

Complementation of the *fadA* Mutation in *Fusobacterium nucleatum* Demonstrates that the Surface-Exposed Adhesin Promotes Cellular Invasion and Placental Colonization[∇]

Akihiko Ikegami,¹ Peter Chung,¹ and Yiping W. Han^{1,2,*}

Department of Periodontics, School of Dental Medicine,¹ and Department of Pathology, School of Medicine,²
Case Western Reserve University, Cleveland, Ohio 44106-4905

Received 23 February 2009/Returned for modification 28 March 2009/Accepted 19 April 2009

***Fusobacterium nucleatum* is a gram-negative oral anaerobe implicated in periodontal disease and adverse pregnancy outcome. The organism colonizes the mouse placenta, causing localized infection and inflammation. The mechanism of placental colonization has not been elucidated. Previous studies identified a novel adhesin from *F. nucleatum*, FadA, as being involved in the attachment and invasion of host cells. The *fadA* deletion mutant *F. nucleatum* 12230 US1 was defective in host cell attachment and invasion in vitro, but it also exhibited pleiotropic effects with altered cell morphology and growth rate. In this study, a *fadA*-complementing clone, *F. nucleatum* 12230 USF81, was constructed. The expression of FadA on USF81 was confirmed by Western blotting and immunofluorescent labeling. USF81 restored host cell attachment and invasion activities. The ability of *F. nucleatum* 12230, US1, and USF81 to colonize the mouse placenta was examined. US1 was severely defective in placental colonization compared to the wild type and USF81. Thus, FadA plays an important role in *F. nucleatum* colonization in vivo. These results also represent the first complementation studies for *F. nucleatum*. FadA may be a therapeutic target for preventing *F. nucleatum* colonization of the host.**

Fusobacterium nucleatum is a gram-negative filamentous anaerobe and a commensal organism of the oral cavity. It is an opportunistic pathogen that has been implicated in various forms of periodontal disease and in infections and abscesses in other parts of the body. *F. nucleatum* is highly prevalent in intrauterine infections causing adverse pregnancy outcome including spontaneous miscarriage, preterm birth, and stillbirth (1, 2, 6, 9; unpublished results). It was previously suggested that the organism can translocate from the oral cavity to the uterus through hematogenous transmission (5, 8). *F. nucleatum* adheres to and invades epithelial and endothelial cells (5, 7), which may be a mechanism for transmission. We have shown using mice that once in the blood circulation, *F. nucleatum* colonizes specifically in the placenta, causing Toll-like receptor 4-mediated localized inflammatory responses leading to preterm or term stillbirth without causing systemic infections (5, 10). The pattern of infection and inflammation mimics that in humans (5). It was unclear, however, which virulence factors allowed the bacteria to colonize the placenta. One possible candidate is the FadA adhesin, the only adhesin identified from *F. nucleatum* so far to be involved in tissue culture cell attachment and invasion (4).

FadA is a small α -helical peptide consisting of 129 amino acid residues in its nonsecreted form (pre-FadA) and 111 residues in the secreted form (mFadA). It is a unique adhesin in that both pre-FadA and mFadA are required for oligomerization and function (13). The crystal structure of FadA reveals a filamentous structure with the monomers linked together in a

head-to-tail pattern via a novel leucine chain motif (11). A *fadA* deletion mutant, *F. nucleatum* 12230 US1, was defective in the attachment and invasion of KB oral mucosal carcinoma cells and Chinese hamster ovary (CHO) cells (4). However, the disruption of *fadA* also exhibited pleiotropic effects, with alterations in bacterial morphology and growth rate (4; unpublished results). Thus, it is necessary to perform genetic complementation studies to confirm the function of FadA. Due to the difficulty in the genetic manipulation of *F. nucleatum*, no complementing clones in *F. nucleatum* have been made until now.

