The disA gene encodes a putative amino acid decarboxylase that inhibits swarming in Proteus mirabilis. 5’ rapid amplification of cDNA ends (RACE) and deletion analysis were used to identify the disA promoter. The use of a disA-lacZ fusion indicated that FlhD4C2, the class I flagellar master regulator, did not have a role in disA regulation. The putative product of DisA, phenethylamine, was able to inhibit disA expression, indicating that a negative regulatory feedback loop was present. Transposon mutagenesis was used to identify regulators of disA and revealed that umoB (igaA) was a negative regulator of disA. Our data demonstrate that the regulation of disA by UmoB is mediated through the Rcs phosphorelay.

This study further defines the disA locus by identifying the transcriptional start site and begins the process of elucidating the regulation of disA. 5’ rapid amplification of cDNA ends (RACE) analysis and transcriptional lacZ fusions demonstrate that disA transcription begins at a thymine residue 70 bp upstream of the disA start codon. Use of a disA-lacZ fusion demonstrated that FlhD4C2 does not have a significant role in disA expression. Transposon mutagenesis was used to identify UmoB as a negative regulator of disA. The umoB gene product is a negative regulator of the Rcs phosphorelay and has been previously implicated by our lab and others in swarming regulation (15, 18, 19, 20, 21, 22, 23, 24, 25). Our data indicate that the effect of the umoB mutation on disA expression is dependent upon the Rcs phosphorelay system. Taken together, our data indicate that a complex network is responsible for regulation of disA, allowing the cell more-precise control over the energy-intensive process of swarming.

MATERIALS AND METHODS

Bacterial growth conditions. The bacterial strains and plasmids utilized are listed in Table 1. P. mirabilis and Escherichia coli were grown in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g sodium chloride per liter) at 37°C with shaking at 250 rpm. For plate growth, E. coli and nonswarming P. mirabilis strains were grown on 1.5% agar; swarming strains of P. mirabilis were plated on 3% agar to inhibit motility. Concentrations of antibiotics for selection for E. coli were as follows: 25 μg/ml for streptomycin and chloramphenicol, 20 μg/ml for kanamycin, and 100 μg/ml for ampicillin. Concentrations of antibiotics for selection for P. mirabilis were as follows: 35 μg/ml streptomycin, 100 μg/ml chloramphenicol, 300 μg/ml ampicillin, 20 μg/ml kanamycin, and 15 μg/ml tetraacycline.

Received 16 January 2013 Accepted 10 May 2013
Published ahead of print 17 May 2013
Address correspondence to Philip N. Rather, prather@emory.edu.
Copyright © 2013, American Society for Microbiology. All Rights Reserved.
TABLE 1 Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain BB1</td>
<td>pRcsC pKNG101 R6K-derived suicide vector; Str r 26</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>Strain BB2</td>
<td>pRcsB pKNG101 R6K-derived suicide vector; Str r 26</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>Strain BB3</td>
<td>pBB1 pQF50 R6K-derived suicide vector; Str r 26</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>Strain BB4</td>
<td>pBB3 pQF50 R6K-derived suicide vector; Str r 26</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>Plasmids</td>
<td>pQF50 Low copy number; Amp'</td>
<td>ATCC</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Low copy number; Chl'</td>
<td>ATCC</td>
</tr>
<tr>
<td>pFDC1H1</td>
<td>pACYC184 + blbDC</td>
<td>ATCC</td>
</tr>
<tr>
<td>pKNG101</td>
<td>R6K-derived suicide vector; Str'</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>pPB1</td>
<td>pQF50 + P_dna -1206 to +39-lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pPB2</td>
<td>pQF50 + P_dna -69 to -1-lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pPB3</td>
<td>pQF50 + P_dna -1260 to +39-lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pUmoB</td>
<td>pACYC184 + umoB</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>pPB4</td>
<td>pKNG101 + blbDC mutant (frameshift mutation)</td>
<td>This study</td>
</tr>
<tr>
<td>pUBK</td>
<td>pKNG101 + umoB; Kan'</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>pKCB</td>
<td>pKNG101 + rcsB; Str'</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>pKCo</td>
<td>pKNG101 + rcsC (internal fragment)</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>pDias</td>
<td>pKNG101 + disA (internal fragment)</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>pMDA</td>
<td>pACYC184 + disA</td>
<td>Laboratory stock</td>
</tr>
</tbody>
</table>

