-free synthesis of exopolysaccharide in the symbiotic nitrogen fixing organism *Rhizobium meliloti*. A general description of the method is as follows: an overnight culture ($A_{600} = 1$) of 50-100 ml of each strain was harvested by centrifugation, washed and resuspended in Tris-EDTA buffer. Cell preparations were permeabilized by freezing and thawing three times and stored at -70C. For each reaction, 70 to 100 ml of permeabilized cells were incubated for 1 h at room temperature in the presence of UDP-[^{14}C]-galactose or UDP-[^{14}C]-glucose together with acetyl SCoA, succinyl SCoA, phosphoenol pyruvate, MgCl_2 , glucose 6-phosphate, NADP, and non-radioactive UDP-glucose, UDP-galactose, UDP-glucuronic acid and GDP-D-mannose. Reactions were stopped by the addition of ice-cold Tris-EDTA and the cell pellets were washed and extracted twice with a mixture of chloroform-methanol-water (1:2:0.3). A portion of the extract was counted in a liquid scintillation counter to determine the incorporation of radioactive sugars into lipid-linked material, while the remainder was dried and hydrolyzed in 0.01 N trifluoroacetic acid at 90C for 20 min to remove the oligosaccharides from the lipid carriers in the membrane. The solutions were then neutralized and the released oligosaccharides were incubated at 37C overnight in the presence of alkaline phosphatase to remove phosphate from the sugars. Water-soluble oligosaccharides were separated into the aqueous phase after extraction with chloroform-methanol (2:1), and subjected to thin layer chromatography on silica-gel 60 plates. The plates were developed in a solvent of n-propanol-nitromethane-water (50:20:20). Since the

oligosaccharides move very slowly in this system, the plates were allowed to develop, air dried, and then allowed to develop for an additional two ascents in order to insure sufficient migration. The spots were visualized either by autoradiography with Kodak film and an intensifying screen at -60C or exposed directly using a phosphorimager. Fluorescent coumarin-labeled oligosaccharide TLC markers (monomer to heptamer) were used as sizing standards and could be visualized under as fluorescent spots under UV light. In order to identify the sugar components, oligosaccharide spots of interest were scraped off the TLC plates and hydrolyzed in 2 M HCl for 2.5 h at 100C. The released sugars were then subjected to TLC on either cellulose or silica-gel plates using a variety of solvent systems. More recently we have been trying high voltage electrophoresis on TLC plates. The striking observation was that no spots were seen in strains VS12006,7, and 8, which are capsule-negative strains resulting from the insertion of kanamycin resistance cassettes. These results indicate that the labeling pattern is not due the synthesis of LPS; which might have yielded similar spots. The Lon⁻ strain JT4000, which produces copious amounts of capsule, shows a weak pattern of oligosaccharides. This strain produced a small amount of what appears to be a heptasaccharide. A similar pattern was obtained with the wild-type Lon⁺ strain SG20250 that produces very little capsule. Four non-mucoid mutants of *E. coli* isolated in our laboratory were also analyzed and shown to accumulate lipid-linked oligosaccharide intermediates using either UDP-[¹⁴C]-glucose or UDP-[¹⁴C]-galactose. As mentioned above we are currently characterizing the composition of the sugar constituents in the major spot for each mutant. Preliminary results indicate that the major oligosaccharide spots contain glucose, galactose, fucose, glucuronic acid, mannose and an unidentified sugar. During the third year, we used these techniques to study the accumulation of lipid-linked oligosaccharide intermediates in a series of non-polar *cps* mutants of *E. stewartii* described above.

BIOSYNTHESIS OF STEWARTAN (ISRAEL)