In this study, a *fadA*-complementing clone, *F. nucleatum* 12230 USF81, was constructed. The ability of *F. nucleatum* 12230, US1, and USF81 to adhere to and invade epithelial cells was assessed in vitro, and their ability to colonize the mouse placenta was examined in vivo.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *F. nucleatum* was cultivated using Columbia blood agar supplemented with 5 μ g/ml hemin (Sigma), 1 μ g/ml menadione (Sigma), and 5% defibrinated sheep blood (Cleveland Scientific, OH) and incubated at 37°C with 5% CO₂, 10% H₂, and 85% N₂. *Escherichia coli* TOP10 cells were cultivated using LB agar or broth. The *fadA* gene with a Shine-Dalgarno sequence was amplified using primers *fadASDF* and *fadASDR*, creating a 436-bp fragment with a BamHI site at the 5' end and a SalI site at the 3' end. The *ermF* gene was amplified using primers *erm1F* and *erm1R*, creating a 622-bp fragment with a BamHI site at the 3' end. The *ermAM* gene was amplified using primers *erm2F* and *erm2R*, creating a 643-bp fragment with a SalI site at the 5' end. Following digestion with BamHI and/or SalI, these three fragments were ligated, and the *ermF-fadA-ermAM* fragment was amplified using primers *erm1F* and *erm2R* and cloned into pCR2.1 (Invitrogen). The resulting plasmid, pYH1479, was digested with SalI, and the *catP* gene from plasmid pJIR418 (12) was inserted into this site to generate pYH1480.

Construction of the *fadA*-complementing clone. Log-phase *F. nucleatum* US1 cells were washed and resuspended in phosphate-buffered saline (PBS) supplemented with 0.1 mM CaCl₂ and 0.1 mM MgCl₂. The bacterial suspension (approximately 1 \times 10⁹ cells) was mixed with 50 μ g plasmid pYH1480 and 50 μ l

* Corresponding author. Mailing address: Department of Periodontics, School of Dental Medicine, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-4905. Phone: (216) 368-1995. Fax: (216) 368-0145. E-mail: yiping.han@case.edu.

[∇] Published ahead of print on 27 April 2009.

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Bacterial strain, plasmid, or primer	Description or sequence (5'-3')	Reference or source
Bacterial strains		
<i>F. nucleatum</i>		
12230	Wild type	7
US1	<i>fadA</i> deletion mutant (12230 Δ <i>fadA::ermF-ermAM</i>)	4
USF81	<i>fadA</i> -complementing strain (12230 US1 <i>ermF-ermAM::fadA-catP</i>)	This study
<i>E. coli</i> TOP10		
	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> <i>galU</i> <i>galK</i> Δ (<i>ara-leu</i>)7697 <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
Plasmids		
pCR2.1	Cloning vector for PCR fragments	Invitrogen
pJIR418	Carrying <i>catP</i> , conferring thiamphenicol resistance	12
pYH1479	Carrying the <i>ermF-fadA-ermAM</i> fragment with the Sall site between <i>fadA</i> and <i>ermAM</i>	This study
pYH1480	<i>catP</i> from pJIR418 cloned into the Sall site in pYH1479, used for sonoporation to generate a <i>fadA</i> -complementing clone	This study
Primers		
<i>fadASDF</i>	TTTGATCCTAAATAAATAATTTGGGAGGTAACAAAAATG	This study
<i>fadASDR</i>	TTTGTGACTTAGTTACCAGCTCTTAAAGC	This study
<i>erm1F</i>	TGCATACCTTTGTTCTCCGG	This study
<i>erm1R</i>	TTTTTTGGATCCGAAGGACAATGGAACCTCCC	This study
<i>erm2F</i>	TTTTTTGTCGACAAAATTGGAACAGGTAAAGGGC	This study
<i>erm2R</i>	CTCATAGAATTATTTCTCCCG	This study

Definity:Perflutren lipid microsphere (Bristol-Myers Squibb) in 0.4-ml wells of an eight-well flat-bottom immunomodule with a frame (Nalge Nunc International). The mixture was treated by sonoporation as previously described (3). The sonoporated suspension was plated onto Columbia blood agar plates and incubated without selection for 24 h at 37°C under anaerobic conditions, followed by replication on Columbia blood agar plates containing 5 μ g/ml thiamphenicol. The plates were incubated for approximately 3 to 5 days at 37°C. The thiamphenicol-resistant colonies were isolated and inoculated in Columbia broth containing 5 μ g/ml thiamphenicol.

