INFECTION AND IMMUNITY, Dec. 2002, p. 7120–7125 Vol. 70, No. 12
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.12.7120–7125.2002

NOTES

Construction of an *Actinobacillus pleuropneumoniae* Serotype 2 Prototype Live Negative-Marker Vaccine

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Received 11 June 2002/Returned for modification 22 July 2002/Accepted 4 September 2002

Deletions were introduced into the *ureC* and *apxIIA* genes of an *Actinobacillus pleuropneumoniae* serotype 2 strain by homologous recombination and counterselection. The double-mutant contains no foreign DNA, is highly attenuated, protects pigs from homologous challenge upon a single aerosol application, and facilitates the serological discrimination of immunized and infected herds.

Vaccination against bacterial pathogens is increasingly relevant in the livestock industry because of the growing problems of drug resistance and antimicrobial residues (22). Frequently, vaccination is directed against pathogens already present in the herd (3). This metaphylactic strategy can decrease the frequency of clinical disease and thereby significantly lower the need for antibiotic therapy. However, bacterial vaccines currently in use do not allow a serology-based discrimination between vaccinated and infected animals in a routine test. This discrimination, however, is of major importance for generating and maintaining specified pathogen-free herds which are the optimum choice with respect to long-term animal health and consumer protection (11).

Actinobacillus pleuropneumoniae, the cause of porcine pleuropneumonia, is a major economic problem in the swine industry worldwide (7). Treatment of the disease, characterized by hemorrhagic, fibrinous, and necrotic lesions, is increasingly difficult due to the occurrence of antibiotic-resistant strains (4). Vaccination is complicated by limited cross-serotype protection upon immunization with bacterin preparations (14). In addition, bacterin vaccines do not prevent colonization, thereby potentially facilitating the development of healthy carrier animals; in contrast, convalescent pigs are completely protected against infection with a homologous serotype (5).

Here we describe the development of a prototype live attenuated *A. pleuropneumoniae* serotype 2-negative marker vaccine carrying deletions in the *apxIIA* and *ureC* genes. We investigate whether this strain is attenuated and whether it can prevent not only clinical disease but also colonization upon a single application. An *A. pleuropneumoniae* serotype 2 strain was chosen since it is the most frequently isolated serotype in northern Europe. The *apxIIA* gene was deleted as it encodes a highly immunogenic virulence factor expressed by all *A. pleuropneumoniae* serotypes except serotype 10; it has been used for serodiagnosis (16) and, therefore, could be used for discrimination of immunized and infected herds in routine diagnostics. The *ureC* gene was deleted in order to potentially reduce shedding of the vaccine strain (2); in additon, it can serve as a reliable phenotypic marker to discriminate between the vaccine and the wild-type strain.

Construction of a mutant strain. To construct the *A. pleuropneumoniae* serotype 2 isogenic mutant, 12 clinical isolates were tested initially with respect to their amenability to genetic manipulation via conjugation and cointegration of pBMKUΔ1 (Table 1). One isolate, designated *A. pleuropneumoniae* C5934, formed stable cointegrates upon conjugation (19) as assessed by DNA colony blots (21) and was used for further manipulations. For sucrose counterselection, which is required to obtain unmarked deletion mutants, a single kanamycin-resistant colony was cultured in 1 ml of supplemented PPLO medium (Difco, Detroit, Mich.) at 37°C for 2 h with shaking. Then, an equal volume of counterselection medium (0.4 volumes of 2× medium without added NaCl [46 g of Bacto Beef Heart for Infusion/liter, heated and filtered as recommended by the manufacturer, plus 9.25 g of Bacto Peptone/liter, both purchased from Difco], 0.5 volume of 40% sucrose, 0.1 volume of equine serum) was added, and the incubation was continued for 6 h. Ten sterile glass beads (2 mm in diameter) were added, and bacterial clumps were broken by vortexing for 2 min. Aliquots were plated and further investigated by PCR analyses (1) with the appropriate primers (Table 1); the PCR consisted of an initial denaturation (94°C, 30 s), 32 amplification cycles (denaturation [94°C, 30 s], annealing [53°C, 40 s], and extension [72°C, 2 min]), and a final extension (72°C, 10 min). Colonies with the correct PCR profile (Fig. 1A) were confirmed by Southern blot analyses upon capillary transfer (21) with the PCR products obtained from the respective deletion mutants as a probe (Fig. 1B). The absence of gross chromosomal rearrangements was shown by pulsed-field gel electrophoresis (Fig. 1C) performed as previously described (18). The resulting *A. pleuropneumoniae* double mutant was urease neg-