For PCR amplification of selected genes, Phusion Hot Start II high-fidelity DNA polymerase (Thermo Scientific) was used. Validation of vector constructs and identification of transposon insertion sites were done through Beckman Coulter QuickLane sequencing according to the recommendations of the manufacturer. All primers are listed in Table 2.

To construct pBB1, primers PdiSa Fwd and PdiSa Rev were used to amplify the 1,245-bp region upstream of disk. The product was ligated into the Smal site of pQF50, verified, and used to transform PM7002. Plasmid pBB2 was constructed by annealing 1 μg diSPro Full Fwd and disPro Full Rev at 72°C for 10 min. The product was digested with Sall and BamH1 and ligated into pQF50 digested with the same enzymes. The plasmid was validated by sequencing before transformation into PM7002. Plasmid pBB3 was constructed by performing site-directed mutagenesis on pBB1 using the primers dis-10Fwd and dis-10Rev. The QuikChangeII site-directed mutagenesis kit (Agilent Technologies) was used to mutate the eight base pairs at the putative — 10 region upstream (TATTATCAT to CGCGCGGCC) as described in the user manual with the following modifications: 54 ng of pBB1 isolated from XL1 was used as a double-stranded DNA (dsDNA) template. Eighteen rounds of amplification were performed at 68°C with 1 min of extension per 500 bp (16.5 μl). A total of 1 μl of 10 μM dis-10Fwd and dis-10Rev primers was used in the reactions. EC100D was transformed with 1 μl of the mutagenesis product and plated on 1.5% LB agar plates containing 100 μg/ml ampicillin. Plasmid pBB4 was constructed by digesting pBc plus flbDC with EcoNI, which digests once in the middle of the flbDC gene. The product was blunt ended with T4 polymerase (Roche) as described by Promega. The blunt-ended vector was ligated to itself and digested a second time with EcoNI to enrich the sample for plasmids that had been successfully religated in the first reaction. This product was transformed into XL1 and selected by plating on chloramphenicol. Sequencing validated the presence of the single base pair insertion in flbDC that resulted from religation. pBc with the flbDC mutant was digested with PvuII and Sall, and the excised product was ligated to the suicide vector pKNG101 digested by Sall with Sall. The proper flbDC mutation in pBB4 was validated by sequencing before transformation into PM7002.

Strain BB1 was obtained by transposon mutagenesis of PM7002/pBB1 as described in “Transposon mutagenesis.” BB4 was constructed by conjugating PM7002 with SM10 harboring pBB4. Exconjugants were plated on tetracycline and streptomycin to select for P. mirabilis containing a Campbell-type insertion of pBB4. A colony was grown in LB broth in the absence of antibiotics and plated on 10% sucrose to identify colonies that had successfully excised pKNG101. Recombinants containing the flbDC mutant allele were validated by Southern blotting and transformed with pBB1. Strain BB1 was cured of pBB1 and then mated with SM10 containing plasmid pRcsB or pRcsC to obtain the umaB rcsB and umaB rcsC double mutants. Mutants were transformed with pBB1 to yield BB2 and BB3.

5’ RACE. 5’ RACE was performed on 5 μl PM7002 RNA according to the 5’ RACE system for rapid amplification of cDNA ends, version 2.0 (Invitrogen), methods section with a few modifications. cDNA was synthesized according to the alternative protocol for first-strand cDNA synthesis. Nested PCR was performed using the AAUP primer provided and GSP3 in 40 cycles with 30 s of annealing at 59°C and 45 s of extension using Phusion Hot Start II high-fidelity DNA polymerase. DNA was ligated to the EcoRV site of pBC and transformed into DH5a for blue/white screening. White colonies were cultured, and insertion of 5’ RACE products was verified by restriction digestion. Clones harboring inserts were sequenced using the universal T7 primer to identify potential transcriptional start sites. pBB2 and pBB3 were constructed based on the sequences of the cloned 5’ RACE products to determine the transcriptional start site.