Western blotting analysis. Western blotting analysis was performed as previously described (13), with slight modifications. Whole-cell *F. nucleatum* cultures, at approximately 10⁸ CFU, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by transfer to polyvinylidene difluoride membranes (Millipore). Anti-FadA monoclonal antibody (MAb) 5G11-3G8 (13) was used as the primary antibody at a 1:5,000 dilution, and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Pierce) was used as a secondary antibody at a 1:2,000 dilution, followed by chemiluminescence development.

Immunofluorescent staining of FadA. Immunofluorescent staining of FadA was performed as previously described, with modifications (11). *F. nucleatum* cells were harvested by centrifugation and resuspended in PBS. The suspension was spotted onto a glass slide and allowed to air dry. After methanol fixation, the bacteria were first incubated with 5% skim milk in PBS for 1 h at room temperature, followed by incubation with anti-FadA MAb 5G11-3G8 (13) at a 1:50 dilution or its mouse IgG1 isotype for 30 min at 37°C. Following washes with PBS, the bacteria were incubated for 1 h at 37°C with Alexa Fluor 594 chicken anti-mouse IgG (Invitrogen) at a 1:100 dilution. For controls, conjugated secondary antibodies were incubated with the bacteria without prior incubation with anti-FadA MAb.

Tissue culture attachment and invasion assays. Tissue culture attachment and invasion by *F. nucleatum* were determined as previously described (7). Briefly, CHO (ATCC CRL10154) and immortalized human oral keratinocyte OKF6/Tert (Tert) cells (13) were grown to near confluence in 24-well trays, followed by the addition of bacteria at a multiplicity of infection of approximately 50:1 to 100:1 (bacteria:cells). For attachment assays, the infected monolayers were incubated at 37°C in 5% CO₂ for 1 h and then washed four times with PBS. The cells were lysed by water, and serial dilutions were plated onto blood agar plates to enumerate cell-associated bacteria. For invasion assays, the infected monolayers were incubated at 37°C for 3 to 4 h, followed by washes with PBS. Fresh media containing 300 μ g/ml gentamicin and 200 μ g/ml metronidazole were

added to the monolayer and incubated for an additional hour to eliminate extracellular bacteria. The cells were washed again and lysed with water. The serial dilutions were plated to enumerate the invaded bacteria. The level of attachment (or invasion) was expressed as the percentage of cell-associated (or invaded) bacteria over the total number of bacteria initially added to the monolayers.

***F. nucleatum* colonization in the mouse placenta.** *F. nucleatum* colonization in the mouse placenta was determined as previously described (5). Briefly, approximately 2 \times 10⁷ CFU of *F. nucleatum* were injected into each pregnant outbred CF-1 mouse via the tail vein on day 16 of gestation. The placentas were harvested at 6 or 24 h postinjection, followed by homogenization. Serial dilutions were plated onto blood agar plates to enumerate the live bacteria. Live bacterial titers in the placentas were expressed as log(CFU/gram tissue).

Statistical analysis. The Student *t* test was used for data analysis. A *P* value of <0.05 was considered to be significant.

RESULTS

Construction of the *fadA*-complementing strain. *fadA* deletion mutant strain US1 was constructed via sonoporation by replacing *fadA* on the chromosome of *F. nucleatum* 12230 with the *ermF-ermAM* cassette, conferring clindamycin resistance (4). To complement the *fadA* deletion, an exogenous wild-type copy of *fadA* needs to be introduced into US1. Complementation by shuttle plasmids was not a viable option because to date, no shuttle plasmid has been successfully introduced into *F. nucleatum* 12230. Taking advantage of the important characteristic of sonoporation, i.e., one-step double-crossover recombination, and the fact that *fadA* is monocistronic and the replacement of *fadA* with the *ermF-ermAM* cassette did not affect the transcription of the downstream genes (3, 4), we constructed the complementing clone by inserting wild-type *fadA* back onto the chromosome in the *ermF-ermAM* cassette. Suicide plasmid pYH1480 was generated, carrying the *ermF-fadA-catP-ermAM* fragment, with wild-type *fadA* and *catP*