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TABLE 1. Characteristics of bacterial strains, plasmids, and primers used in this study

Strains, plasmids, and primers	Characteristics or sequence[a]	Source or reference
Strains		
E. coli DH5αF′	F′ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*) *U169 deoR* [ϕ80d*lac*δ*(lacZ)*M15]	20
E. coli HB101	F′ Δ(*gpt-proA)62 leuB6 supE44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20* (Str^r) *xyl-5 mtl-1 recA13*	21
E. coli β2155	*thrB1004 pro thi hsdS lacZ*ΔM15 (F′ *lacZ*ΔM15 *lacI*^{q} *traD36 proA*$^+$ *proB*$^+$) Δ*dap*::*erm* (Erm^r)	6
11 *A. pleuropneumoniae* serotype 2 isolates	Strain collection; Institut für Mikrobiologie und Tierseuchen; all strains are obtained from diseased pigs in Northern Germany	This work
A. pleuropneumoniae C5934	*A. pleuropneumoniae* serotype 2 clinical isolate from the lung of a diseased pig in northern Germany	This work
A. pleuropneumoniae C5934Δ*ureC*	Unmarked urease-negative knockout mutant of *A. pleuropneumoniae* C5934	This work
A. pleuropneumoniae C5934Δ*ureC*Δ*apxIIA*	Unmarked ApxIIA-negative knockout mutant of *A. pleuropneumoniae* C5934Δ*ureC*	This work
Plasmids		
pBluescript SK	*E. coli* cloning vector carrying an ampicillin resistance determinant	Stratagene[b]
pGH432	*E. coli* cloning vector carrying an ampicillin resistance determinant and a *tac* promoter transconjugation vector based on pBluescript SK with *mob*RP4, polycloning site, kanamycin resistance, and transcriptional fusion of the *omlA* promoter with the *sacB* gene	9
pBMK1		19
pBMKUΔ1	pBMK1 carrying the *ureC* gene with an internal *Sph*I-*Bst*E deletion	19
pBMKAΔ1	A PCR fragment obtained with the primers apxIIAU and apxIIAL was restricted with *Bam*HI and *Sad*I and ligated into pBluescript tSK, resulting in plasmid pBAPX3; plasmid pBAPX3 was restricted with *Nco*1 and *Bgl*II, blunt ended with Klenow fragment, and religated; the truncated *apxII*A gene was removed	This work
Primers		
ureC2	GTAAGG ATCC ATTAACAATC CCACGCAGTC AGTAT; primer with internal *Bam*HI site (underlined) comprising positions 997 to 1022 of the urease operon	19
ureX	TCATGTCGAC TAGAACAAGA AATAACGCTG TGCAA; primer with internal *Sal*I site (underlined) comprising positions 2686 to 2711 of the urease operon	19
apxIIAU	CTTTGGATCC AAAAATCACT TTGTCATCAT; primer with internal *Bam*HI site (underlined) comprising positions 782 to 811 of the *apxIICA* genes	This work
apxIIAL	CAATGTCGAC ATTTACACCA TAGGATTGCT; primer with internal *SalI* site (underlined) comprising positions 3670 to 3699 of the *apxIICA* genes	This work

[a] Nal^r, nalidixic acid resistant; Erm^r, erythromycin resistant; Str^r, streptomycin resistant.
[b] Stratagene Europe, Amsterdam, The Netherlands.

ative and showed a CAMP-like hemolytic activity on Columbia sheep blood (CSB) agar plates with *S. aureus* (Fig. 1D). These results show that some isolates of *A. pleuropneumoniae* serotype 2 are amenable to genetic manipulation by a conjugation system previously described for *A. pleuropneumoniae* serotype 7.