Transposon mutagenesis. Strain BB1 was transformed by electroporation with EZ-Tn5 (<Kan-2> Tnp Transposome (Epiconc), Transformants were selected on 3% LB containing ampicillin, kanamycin, and X-Gal (60 μg/ml). Colonies with increased or decreased blue color were cultured as described above and assayed for β-galactosidase activity (see below). Southern blotting was performed to identify the segment of the
TABLE 2 Primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′–3′)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSP1</td>
<td>GGATGACGCCGTCAAATCGCCATCGGCAG</td>
<td>5′ RACE</td>
</tr>
<tr>
<td>GSP2</td>
<td>GCTGATAAATGTTTTTTAACC</td>
<td>5′ RACE</td>
</tr>
<tr>
<td>GSP3</td>
<td>CCAACGCTAATGAAAT</td>
<td>5′ RACE</td>
</tr>
<tr>
<td>disPro Full Fwd</td>
<td>GCAAGGATCCATATATATAATTTTTGTTATCAATTATTGAG</td>
<td>P.disA-60′-lacZ</td>
</tr>
<tr>
<td>disPro Full Rev</td>
<td>GCCAAGGATCCATATATATAATTTTTGTTATCAATTATTGAG</td>
<td>P.disA-60′-lacZ</td>
</tr>
<tr>
<td>PdisA Fwd</td>
<td>CCCGAGGCTGAGCTGATTACATTTGAG</td>
<td>P.disA-1206′-lacZ</td>
</tr>
<tr>
<td>PdisA Rev</td>
<td>GCCAAGGATCCATATATATAATTTTTGTTATCAATTATTGAG</td>
<td>P.disA-1206′-lacZ</td>
</tr>
<tr>
<td>pdisA Fwd</td>
<td>ATCAGATCTGGATGTTACAGGGCGCCATTTCAAGACTAATTATTGAG</td>
<td>P.disA-1206′-lacZ</td>
</tr>
<tr>
<td>pdisA Rev</td>
<td>ATCAGATCTGGATGTTACAGGGCGCCATTTCAAGACTAATTATTGAG</td>
<td>P.disA-1206′-lacZ</td>
</tr>
<tr>
<td>dis-10Fwd</td>
<td>GATTTTTTACCGTGAGGCTTGG</td>
<td>P.disA-39′-lacZ</td>
</tr>
<tr>
<td>dis-10Rev</td>
<td>AAATAATTAGTCCTTTTTAGGGCCGGGTCTAGATGGTAAGAATGCATAGT</td>
<td>P.disA-39′-lacZ</td>
</tr>
</tbody>
</table>

chromosome where the transposon was inserted. This region was sub-
cloned into pBC and sequenced using primers provided with the transpos-
some to identify the specific site of insertion.

**Construction of an umboB disruption by a Campbell-type insertion.** An umboB disruption was constructed in PM2199 disA::mini-Tn5lacZ by cloning a PCR-derived fragment internal to the umboB coding region ob-
tained using the primers 5′-CGTCACTTAGACGGGTAGAGATCCATAGATTAGAATACGTAACCATG into the suicide plasmid pKNG101 (26). The construct was then mobilized into PM2199 by a filter mating with E. coli SM10 containing the plasmid. Exconjugants were selected on LB plates containing 35 μg/ml streptomycin and 15 μg/ml tetracycline. The correct disruption of umboB was verified by Southern blotting.