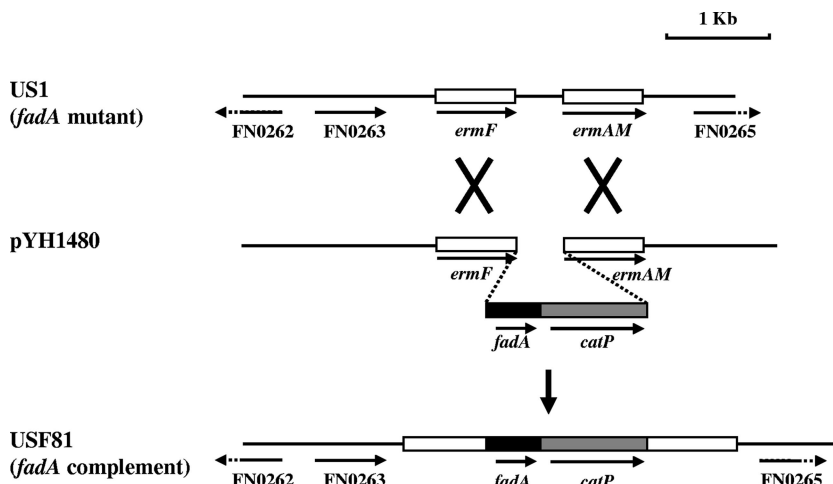


FIG. 1. Construction of *fadA*-complementing clone USF81. Suicide plasmid pYH1480 carrying *ermF-fadA-catP-ermAM* was sonoporated into *fadA* deletion mutant US1 ($\Delta fadA::ermF-ermAM$), in which the *fadA* gene was almost completely replaced by the *ermF-ermAM* cassette. Through double-crossover homologous recombination in *ermF* and *ermAM*, the wild-type *fadA-catP* fragment was inserted into the chromosome of US1, generating thiamphenicol-resistant, *fadA*-complementing clone USF81.

flanked by a 622-bp fragment of *ermF* upstream and a 643-bp *ermAM* fragment downstream, respectively (Fig. 1). All four genes or gene fragments were aligned such that they transcribed in the same direction as the *ermF-ermAM* cassette on the chromosome of US1. The >600-bp flanking fragments of *ermF* and *ermAM* provide enough homology for the double crossover to take place. Plasmid pYH1480 was delivered into US1 by sonoporation using established protocols (3). Thiamphenicol-resistant transformants, conferred by *catP*, were selected. As reported previously, the transformation efficiency was low, at approximately 0.02 transformants/ μ g DNA (4). Nevertheless, PCR examination of the transformants showed that, as expected, they were double-crossover constructs with wild-type *fadA* and *catP* inserted between *ermF* and *ermAM* on the chromosome of US1. One such clone was designated USF81.

Expression of FadA in the *fadA*-complementing strain. The expression of FadA in USF81 was examined by Western blotting analysis and immunofluorescent labeling using anti-FadA MAbs 5G11-3G8 (13) (Fig. 2). *F. nucleatum* 12230 and US1 were included as positive and negative controls, respectively. While the results from the Western blotting analysis indicated that FadA was expressed at a lower level in USF81 than in the wild type, the results from immunofluorescent labeling were comparable between the two strains. Furthermore, since the immunofluorescent staining was performed using non-detergent-treated cells, the positive staining of USF81 indicates that, like in *F. nucleatum* 12230, FadA is exposed on the bacterial cell surface.

FadA complementation restores wild-type levels of attachment and invasion of host cells. We have previously shown that US1 was defective in attachment to host cells. However, since morphological and growth rate changes were observed in US1, the question remained as to whether or not the decreased adherence was due to the absence of FadA. Such a question can now be addressed using USF81. USF81 adhered to both CHO and Tert cells to levels comparable to those of *F. nucleatum* 12230, confirming that FadA was responsible for re-

storing the attachment activity (Fig. 3A). Furthermore, previous studies showed that the purified FadA protein could be internalized into host cells (13), but it was unclear if FadA was a true invasin responsible for the internalization of the bacteria