Virulence studies. The degree of attenuation of the *A. pleuropneumoniae* double mutant was investigated by using three groups of eight pigs each as described previously for *A. pleuropneumoniae* serotype 7 (2). The results showed a significant reduction in clinical symptoms and pathology even with the higher dose (Fig. 2A). The bacteriological examination at the end of the experiment revealed that *A. pleuropneumoniae* could be consistently reisolated in high numbers from the lung lesions of pigs challenged with the wild-type strain (Fig. 2A). In pigs challenged with the double mutant, *A. pleuropneumoniae* could be reisolated from intact lung tissue in small numbers in the low-dose challenge group and from lesions in the high-dose challenge group (Fig. 2A). In all pigs, an immune response could be detected in the detergent extract enzyme-linked immunosorbent assay (ELISA) (10), and only in the wild-type group six of seven pigs showed elevated levels (>10 ELISA units [EU]) in the ApxIIA ELISA (16) 3 weeks after challenge (Fig. 2B). These results showed that the *A. pleuropneumoniae* double-mutant strain is highly attenuated and that the group infected with the mutant strain can be discriminated from the wild-type-infected group based on the ApxIIA ELISA; the antigenic relationship among *A. pleuropneumoniae* Apx toxins described previously (17) did not cause any false-positive reaction in the recombinant ApxII-ELISA used.

Protection studies. Protection experiments were performed by using the *A. pleuropneumoniae* serotype 2 double mutant (*A. pleuropneumoniae* C5934Δ*ureC*Δ*apxIIA*) as a live vaccine in a single aerosol immunization. Three weeks later the immunized group and a nonimmunized control group were challenged with the virulent parent strain. No clinical symptoms were observed in immunized pigs, whereas six of seven pigs in the control group developed severe disease with two pigs dying on the second day after challenge. Postmortem analysis on day 7