The procedure outlined above was attempted with *E. stewartii*. However, although lipid linked intermediates were found in this procedure, there was no difference in the presence of either the intermediates or the individual sugar components between preparations from the parental strain, strains carrying putative

mutations in the various *trans* glycosylases, or the negative control carrying the deletion of *cpsB-galE* (strain GAL8) which should not have yielded any bands. Attempts were made to improve the radioactive uptake in the cell-free system by introducing a plasmid carrying a second copy of the RcsA protein. Although incorporation of label from radioactive galactose or glucose was enhanced, the pattern remained the same, suggesting that the labeling was due to lipopolysaccharide production rather than stewartan synthesis. Similar results were found when the cells were prepared on media limited for nitrogen (in order to enhance polysaccharide synthesis). In addition, Dr. Coplin introduced a *galE* mutation into the various mutants in order to prevent reconversion of labeled galactose back to glucose. Finally, we did a series of experiments to test whether the EDTA treatment was causing damage to the cell-free system. Once again, we saw little if any difference between incorporation into mutants and into wild-type. We therefore modified the experimental approach described above for the cell-free synthesis of stewartan. In this method we used stationary phase cultures prepared in 2 ml of CPG media from which 0.1 ml of inoculum was spread on CPG agar plates to generate thick lawns. The bacterial lawns were scraped off the plates and suspended in 0.5 ml liquid media. Cell pellets were prepared by centrifugation and resuspended in 0.2 ml chloroform:methanol:water (1:2:3) and stirred vigorously to extract the lipid soluble fraction. The organic phase was dried in-vacuo and resuspended in 90 ml 13 mM trifluoroacetic acid (TFA). The suspension was heated at 90C for 20 minutes. The hydrolysate was run on TLC in a solvent containing n-butanol-ethanol-water (5:3:2) for 3.5 h, air dried and run a second time before spraying with anisaldehyde spray reagent according to the procedure of Krispin and Allmansberger (J.Bacteriol. 180:2265-2270). After spraying, the plates were baked at 90C for 15 min. which allowed visualization of the colored sugar bands. Here we found that there was a TLC band missing from those mutant extracts in which transglycosylases were defective. However, once again, the negative control, carrying the *cpsB-galE* deletion, exhibited the same chromatographic profile as did the wild-type strain. Surprisingly, when the organic extract was hydrolyzed in the presence of 2 N TFA at 90C for 2 h to release the individually bound sugars, the same TLC pattern was obtained. The experiments were repeated with the same strains, however, this time radioactive sugars replaced the unlabeled substrates. In addition, the extracts were subjected to both strong and weak acid hydrolysis in the presence of TFA, and

negative controls were used which included boiled cell suspensions prior to labeling. While no labeling was observed in the case of this negative control, no changes in TLC profiles were observed between extracts subjected to either strong or weak hydrolysis. While time did not permit further development of this system, we suspect that the determination of stewartan synthesis *in vitro* is complicated by LPS synthesis. Accordingly, we hope, in the future, to obtain mutants defective in the latter process in order to eliminate the possibility of complicating artifacts.

DESCRIPTION OF THE COLLABORATION

The work described in this proposal was a three-way collaboration between laboratories in Israel (Drs. Gutnick and Manulis), the USA (Dr. Coplin), and Germany (Dr. Klaus Geider from Max Planck Institute in Heidelberg). Although Dr. Geider was not eligible to receive BARD funds, his contribution was a valuable part of this proposal. The responsibilities of each laboratory were as follows: Dr. Coplin subcloned the *E. stewartii* *cps* genes and constructed defined non-polar *cps* mutants. Dr. Gutnick provided cloned *cps* genes from *E. coli*, investigated the role of capsules in survival to desiccation, and worked on the isolation of oligosaccharide intermediates from *E. coli* and the *E. stewartii* mutants created in Dr. Coplin's lab. These *E. stewartii* and *E. coli* *cps* genes were then introduced into characterized *E. amylovora* *ams* mutants provided by Dr. Geider, who also arranged for the structural and molecular weight analyses of the polysaccharides produced by wild-type and mutant erwinias to be done by his colleagues in Germany. Parallel studies on the genetics and biochemistry of amylovoran synthesis were done independently by Dr. Geider and we exchanged information in a timely manner. Dr. Manulis conducted pathogenicity tests and blossom colonization studies with several transgenic *E. amylovora* strains.