**β-Galactosidase assays.** Overnight cultures were grown, and optical densities (ODs) were normalized to the lowest density culture. A total of 200 μl of normalized culture was spread on a 1.5% LB plate and incubated at 37°C for 2 or 4 h. These time points were chosen to assess expression before (T2) and at the peak of (T4) swarming. Cells were collected from these potential start sites, various fragments were cloned into pQF50 (28) to create transcriptional RACE PCR products returned two potential transcriptional start sites, one located 8 bp upstream of the lacZ moterless gene in pQF50. (C) Plasmid pBB2 contains the region from 39 bp into the lacZ gene to 1,206 bp upstream of the disA ORF. The open arrow (−8) is located eight base pairs upstream of the disA ORF. (D) Plasmid pBB3 is identical to pBB2 but contains an 8-bp substitution at the putative −10 region, changing the sequence from TATATCAT to GGCCGGCC (see Materials and Methods) (Table 1). (E) Sequence upstream of the disA open reading frame. The start codon is underlined, and the putative transcriptional start sites located 8 bp and 70 bp upstream of the ATG start codon are shown in bold font. The proposed −10 promoter element is shaded. These Rs binding sites with homology to the E. coli consensus sequence (TAAGAAAATCTCCTA) are underlined, and mismatched bases are noted with lighter font.

**RESULTS**

**Identification of the disA promoter.** The 5′ end of the disA tran-
script was identified by 5′ RACE performed on total RNA har-
vested from wild-type PM7002. Sequencing of the 5′ RACE PCR
products returned two potential transcriptional start sites, one located 8 bp upstream of the disA open reading frame (ORF) (Fig. 1A, open arrow, designated −8) and a second site located 70 bp upstream of the disA ORF (Fig. 1A, closed arrow, designated −70). To determine if active promoters were present upstream of these potential start sites, various fragments were cloned into pQF50 (28) to create transcriptional lacZ fusions. These con-
structs were transformed into PM7002 and assayed for β-galacto-
sidase activity (Fig. 2).

Plasmid pBB1 contains a fragment extending from −1,206 to +39 relative to the ATG start of the disA gene (Table 1; Fig. 1B). This fragment contains both potential transcriptional start sites as well as part of the disA and PMI1208 open reading frames (Fig. 1B). The expression of β-galactosidase from pBB1 was 816-fold and 50-fold higher than in cells containing the pQF50 vector with no promoter inserted at 2 and 4 h after plating, respectively.

**Construction of transcriptional lacZ fusions.** (A) The region upstream of disA is shown with the noncoding region comprised of 511 bp, designated −511 to +1 with the disA ORF beginning at +1. The bent arrows indicate potential transcriptional start sites identified by 5′ RACE. The black arrow (−70) is located 70 bp upstream of the disA ORF. The open arrow (−8) is located eight base pairs upstream of the disA ORF. (B) Plasmid pBB1 contains a region extending from 39 bp into the disA gene to 1,206 bp upstream of the disA ORF (including 695 bp from the PMI1208 gene) that is fused to a pro-

**RACE** performed on total RNA har-
vested from wild-type PM7002. Sequencing of the 5′ RACE PCR
products returned two potential transcriptional start sites, one located 8 bp upstream of the lacZ moterless gene in pQF50. (C) Plasmid pBB2 contains the region from 39 bp into the lacZ gene to 1,206 bp upstream of the disA ORF. The open arrow (−8) is located eight base pairs upstream of the disA ORF. (D) Plasmid pBB3 is identical to pBB2 but contains an 8-bp substitution at the putative −10 region, changing the sequence from TATATCAT to GGCCGGCC (see Materials and Methods) (Table 1). (E) Sequence upstream of the disA open reading frame. The start codon is underlined, and the putative transcriptional start sites located 8 bp and 70 bp upstream of the ATG start codon are shown in bold font. The proposed −10 promoter element is shaded. These Rs binding sites with homology to the E. coli consensus sequence (TAAGAAAATCTCCTA) are underlined, and mismatched bases are noted with lighter font.
FIG 2 Transcriptional activity of promoter fragments. 5’ RACE returned two potential transcriptional start sites, −70 and −8 (Fig. 1). Various promoter fragments were cloned upstream of a promoterless lacZ gene in pQF50 as shown in Fig. 1. LB agar plates were inoculated with overnight cultures of each strain that were adjusted to identical optical densities, and cells were harvested off plates at 2 and 4 h after plating. The data shown are representative of two independent experiments, with samples assayed in triplicate. An asterisk indicates a P value of <0.05.