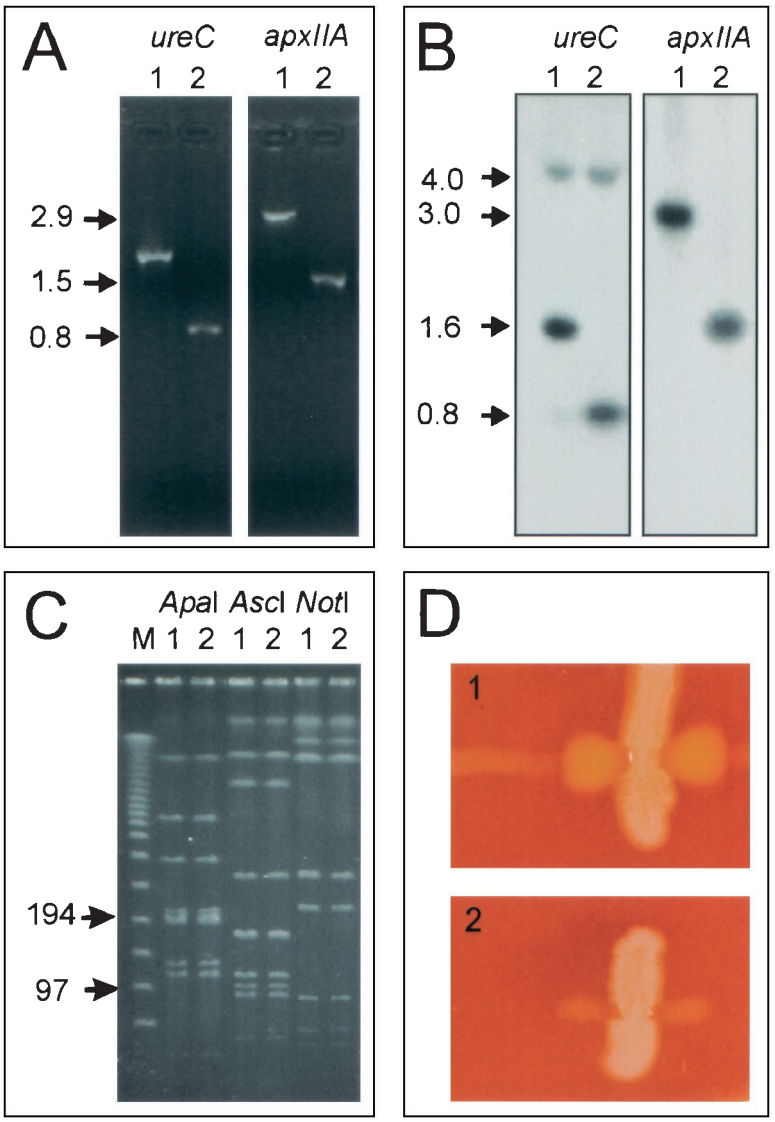

FIG. 1. Analysis of *A. pleuropneumoniae* C5934wt (lanes 1) and *A. pleuropneumoniae* C5934Δ*ureC*Δ*apxIIA* (lanes 2). (A) PCR with the primers ureC2 and ureX (left) and apxIIAU and apxIIAL (right). The reduction of the size of the PCR fragments obtained from the double mutant is due to the deletion of an 870-bp *Sph*I-*Bst*EII fragment in the *ureC* gene (19) and a 1,518-bp *Bgl*II-*Nco*I fragment in the *apxIIA* gene (Table 1). (B) Southern blot analyses by using the truncated *ureC* gene (left) and the truncated *apxIIA* gene (right) as probes; chromosomal DNA was digested with *Bst*EII (*ureC* blot) and *Nde*I (*apxIIA* blot); the reduction of the size in the hybridizing restriction endonuclease fragments obtained in the mutants is as calculated based on the size of the deleted fragments. (C) Pulsed-field gel electrophoresis of *Apa*I-, *Asc*I-, and *Not*I-digested DNA showing that no gross rearrangements have occurred. (D) Hemolysis and CAMP-like phenomenon of the *A. pleuropneumoniae* double mutant due to a *Staphylococcus aureus* strain on CSB agar.

postinfection revealed no pathological changes in six of seven immunized pigs (Fig. 3A). The pathological changes in the remaining pig were due to the vaccine strain since only this strain could be isolated; the vaccine strain could not be isolated from any of the other pigs. The challenge strain (*A. pleuropneumoniae* C5934wt) was isolated in low numbers from unaltered lung tissue of one of the seven pigs. In the control group, confluent growth of the challenge strain was obtained from the lesions of six of seven pigs (Fig. 3A). None of the pigs in either group showed a detectable immune response in the ApxIIA ELISA before challenge. The lack of a detectable response in the wild-type group 1 week after challenge was to be expected, since a minimum of 10 days is required to obtain a detectable serum activity (data not shown). In the detergent extract ELISA, all vaccinated pigs and none of the controls responded before challenge (Fig. 3B). At 1 week after challenge, the specific titer in the sera of immunized pigs had increased slightly, and also all nonimmunized pigs had developed very low titers in the detergent extract ELISA (Fig. 3B). The results show that a single aerosol application of the attenuated double mutant resulted in protection from clinical disease comparable to that obtained with two applications by using a conventional bacterin vaccine. In addition, immunized pigs were protected significantly from colonization of