In August, 1995, Drs. Coplin, Gutnick, Manulis and Geider met for two days before the American Phytopathology Society meeting in Pittsburgh. We exchanged data and ideas and planned collaborative experiments. In February 1997, Dr. Coplin visited Dr. Gutnick's laboratory for two weeks. During this time, we worked on the techniques for isolating and characterizing lipid-bound oligosaccharide intermediates from membrane fractions to characterize the intermediates that accumulate in

non-polar *cps* mutants of *E. stewartii*. Finally, in April 1997, Dr. Gutnick visited Dr. Coplin's laboratory to exchange information on the mutants being constructed for his biochemical studies. During this entire period all of the groups were in constant contact through telephone, fax and e-mail communication.

EVALUATION

Although we were initially successful in partially restoring EPS production to various *ams* mutants with the entire EPS gene cluster from *E. stewartii*, we did not produce any strains that produced both an altered amylovoran and normal amounts of that EPS. These strains did not show significant differences in blossom colonization and were completely avirulent in tree inoculations. Consequently, we felt that they were compromised in their pathogenic ability. As a result further work along these lines was postponed. During the second year of the project, the complete sequences of the polysaccharide gene clusters for all three bacteria and the corresponding chemical structures of their EPSs became available. The structures of amylovoran and stewartan differed by only three bonds and the *ams* and *cps* gene clusters differed by only three enzymes, thereby accounting for the difference in structure. On the other hand, colanic acid and its gene cluster in *E. coli* were very different in structure and the genes present. We then realized that further efforts to alter EPS synthesis would have to come from introduction of specific *E. stewartii cps* genes into *E. amylovora* and that further characterization of the *E. coli cps* gene cluster would not be directly relevant. At the time we wrote the proposal, we were aware that a simple approach to altering EPS structure might not work. Consequently, we included a complete characterization of the genetics and biochemistry of stewartan synthesis as Objective 4 so that we could take a rationale and defined approach to engineering changes in amylovoran if an empirical one did not work. This objective became the major focus of the second half of the project. We have accomplished most of Objective 4 and even expanded it to include some aspects of the regulation of colanic acid and stewartan synthesis. In terms of the role of the capsule in both *Erwinia* and *E. coli* we have confirmed previous findings illustrating the effect of the water soluble EPS envelope in protecting the cells from desiccation. This was further confirmed in mutants of *E. amylovora* defective in amylovoran synthesis which were

complemented with heterologous genes from *E. stewartii*. The results from the desiccation experiments with *E. coli* suggest that the expression from the *cps* promoter(s) is sensitive not only to desiccation but to other environmental signals as well. This coupled with the negative quorum sensing control over EPS synthesis may offer new avenues for investigation of the precise timing of enhanced EPS synthesis in-planta. In terms of the biosynthesis, we were able to get labeled sugar incorporated into membrane bound oligosaccharide intermediates, but thus far have not been able to correlate these accumulated products with defects in specific glycosylases. This problem is most likely due to the similar pathways and intermediates associated with LPS synthesis in these mutants. Thus, a tested model for biosynthesis of the pathway will be forthcoming when these technical problems are overcome.

CONCLUSIONS

Extracellular polysaccharide slime and capsules are important for the virulence of bacterial pathogens of plants and animals and to enhance their survival and dissemination outside of the host. The goals of this project were to examine the importance of polysaccharide structure on the pathogenicity and survival properties of three pathogenic bacteria: *Erwinia amylovora*, *Erwinia stewartii* and *Escherichia coli*. The project was a collaboration between the laboratories of Dr. Gutnick (PI, *E. coli* genetics and biochemistry), Dr. Coplin (co-PI, *E. stewartii* genetics) and Dr. Geider (unfunded collaborator, *E. amylovora* genetics and EPS analysis). Structural analysis of the EPSs, sequence analysis of the biosynthetic gene clusters and site-directed mutagenesis of individual *cps* and *ams* genes revealed that: i) the three gene clusters shared common features for polysaccharide polymerization, translocation, and precursor synthesis; ii) the structure of amylovoran and stewartan were very similar and this was reflected in the organization and functional equivalence of the genes in the *E. amylovora* *ams* and *E. stewartii* *cps* clusters; and iii) the structure of colanic acid and the *cps* genes for its biosynthesis in *E. coli* were very functionally different from those in *Erwinia*. Stewartan differs from amylovoran in that the backbone contains a glucose residue and the major sidechain terminates in a glucose rather than pyruvate. These differences can be accounted for by three