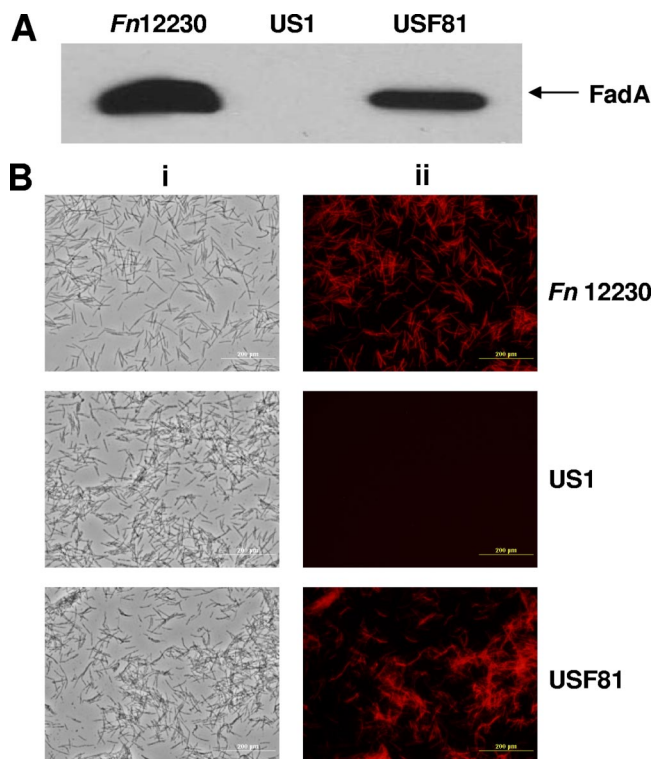


FIG. 2. Expression of FadA in *F. nucleatum* 12230, US1, and USF81. (A) Western blotting analysis using MAb 5G11-3G8. The arrow points to FadA detected in *F. nucleatum* 12230 (Fn12230) and USF81. (B) Immunofluorescent labeling of FadA on non-detergent-treated bacteria using MAb 5G11-3G8. The same fields were captured by both light (i) and fluorescent (ii) microscopes using a 40 \times objective.

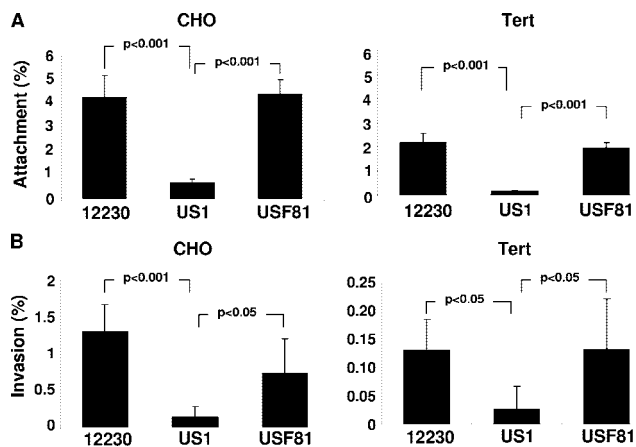


FIG. 3. Attachment (A) and invasion (B) of CHO and immortalized human oral keratinocyte OKF6/Tert (Tert) cells by wild-type *F. nucleatum* 12230, *fadA* deletion mutant US1, and the *fadA*-complementing clone USF81. The results shown are averages of data from at least four independent experiments, with each experiment performed in triplicate. The standard deviations are expressed as lines above the bars. In all experiments, no significant difference was found between *F. nucleatum* 12230 and USF81.

into the host cells. Thus, *F. nucleatum* 12230, US1, and USF81 were tested for invasion of CHO and Tert cells (Fig. 3B). While US1 was severely defective in the invasion of both cell types, USF81 restored the invasion levels to wild-type levels. The results shown here represent the first demonstration of the direct involvement of FadA in *F. nucleatum* invasion.

FadA is involved in bacterial colonization in mouse placenta. To determine if FadA is involved in bacterial colonization in vivo, we took advantage of the previously established pregnant murine model (5) by injecting *F. nucleatum* 12230, US1, and USF81 into the mouse tail veins on day 16 of gestation. Bacterial titers in the mouse placenta were determined at 6 and 24 h postinjection according to our established protocol (5). At 6 h, the bacterial titers for all three strains were similar to each other (Fig. 4). This result confirms that the doses of the bacterial strains were comparable and that all three strains survived in mice equally well during that period. At 24 h, the titer of US1 was >1,000-fold lower than those of *F. nucleatum* 12230 and USF81 (Fig. 4), indicating that FadA may be responsible for *F. nucleatum* colonization in vivo. FadA is the first *F. nucleatum* component identified to be involved in intrauterine infection in the mouse model.