(FIG 2). PM7002 containing plasmid pBB2 with the disA region from −69 to −1 exhibited no β-galactosidase activity, indicating the absence of a promoter upstream of the −8 transcriptional start site (Fig. 1C and 2). This indicated that the functional disA promoter region was upstream of the transcriptional start site originating 70 bp upstream of the disA ATG start codon. To verify this, site-directed mutagenesis was used to change base pairs at the −10 sequence from TATATCAT to CGCCGGCC. The resulting plasmid pBB3 contains these altered base pairs in the context of the full-length disA region present in pBB1. Plasmid pBB3 exhibited a 4.3-fold reduction and a 10.7-fold reduction in β-galactosidase activity 2 and 4 h after being plated on agar surfaces, respectively, compared to pBB1, indicating that altering nucleotides in the −10 region severely decreased the overall promoter activity.

FlhD4C2 does not regulate disA. The class I activator FlhD4C2 has a central role in activating gene expression during swarming, and our lab previously demonstrated that disA expression increases during swarming (16). To address the role of FlhD4C2 in regulating disA expression, a null allele in flhC was constructed as described above (Materials and Methods), resulting in strain BB4. As expected, BB4 was unable to swarm (data not shown). The loss of flhC did not have a statistically significant effect on the expression of a disA-lacZ fusion (pBB1) when cells were assayed either 2 or 4 h after plating on agar surfaces (data not shown). Furthermore, when flhDC was overexpressed from a medium-copy-number plasmid, disA expression was not altered in a statistically significant manner (data not shown). These data indicate that FlhD4C2, the master regulator of swarming, does not have a role in regulating disA expression.

Role of phenethylamine and autoregulation in disA expression. The predicted product of the DisA decarboxylase is phenethylamine (PEA), and previous work demonstrated that exogenous PEA inhibited swarming and flagellar gene expression in a manner similar to disA overexpression (16). We assessed the effect of PEA on disA expression in PM2199, containing a single-copy transcriptional disA-lacZ fusion generated by the insertion of mini-Tn5lacZ1 into the chromosomal copy of disA. This strain was used because the disA gene is inactivated, thereby reducing the intracellular levels of the putative product phenethylamine and allowing for a more sensitive assessment of the effects of exogenous phenethylamine. The presence of various concentrations of phenethylamine decreased disA expression in a dose-dependent manner at T4, with 3.1-fold repression seen at 25 mM, 2.7-fold repression at 16 mM, 1.7-fold repression at 8 mM, and 1.3-fold repression at 4 mM (Fig. 3A). The presence of phenethylamine had little effect in cells at T2 (Fig. 3). To determine if disA expression was subject to autoregulation, the disA gene was overexpressed in trans (Fig. 3B). The overexpression of disA in PM2199 decreased disA-lacZ expression 1.3-fold at 4 h after plating on agar but had no effect at 2 h.

UmoB is a negative regulator of disA. To identify potential regulators of disA, transposon mutagenesis was used to create random mutations in PM7002/pBB1 and colonies were screened on X-Gal plates for those with increased expression from the disA-
lacZ fusion. This yielded an insertion in the umoB (igaA) gene encoding an integral membrane protein that has been shown in members of the Enterobacteriaceae to act as an inhibitor of the Rcs phosphorelay (reviewed in reference 29). The transposon insertion in umoB resulted in a 2.5- to 3-fold increase in disA expression (Fig. 4). The levels of disA expression were reduced to those of the wild type in the presence of a plasmid containing the cloned umoB gene (data not shown). An umoB mutation (umoB::Str) was also independently constructed in PM2199, in which the disA-lacZ fusion is in a single copy, and a similar 2.5-fold increase in expression was observed (Fig. 4).