different enzymes. Comparison of the *cps* and *ams* clusters revealed that these enzymes could be encoded by *cpsD*, *cpsF*, and *cpsG* and *amsE*, *amsC* and *amsC*, respectively. (The complete sequencing, subcloning and directed mutagenesis of the *E. stewartii* *cps* cluster was completed on BARD funding.) In our initial experiments, we showed that by transferring entire *cps* and *ams* gene clusters we could make *E. amylovora* synthesize stewartan, with an accompanying loss of virulence, and make *E. stewartii* produce amylovoran, with no loss of virulence. Later experiments in Dr. Coplin's laboratory were directed at moving selected *E. stewartii* *cps* genes into *E. amylovora* *ams* mutants. We were successful in complementing an *amsE* mutant with *cpsFG* and thereby exchanging a glucose for a galactose in the backbone of the amylovoran. This change affected the amount of EPS produced in culture, but not in planta, and did not alter pathogenicity. We were also successful in altering the amount and size of the EPS produced by *E. stewartii*. Non-polar mutations in *cpsH* and *cpsJ* resulted in production of four to five times more stewartan that was four to six times greater in molecular size; this change, however, did not affect virulence. In biochemical studies, Dr. Geider was able to characterize a number of the intermediates in amylovoran synthesis. From his data, the structures of the EPSs and our genetic analysis we were able to propose a common model for the synthesis of amylovoran and stewartan (Fig. 1). Techniques for the characterization of the intermediates in colanic acid synthesis were developed in Dr. Gutnick's laboratory and are currently being adapted for the characterization of stewartan synthesis.

The role of EPS in the survival of erwinias and *E. coli* to desiccation and the response of EPS regulatory systems to environmental conditions was a second part of the project. Research in Dr. Gutnick's laboratory demonstrated a clear role for amylovoran, stewartan and colanic acid in enabling bacteria to survive desiccation. Interestingly, stewartan appeared to be less effective than other polysaccharides in protecting *E. stewartii* and transgenic *E. amylovora* strains than amylovoran and colanic acid. This may be due to the fact that *E. stewartii* is not required to survive outside of a plant host or insect vector. In *E. coli*, the effect of adverse conditions on EPS synthesis during colony development was studied using a new microplate technique for monitoring *lux* reporter genes that was developed by Dr. Gutnick. In these tests, *cps* transcription was stimulated by drying and chemical agents in an *rscC* dependent manner. Dr. Coplin collaborated with Dr. VonBodman's laboratory to investigate the regulation of stewartan synthesis by a homoserine lactone mediated

autoinduction system. (A similar system was not found in *E. amylovora* and *E. coli*.) This system turned out to be unique in that the LuxR homolog acts as a repressor and mutants are derepressed for EPS synthesis at low cell densities. Such early EPS production resulted in decreased virulence, indicating that EPS, although required for pathogenicity, is not always advantageous and pathogens must regulate its production carefully.

PUBLICATIONS

Journal Articles:

Bernhard, F., Schullerus, D., Belleman, P., Nimtz, M., Coplin, D.L., and Geider, K. 1996. Genetic transfer of amylovoran and stewartan synthesis between *Erwinia amylovora* and *Erwinia stewartii*. Microbiology (UK) 142:1087-1096.

Nimtz, M., Mort, A., Wray, V., Domke, T., Zhang, Y., Coplin, D.L., and Geider, K. 1996. Structural analysis of stewartan, the capsular exopolysaccharide from the corn pathogen *Erwinia stewartii*. Carbohydrate Res. 288:189-201.

Nimtz, M., Mort, A., Domke, T., Wray, V., Zhang, Y., Qiu, F., Coplin, D., and Geider, K. 1996. Structure of amylovoran, the capsular exopolysaccharide from the fire blight pathogen *Erwinia amylovora*. Carbohydrate Res. 287:59-76

Markus Wehland, M., Clemens Kiecker, C., Coplin, D.L., Kelm, O., Saenger, W. and Bernhard, F. 1998. Evaluation of a RcsA/RcsB recognition motif in the promoters of exopolysaccharide biosynthetic operons from *Erwinia amylovora* and *Erwinia stewartii*. Molec. Microbiol. (submitted).

