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Final Report

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Lipopolysaccharide Structures and Genes Required for Root Nodule Development
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FINDINGS/ACCOMPLISHMENTS

1. The nucleotide sequence of the O-antigen gene cluster (28 kilobases) of *Rhizobium etli* was determined.
2. All 25 ORFs (genes) of this cluster were mutated.
3. The effects of the mutations on symbiosis, LPS synthesis, and LPS structure were determined.
4. Genes were correlated with proposed biosynthetic steps and specific portions of the LPS structure.
5. LPS hybrid structures were constructed by mixing genes from two different *Rhizobium* species.
6. Certain structural features were shown to be important for symbiosis (by studying hybrid LPSs and the effects of specific mutations).
7. The model proposed for regulating the O-antigen length in E. coli was shown not to apply in *R. etli*.
8. A method for manipulating the expression of an *R. etli* gene during nodule development was developed and tested with an *lps* gene of *R. etli*.
9. It was discovered that catalase is required for surviving high concentrations of compounds that induce LPS changes. Interestingly, catalase also is required for at least one of the LPS changes that are induced by low pH.
10. Genes that control conditional methylation in R. etli were identified and the mutant effects studied. Mutations that eliminated 2-O-methylfucose delayed symbiotic development.
11. The content of an unusually long fatty acyl residue of the lipid A was doubled in response to growth at low pH. This finding complemented the discovery in another lab that this fatty acyl moiety appears to be essential for growth at low pH.

SIGNIFICANCE

This project dealt with rhizobial O antigen, a complex factor that is absolutely required in almost all symbioses between leguminous plants and rhizobial bacteria. The significance of the work

lies in two areas. It substantially advanced the understanding of 1) the infection process of the rhizobium-legume symbiosis and the role of O-antigen structure in this process, and 2) the genetic basis and biosynthesis of this O-antigen, to the extent that it now can serve as a model for others of its type.

This symbiosis is extremely important in nature, believed to account for at least one half of all nitrogen fixation on land. Its greater use, and its introduction into other plants, should reduce energy costs associated with agriculture. In addition, supplanting nitrogen fertilizer with symbiotic nitrogen fixation would greatly reduce nitrogen pollution that causes dead zones and other problems in watersheds that collect drainage from agricultural fields. This problem already threatens ecosystems of the 21st century, but it will become more of issue as plants are used as biofuels. Therefore, research in this area goes hand in hand with others at the core of the DOE mission in biology.

We had established in previous work that the O-antigen was essential for infection. These prior studies involved extensive isolation and testing of O-antigen mutants. We built upon this prior work to compile a complete accounting of all the genes required to synthesize the O antigen.

This approach allowed us to target particular structural features by mutation of specific genes and test whether these features within its overall structure were important in the interactions with host plants. This type of question has been raised about O-antigens in many bacteria but seldom tested. In particular, we targeted O-methylation of O-antigen sugar residues. Previously we had established that antigenic changes occurred in response to plant anthocyanins, and we had isolated mutants that did not exhibit these changes. Investigating these mutants in this grant period, we first discovered that up-methylation at the 2-hydroxyl of O-antigen fucoses was an important part of induced changes. Mutations that eliminated this methylation caused delay in infection. This first analysis used transposon mutations that were polar and had complex LPS phenotypes (Noel et al 2004). Once we had total the genetic sequence, we targeted the gene of interest, *lpsM*, with site-specific mutagenesis. This provided a much more convincing case because the only difference in the LPS structure in the resulting mutants was this 2-O-methylation, and it still shows the delay in infection (Ojeda et al, soon to be submitted). Sugar methylation of O antigens is prevalent in this genus and in related bacteria, begging the question of why; this work is the first to show functional significance of this type of modification.

We have attacked the question of whether specific structure is important symbiotically by a more extreme approach: constructing genetic hybrids that attach a different O antigen from the closely related strain *R. leguminosarum* 3841. This had severe effects on the symbiosis. Using the genetic sequence, we constructed mutations that we predicted would target portions of the structure that might allow the rest of the O antigen to be synthesized. In particular the branch sugar of the O-antigen repeat unit, 3-O-methyldeoxytalose. However, mutations affecting this branch sugar resulted in severely truncated O-antigen. It appears then that, by the approach of null mutations, the only ones that do not result in truncated O antigen are the ones already isolated in genes responsible for the two known features that change during interactions with the plant.