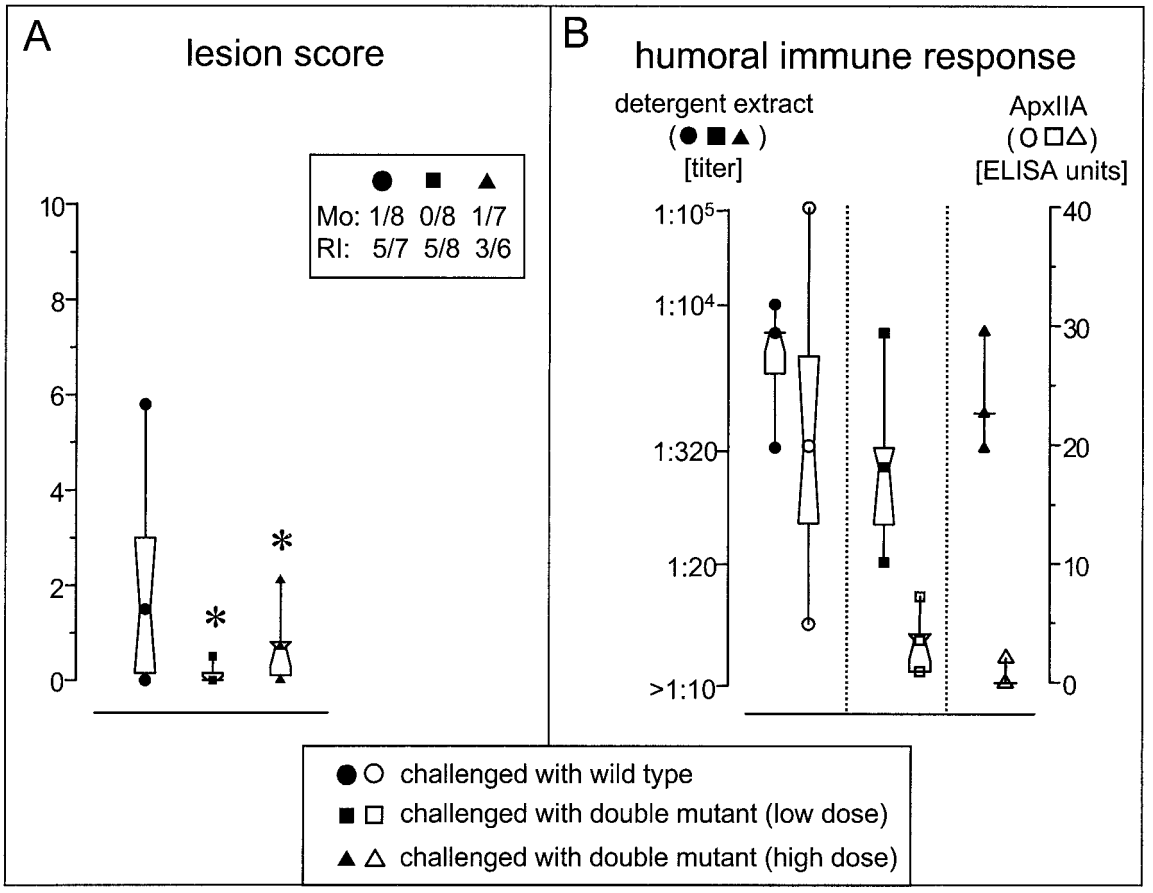

FIG. 2. Lung lesion score (A) and humoral immune response (B) at 21 days after challenge with the *A. pleuropneumoniae* parent strain and isogenic double mutant. Pigs were aerosol infected with a total of 1.3×10^5 *A. pleuropneumoniae* C5934wt and a total of 1.3×10^5 or 6.5×10^5 *A. pleuropneumoniae* C5934Δ*ureC*Δ*apxIIA*. The aerosolization of a total of 10^5 bacteria results in 10^2 *A. pleuropneumoniae* per liter of aerosol in the chamber. Lung lesion scores were determined as described by Hannan et al. (12), and the bacteriological examination was done as previously described (2). Mortality (Mo) and reisolation frequency from lung tissue (RI) results for the different groups are given. The antibody response was assessed with two ELISAs with a detergent extract (solid symbols) and the recombinant ApxIIA protein (open symbols) as the solid-phase antigen. The response was quantified as the serum titer in comparison to an internal negative control (detergent extract ELISA) and in EU (based on an external standard) in the ApxIIA ELISA. For the standardized ApxIIA ELISA, activities of ≤10 EU in the sera are considered negative, 11 to 25 EU is intermediate, and >25 EU is a positive result. The central square within the hourglass shape represents the geometric mean, the hinges present the values in the middle of each half of data, and the top and bottom squares mark the maximum and minimum values, respectively. The asterisk denotes statistical significance ($P < 0.05$) for the lung lesion score (Wilcoxon test).