DISCUSSION

F. nucleatum is refractory to genetic manipulation, possibly due to the numerous restriction and modification systems which the organism encodes (4). Using sonoporation, we constructed the first allelic exchange mutant, US1, in *F. nucleatum* (4). In this study, a *fadA*-complementing clone, USF81, was constructed by inserting a wild-type copy of *fadA* onto the chromosome of US1. The original deletion was not restored. Thus, our approach fulfills the *cis-trans* testing criteria of complementation. An advantage of inserting the complementing gene onto the chromosome is that only one copy of the gene is provided, as in the wild type, compared to multiple

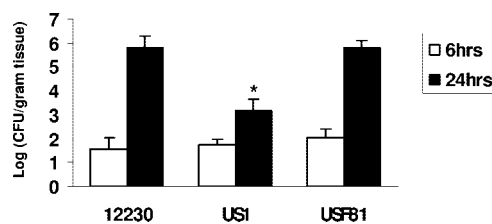


FIG. 4. Colonization of *F. nucleatum* 12230, US1, and USF81 in the mouse placenta. Approximately 2×10^7 CFU of different *F. nucleatum* strains were injected into each pregnant CF-1 mouse via tail vein on day 16 of gestation. The placentas were harvested 6 or 24 h later. Live bacterial titers in the placentas were determined and expressed as log(CFU/gram tissue). The results shown are averages of data from three to seven mice per strain. The standard deviations are expressed as lines above the bars. *, $P < 0.05$ compared to *F. nucleatum* 12230.

copies provided by plasmids. To the best of our knowledge, this is the first genetic complementation reported for *F. nucleatum*. The study reported here confirms that FadA is expressed on the bacterial cell surface. The loss of FadA detection by fluorescent labeling in US1 was not due to changed cell morphology. Furthermore, complementation of FadA restored the ability of the bacteria to adhere to and invade CHO and oral keratinocyte OKF6/Tert cells to levels similar to those of wild-type *F. nucleatum*. Attachment precedes invasion; thus, the reduced invasion activity observed in US1 may be attributable to its reduced ability to adhere. However, since previous studies have demonstrated both binding and internalization of purified recombinant FadA (13), it is plausible that FadA is required for both attachment and invasion.

We have shown that *F. nucleatum* colonizes and proliferates in the mouse placenta following hematogenous infection (5). Specific colonization in the placenta was observed at 24 h postinfection, when the bacteria were completely eliminated from the liver and spleen (5). It was unclear which virulence factor(s) allowed the bacteria to colonize the placenta. In this study, we have demonstrated that FadA plays a key role in this process. When injected into the tail veins of pregnant mice, *F. nucleatum* 12230, US1, and USF81 were isolated from the placenta in similar quantities at 6 h, confirming that equivalent doses of these strains were administered. In addition, the results at 6 h indicate that all three strains survived equally well in mice during that period. At 24 h postinjection, the amount of US1 recovered was severely reduced compared to amounts of *F. nucleatum* 12230 and USF81, indicating that FadA is involved in placental colonization and proliferation. Previously reported observations revealed that *F. nucleatum* invaded endothelial cells in infected mouse placentas in vivo (5). It is possible that the lack of FadA prevented the bacteria from crossing the endothelial lining in the placenta, rendering them unable to colonize. The results reported here represent the first demonstration of FadA function in vivo. It is also the first virulence factor identified in *F. nucleatum* to be involved in the pathogenesis of intrauterine infection. FadA may be a potential therapeutic target for the prevention and treatment of adverse pregnancy outcome caused by *F. nucleatum* infection.

ACKNOWLEDGMENTS

We thank Yann Fardini for assistance in manuscript preparation and Howard Kuramitsu for reading the manuscript.

This work was supported in part by NIH grants RO1 DE 14924, KO2 DE 16102, and R21 DE17165 to Y.W.H.

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Editor: S. R. Blanke