Aside from these changes induced by host compounds, we know that growth at low pH, a condition expected to hold during all stages of the bacterial infection, also induces changes in the O antigen. During this grant period, we isolated mutants that affect these low-pH induced changes. They are in different genes than the ones that affect the changes induced by plant compounds.

During the course of this grant period, we developed a method of testing the importance of a bacterial component at stages of nodule development beyond the step that is blocked by null mutation. We have successfully applied this method to study of the symbiotic role of O antigen, but it also has implications for testing roles of Nod factor and other important bacterial factors at multiple stages of nodule development.

ORF analysis of the gene cluster encoding the O antigen revealed it to be of the type that uses an ABC transporter for translocation of the O antigen to the periplasmic side of the membrane. The mechanism by which this type of O antigen is synthesized is still obscure. A very basic question is how the length of the O antigen is controlled. This is a very intriguing question with this O antigen because it falls within a subset whose length is extraordinally uniform, having in this case exactly five repeat units. With our mutational approach we were able to show that a model proposed based on work with E. coli does not to apply to this rhizobial strain. This becomes an interesting fundamental question to pursue in future work.

Synthesis of this type of O antigen has been studied in only one bacterium, and its synthesis in many respects does not match heteropolymeric O antigens such as that of *R. etli* CE3. We have begun our studies by exploiting the availability of mutations in all of the genes that should be involved. By different types of analysis, we have evidence that gives us strong arguments for the assignment of each gene in a particular predicted step of the biosynthesis. Current and future work is carrying this knowledge into the realm of synthesis in vitro, which would be a first for a heteropolysaccharide of this type.

PUBLICATIONS

BOOK CHAPTER IN PRESS

Noel, K. D. Rhizobia. *Encyclopedia of Microbiology*, 3rd edition, ed. Schaechter M. Chapter 43. [17 book pages] Elsevier. (tentative print date March, 2009)

REFEREED RESEARCH PAPERS

García-de Los Santos A, López E, Cubillas CA, Noel KD, Brom S, Romero D. 2008. Requirement of a plasmid-encoded catalase for survival of Rhizobium etli CFN42 in a polyphenol-rich environment. Appl Environ Microbiol. 74:2398-2403. **(finding #9 above)**

Noel, K. D., J. M. Box, and V. J. Bonne. 2004. 2-O-Methylation of Fucosyl Residues of a Rhizobial Lipopolysaccharide Is Increased in Response to Host Exudate and Is Eliminated in a Symbiotically Defective Mutant. App. Environ. Microbiol. 70:1537-1544. **(finding # 6 and 10)**

D'Haeze, W., C. Leoff, G. Freshour, K. D. Noel, and R. W. Carlson. 2007. *Rhizobium etli* CE3 Bacteroid Lipopolysaccharides Are Structurally Similar but Not Identical to Those Produced by Cultured CE3 Bacteria. J. Biol. Chem. 282:17101-17113. **(finding # 6)**

PAPERS SOON TO BE SUBMITTED FOR PUBLICATION

Noel, K.D., L. Benziger, R. Kowalski, and E. Kannenberg. Effects of transferring a *Rhizobium leguminosarum lps* gene cluster into *R. etli* mutants deficient in O-antigen biosynthesis. (Being revised for submission to *J. Bacteriology*) **(finding # 5)**

Noel, K.D. and J.M. Box. Controlling the expression of bacterial genes during nodule development with elements and inducers of the *lac* operon. (for submission to *Mol. Plant-Microbe Interact.*) **(finding # 8)**

Ojeda, K. J., Box, J. M., Noel, K. D. Genetic Basis for *Rhizobium etli* CE3 O-Antigen O-Methyl Moieties That Vary According to Growth Conditions (for submission to *Journal of Bacteriology*) **(findings # 4-7 and 10)**

Additional manuscripts besides these will be submitted within the next few months. The first of these will summarize our findings 1-4 above. The next one after that will look at our findings regarding changes in the LPS in response to low pH, a condition expected during infection of the plant (findings #9 and #11).