Beck, Von Bodman, S., Majerczak, D.R., and Coplin, D.L. 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii* Proc. Natl. Acad. Sci. USA 95:7687-7692.

Book chapters:

Bernhard, F., Bugert, P., Geider, K., Majerczak, D.R., and Coplin, D.L. 1995. Genetics and complementation of DNA regions involved in amylovoran synthesis of *Erwinia amylovora* and stewartan synthesis of *Erwinia stewartii*. Acta Hort. 411:269-273.

Coplin, D.L., Majerczak, D.R., Bugert, P., and Geider, K. 1995. Nucleotide sequence analysis of the *Erwinia stewartii* *cps* gene cluster for synthesis of stewartan and comparison to the *Erwinia amylovora* *ams* cluster for synthesis of amylovoran. *Acta Hort.* 411:251-255.

Langlotz, C., Ullrich, H., Coplin, D., Huber, A., Nimtz, M., and Geider, K. 1998. Biosynthesis and characterization of exopolysaccharides from *Erwinia amylovora* and *Erwinia stewartii*. *Acta Hort.* (submitted)

Abstracts:

Beck Von Bodman, S., Majerczak, D.R., Farrand, S.K., and Coplin, D.L. 1995. Autoinduction controls extracellular polysaccharide production and watersoaking ability in *Erwinia stewartii*. *Phytopathology* 85:1159.

Bernhard, F., Bugert, P., Geider, K., Majerczak, D.R., and Coplin, D.L. 1995. Genetics and complementation of DNA regions involved in amylovoran synthesis of *Erwinia amylovora* and stewartan synthesis of *Erwinia stewartii*. *Phytopathology* 85:1163.

Coplin, D.L., Majerczak, D.R., Bugert, P., and Geider, K. 1995. Nucleotide sequence analysis of the *Erwinia stewartii* *cps* gene cluster for synthesis of stewartan. *Phytopathology* 85:1159.

Geider, K., Coplin, D.L., Jumel, K., and Nimtz, M. Molecular relations of amylovoran from *Erwinia amylovora* and stewartan from *E. stewartii*. Abst. 697A. Abstracts of Presentations, APS/MSA Joint Annual Meeting. July 27-31, 1996. Indianapolis, IN.

Beck von Bodman, S., Majerczak, D.R., and Coplin, D.L. Extracellular polysaccharide biosynthesis and virulence in *Erwinia stewartii* is regulated by autoinduction. Presentation at the 1996 Annual Meeting of the American Phytopathological Soc. Indianapolis, July 27-31.

¹Ophir, T. Belkin, S. and Gutnick, D.L. Role of desiccation and other environmental signals in induction of capsule synthesis in *E.coli*. Presentation at the International Union of Microbiological Societies. Jerusalem, Aug. 24-31, 1996.

²Ophir, T. and Gutnick, D.L.. Characterization of the promoter regulating capsule gene transcription in *E. coli*.. Presentation at the International Union of Microbiological Societies. Jerusalem, Aug. 24-31, 1996.

³Caspi, T. Berdichevsky, Y. and Gutnick, D.L. A role for a biosynthetic gene in the regulation of capsule synthesis in *E. coli*. Presentation at the International Union of Microbiological Societies. Jerusalem, Aug. 24-31, 1996.

⁴Paley, E. and Gutnick, D.L. A cell-free system for the characterization of mutants defective in capsule biosynthesis in *E. coli* K-12. Presentation at the Annual Meeting of the Israel Microbiological Society. Jerusalem, 1997.

³Ophir, T and Gutnick, D.L. A role for desiccation in the regulation of capsule synthesis in *E. coli*. Cold Spring Harbor Symposium on Microbial Genetics, Cold Spring Harbor, New York, August, 1995.

³Nakar, D. and Gutnick, D.L. A role for protein tyrosine kinase and protein tyrosine phosphatase in capsule biosynthesis in bacteria. Presentation at the Annual Meeting of the Federated Israel Societies of Biology. Eilat, 1998.