The umoB mutation alters disA expression via the Rcs phosphorelay. The effect of UmoB on disA expression, as well as its established role as an inhibitor of the Rcs phosphorelay, led us to investigate if the Rcs phosphorelay was involved in regulating disA. Single mutations in rcsB and rcsC, the Rcs response regulator and sensor kinase, and the rcsB umoB and rcsC umoB double mutants were constructed and designated BB5, BB6, BB2, and BB3, respectively (Table 1). Activity of disA was measured in samples harvested 2 and 4 h after plating by β-galactosidase assays, as described earlier. The data demonstrate that an rcsB or rcsC single mutant had no effect on disA expression; however, the umoB rcsB and umoB rcsC double mutants mitigated the effect of anumbo single mutant, returning disA expression to wild-type levels (Fig. 5).

DISCUSSION

In this study, regulation of the disA locus was characterized by using both single-copy and plasmid-based transcriptional lacZ fusions, which allowed us to (i) identify the promoter region, (ii) determine that FlhD4C2 does not have a role in disA regulation, (iii) address the role of autoregulation via phenethylamine and disA overexpression, and (iv) identify a regulatory mutation in umoB that alters disA expression via the Rcs phosphorelay. Interestingly, the levels of β-galactosidase from the single-copy disA-lacZ fusion in PM2199 were higher than those from the disA-lacZ fusion in multicopy (pBB1) (compare Fig. 2 and 3). There are several possible explanations for this. First, the lacZ gene is translated from different ribosome binding sites (RBS) in each construct; the RBS in mini-Tn5lacZ is from the trp operon of E. coli, and in pBB1 (pQF50 vector), it is derived from the lpp gene. Second, there may be cis-acting regulatory sequences that are missing from the promoter region cloned into pBB1 that are required for full expression.

Mutations in rcsB and rcsC in conjunction with an umoB mutation demonstrated that the increased disA expression observed in an umoB mutant background was completely abrogated when
the Rcs phosphorelay was nonfunctional. The observation that disA expression was not decreased in either an rcsB or rcsC single mutant was unexpected but may be due to the presence of the disA-lacZ fusion in pQE50, where the effect of the Rcs phosphorelay, and specifically the RcsB response regulator, on disA expression may be masked by the multicopy nature of the disA-lacZ fusion. The increased RcsB activity in the rcsB mutant may be masked by the multicopy nature of the disA-lacZ fusion. In support of this, recent data from our lab have identified enough to still see regulatory changes in the multicopy fusion. The increased RcsB activity in the multicopy nature of the disA mutant was unexpected but may be due to the presence of the disA-lacZ fusion.

Previous research and sequence homology led to the prediction that phenethylamine, the decarboxylated form of phenylalanine, or a similar molecule was the product of DisA (16). We have provided evidence that a negative feedback loop is present for disA regulation. First, disA expression was decreased in PM2199 when disA was overexpressed (Fig. 3). Second, the addition of exogenous phenethylamine inhibited disA expression (Fig. 3). However, the magnitude of the repression differed under each condition, 3-fold with phenethylamine at 25 mM versus 1.4-fold with 10 mM. Consistent with this, a strong dose-dependent effect on disA repression was observed with phenethylamine concentrations ranging from 4 to 25 mM (Fig. 3).

One purpose of this negative feedback loop may be to downregulate disA expression after the peak levels of expression have been reached at 3 to 4 h into the swarming cycle. The subsequent decrease in DisA activity would then prepare cells for the next cycle of swarming by relieving the inhibition of FlhD_C2. It is also possible that extracellular phenethylamine encountered in the environment may have a role in regulating disA expression. It has been proposed that phenethylamine concentrations in the low millimolar range may be encountered in the intestinal tract and that this may influence swarming in the intestine (30). A similar mechanism for the control of disA expression may exist in the urinary tract. However, this is highly speculative because, to our knowledge, the levels of phenethylamine in the urinary tract are unknown. The ability to fine-tune disA expression by both a negative feedback loop and the Rcs phosphorelay would provide more-precise control of disA expression required for the transition between the swarming and consolidation phases.

ACKNOWLEDGMENTS

This work was funded by Merit Review and Research Career Scientist awards from the Department of Veterans Affairs. We are grateful to Katy Clemmer for the isolation of PM2199 and to Katy Clemmer and Elizabeth Ohneck for their comments on the manuscript.

REFERENCES