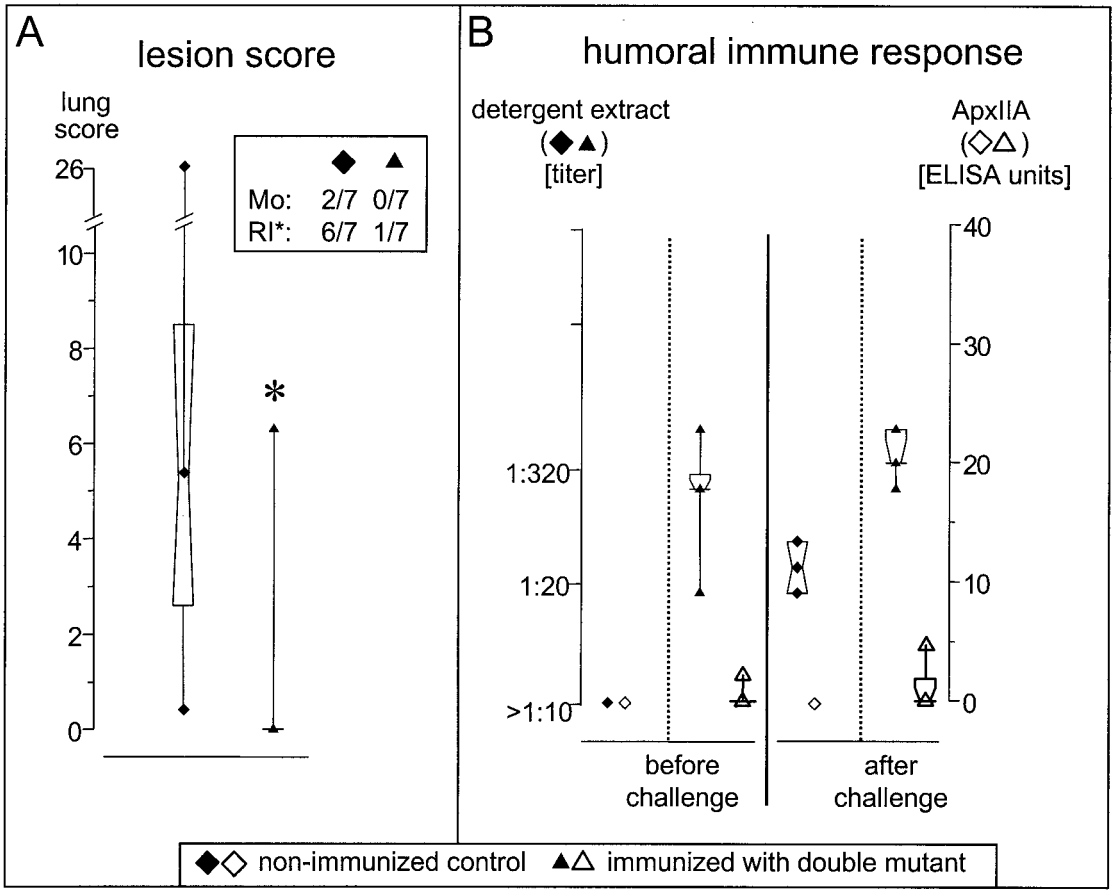

FIG. 3. Lung lesion score (left) and humoral immune response (right) of naive and immunized pigs 7 days after homologous challenge. For immunization, a total of 6.5×10^5 *A. pleuropneumoniae* C5934Δ*ureC*Δ*apxIIA* were aerosolized; for infection, a total of 1.6×10^5 *A. pleuropneumoniae* C5934wt were used. Lung lesion scores were determined as described by Hannan et al. (12), and the bacteriological examination was done as previously described (2); mortality (Mo) and reisolation frequency of the *A. pleuropneumoniae* wild-type strain from lung tissue (RI) results for the different groups are given. The antibody response was assessed with two ELISAs with a detergent extract (solid symbols) and the recombinant ApxIIA protein (open symbols) as the solid-phase antigen. The response was quantified as a serum titer in comparison to an internal negative control (detergent extract ELISA) and in EU (based on an external standard) in the ApxIIA ELISA. For the standardized ApxIIA ELISA serum activities of ≤10 EU are considered negative, 11 to 25 EU is intermediate, and >25 EU is a positive result. The central square within the hourglass shape represents the geometric mean, the hinges present the values in the middle of each half of data, and the top and bottom squares mark the maximum and minimum values, respectively. The asterisks denote statistical significance ($P < 0.05$) for the lung lesion score (Wilcoxon test) and the reisolation rate (χ^2 test).

the lungs. This level of protective efficacy, which is a prerequisite to prevent an evolutionary increase of the intrinsic virulence levels of the pathogen (8), to date has only been obtained with a repeated intramuscular or aerosol application with bacterial ghosts (13, 15).

This work was supported by grant GE522/3-2 from the Deutsche Forschungsgemeinschaft, Bonn, Germany. W.T. is a fellow of the Mahanakorn University of Technology in Thailand.

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Editor: B. B. Finlay