Coplin, D.L., Majerczak, D.R., and Gupta, S. 1997. Characterization of defined mutations in the exopolysaccharide biosynthetic cluster of *Erwinia stewartii*.
Phytopathology 87:S21

Beck von Bodman, S., Majerczak, D.R., and Coplin, D.L. 1997. Quorum sensing controls capsular polysaccharide synthesis and virulence in *Erwinia stewartii*.
Phytopathology 87:S7

In preparation:

Coplin, D.L., and Majerczak, D.R. Organization of the *Erwinia stewartii* *wce* (*cps*) gene cluster responsible for production of the capsular polysaccharide stewartan. For J. Bacteriol.

Coplin, D.L., Majerczak, D.R. and Poetter, K. Extracellular polysaccharide production in *Erwinia stewartii* is regulated by *rscB* and *rscC*. For Mol. Gen. Genet.

Ophir, T and Gutnick, D.L. Expression of the colanic acid biosynthetic regulon during colonial development. For Applied and Environmental Microbiology.

Nakar, D. and Gutnick, D.L. Involvement of protein tyrosine kinase and protein tyrosine phosphatase in the production of exopolysaccharides in bacteria. For

¹ In preparation for Applied and Environmental Microbiology.

² In preparation for Applied and Environmental Microbiology

³ In preparation for J. Bact.

⁴ In preparation for FEBS Letters

Table 1. Sequential steps in the addition of sugars to the repeating unit of amylovoran. Phosphorylated lipid carrier (undecaprenyl-phosphate) is indicated by lip-P. Newly added residuees are in bold. The first three sugar residues form the backbone. The functions of gene products in parenthesis have been deduced from genetic data. From C. Langlotz and K. Geider, personal communication.

<u>Protein</u> Intermediate Best guess	Ams Protein	<u>Equivalent Cps</u> By alignment
lip-P-P-Gal CpsA	AmsG	CpsA
lip-P-P-Gal-Gal CpsG ¹	AmsD	CpsG ¹
lip-P-P-Gal-Gal-Gal CpsF	AmsC	CpsD
lip-P-P-Gal-Gal-Gal-GlcA CpsE	AmsB	CpsE
lip-P-P-Gal-Gal-Gal-GlcA-Gal CpsK	AmsK	CpsK
lip-P-P-Gal-Gal-Gal-GlcA-Gal-pyr CpsD ²	(AmsE)	CpsF ²
lip-P-P-Gal-Gal-Gal-GlcA-Gal-pyr CpsJ -Glc	(AmsJ)	CpsJ

¹In *E. stewartii* this enzyme would add glucose.

²By process of elimination. In *E. stewartii* this enzyme adds a terminal glucose to the sidechain instead of pyruvate.

Table 2. Colonization of pear flowers by *ams* mutants and transconjugants of *E. amylovora*¹

Strain	<i>Ams</i> gene affected	Viable count
D2	<i>amsD</i>	1.2 X 10 ⁸
D2 (pES2144)	<i>amsD</i>	1.1 X 10 ⁸
D4	<i>amsE</i>	>2 X 10 ⁸

D4 (pES2144)	<i>amsE</i>	1.4 X 10 ⁸
D9	<i>amsB</i>	9.6 X 10 ⁷
D9 (pES2144)	<i>amsB</i>	3.7 X 10 ⁸

1.2X10⁴ cells were applied in a 20 µl aliquot to flowers and incubated for 6 days. After six days incubation, the flowers were macerated and the viable cell count determined. Bacteria reisolated from flowers were screened on tetracycline plates to verify the presence of pES2144.

Table 3. Ooze production and necrosis induced by specific transconjugants of *ams* mutants in *E. amylovora*.¹

Strain	Complemented gene	Trial 1	Trial 2	Trial 3
D41(pES2144)	<i>amsE</i>	-	-	++
K4 (pES2144)	<i>amsI</i>	+	+++	+++
K15(pES2144)	<i>amsG</i>	-	++	+++

¹These were the only strains that yielded any results from among a group of 19 strains tested. None of the other complemented strains showed any signs of pathogenicity in the pear slice assay.

Table 4. *cps* inserts subcloned from pES2144.

Genes	Vector1	Fragment	Sequence Coordinates ²
<i>cpsI</i>	pBS, pRK415*	<i>EcoRV</i>	3647-4799
<i>cpsD</i>	pLAFR3	<i>EcoR1-PstI</i>	6540-8233
<i>cpsE</i>	pUC129, pBS	<i>Sph1-BamH1</i>	7686-9093
<i>cpsE</i>	pRK415*, pBS	<i>Sph1-BamH1</i>	7686-9093
<i>cpsFG</i>	pRK415*, pBS	<i>PstI</i>	8233-12143
<i>cpsGH</i>	pBS*	<i>BamH1-HindIII</i>	9093-13730
<i>cpsDEFGH</i>	pRK415, pBS	<i>EcoR1-BglII</i>	6540-13585
<i>cpsJK</i>	pRK415*, pBS	<i>PstI</i>	12300-15749
<i>cpsL</i>	pRK415, pBS	<i>PstI</i>	15848-17433
<i>cpsK</i>	pRK415*	<i>HindIII-PstI</i>	13730-15749
<i>cpsKL</i>	pRK415*, pBS	<i>HindIII</i>	13730-17303

¹ * indicates plasmid constructed in JM107 *lacI*^q

² Sequence coordinates of *cps* ORFs:

cpsA 751-2181 C 4233-6410 F 8914-9672 J 13202-14464 M 17297-18193

cpsB 2637-3776 D 6656-7792 G 9719-10753 K 14469-15692 *galE* 18243-

cpsI 3786-4220 E 7921-8838 H 10842-13052 L 15857-17029

Table 5. Non-polar *cps* insertion mutations constructed with an *aphT* kanamycin resistance cartridge.

Mutant	<i>cps</i> Gene	site	Mucoidy	Complemented by subclone
DM2258	<i>cpsI</i>	3961	-	yes
DM2217	<i>cpsD</i>	6928	-	yes
DM2213	<i>cpsE</i>	7960	-	mixed
DM2191	<i>cpsF</i>	9093	-	yes
DM2192	<i>cpsG</i>	10052	-	yes
DM2237	<i>cpsH</i>	11518	+	ND
DM2211	<i>cpsJ</i>	14265	+	ND
DM2245	<i>cpsK</i>	15069	-	yes

Table 6. Effect of environmental signals on transcription *cps* regulon in lac fusion strains of *E. coli*^a

Strain	Relevant genotype	Treatment			
		None	Cd ⁺⁺	NaCl	Ethanol
SG20250	<i>lon</i> ⁺ <i>rsc</i> ⁺	4	113	99	145
SG20780	<i>lon</i> ⁻ <i>rsc</i> ⁺	457	622	593	485
ZO78093	<i>lon</i> ⁺ <i>rscC</i> ⁻	2	17	49	32
ZO78085	<i>lon</i> ⁻ <i>rscA</i> ⁻	3	0	1	5
ZO78064	<i>lon</i> ⁻ <i>rscB</i> ⁻	0	0	2	0
ZO78011	<i>lon</i> ⁻ <i>rscC</i> ⁻	683	376	720	444

All strains carried a *cps::lac* fusion in the *cpsB* gene of the biosynthetic operon.

^a β -galactosidase assays measured in Miller units

^b Determined on individual colonies desiccated in vacuo

Table 7. Effect of environmental signals on transcription *cps* regulon in *lux* fusion strains of *E. coli*.

Strain	Relevant genotype	Treatment			
		None	Cd ⁺⁺	NaCl	Ethanol
SG20250	<i>lon</i> ⁺ <i>rsc</i> ⁺	4	46	61	32
SG20780	<i>lon</i> ⁺ <i>rsc</i> ⁺	183	244	312	298
ZO78093	<i>lon</i> ⁺ <i>rsc</i> C ⁻	1	22	26	12
ZO78085	<i>lon</i> ⁻ <i>rsc</i> A ⁻	3	0	1	5
ZO78064	<i>lon</i> ⁻ <i>rsc</i> B ⁻	0	0	2	0
ZO78011	<i>lon</i> ⁻ <i>rsc</i> C ⁻	148	198	244	320

All strains carried a *cps::lac* fusion in the *cpsB* gene of the biosynthetic operon. Cells were inoculated into microtiterplates and luminescence was monitored continuously. Luminescence values are reported as "relative luminescence units" (RLU). Environmental treatments were carried out as described for Table 6.

Table 8. Effect of non-titrating promoter mutations on the expression of *cps::lux* fusions

Strain	Promoter mutation	RLU
ZO 7859	None	648
ZO 7860	21	<5
ZO 7862	51	0
ZO 7863	28	11
ZO 7869	20	3

Strains carrying the *cps::lux* fusion plasmids were all *lon*⁻ (Fig. 3). Luminescence was measured as in Table 7.

Table 9. Survival of *E. amylovora* transconjugants to desiccation.

Strain	Phenotype	% Survival
DC 283	mucoid	6.2 +/- 0.8
DM 14101	mucoid	4.3 +/- 0.5
PJD 1910 [<i>gal</i> 8]	non-mucoid	0.15 +/- 0.03
D41 [<i>ams</i> E]	non-mucoid	0.03 +/- 0.01
D41(pES2144)	mucoid	3.3 +/- 0.4
K4 [<i>ams</i> I]	non-mucoid	<0.01
K4(pES2144)	mucoid	5.1 +/- 0.7
K15 [<i>ams</i> G]	non-mucoid	0.07 +/- 0.01
K15(pES2144)	mucoid	4.3 +/- 0.3

Cells were dried for different periods of time as described previously (Ophir, T. and D.L. Gutnick, 1993, Appl. Environ. Microbiol. 60:740-7450). Results are presented as maximum survival rates.

AMYLOVORAN

STEWARTAN

(Structures determined by Manfred Nitz and Andrew Mort)

Organization of *Erwinia stewartii* cps and *Erwinia amylovora* ams EPS Gene Clusters

1 ANGAAAAGTA TGNCATGATT ACGAATTCGA GCTCGGCACC CGGGGATCCT
21
51 CTAGAGTCAA CCTAAAGAAA CTCC[C/T]AAAAA CCATATTGAA TGACACTTAA
5
101 TATAATTCTT AAAAATAGCC AATTACCGAA TTGTTATCTT GCCTGCTATT
+1
151 CCGTTAGCTG TAACACTTCC TCCTGCATTA TTGGAAAGCC AATATTCAAT
201 TACCATAATG TCACCTTATA TTCGGAAGTT AATTTCCGTA ATAACCCTTT
2
251 CTCATCAACG ACTGCATGCT GAGAGGGTGT GAAAAATTAA ACTAAATCAG
1
301 TGTATTGGTA GCTAAAAAGC CAGGGGCGGT AGCGTGTCTG GATGCCTGAA
JUMP start 51
351 AGACCAGTCT GAATTATTCT GTCAATAGCC TGCGGAT[C/G]AA ATCAATTTTT
4
401 TTAGGACTGA TGCCAGTTAA ATTTTATTTC AATTAAGTGC AAGGTAAATG
3
451 TGCATGGCGA CATTGCTATT AATAGTGCAC AGGATAATTA CTCTGCCAAA
501 GTGATAAATA AACAATGATG AAATCCAAAA TGAAATTGAT GCCATTATTG
20 28,58
551 GtGTCAGTAA CCTGGATAAGCGGGTGGACATACTTCCGGGCAG[G/C][T/A]ATAT
601 GTCGACCGGA GGCATGCAAG CTTGGCACTG GCCGNCGGTT TACAACGGCG
6
651 GGGATTGGGA AAACCCTGGG GTANCCAACT TAATCGCCTG GAGGANATCC
701 CCCTTCGCAG TGGGGTATGN GAGGGGGCCG ACCGNNCGCC

GTCAAC **GTCGAC** HincII sites used for cloning into pUC. Sites enclosed in bold brackets are the sites of non-titrating mutants of the putative *cps* promoter region showing the positions of 4 mutations thus far identified. Horizontal arrows show the direction of transcription.

mutant 21- 75

Fig. 4 *Cps* transcription and *lux* expression during colonial development on agar supplemented with polyethylene glycol (PEG).

Fig. 5. *Cps* transcription and *lux* expression during colonial development on agar supplemented with NaCl.

Fig. 6. Effects of specific signals on *lux* expression in a *lon*⁻ strain.

Fig. 7 Effects of specific signals on *lux* expression in a *lon*⁺ strain.