IMPORTANCE OF HEAT-STABLE ENTEROTOXIN B
IN THE INDUCTION OF EARLY IMMUNE RESPONSES IN PIGLETS
AFTER INFECTION WITH ENTEROTOXIGENIC ESCHERICHIA COLI

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INTRODUCTION
Enterotoxigenic Escherichia coli (ETEC) are a major cause of dehydration diarrhea in children and weaned piglets living under suboptimal conditions. After colonization of the small intestine, ETEC produce heat-labile (LT) and/or heat-stable (ST) enterotoxins. However, the relative importance of the different enterotoxins in the pathogenesis of ETEC infection has been poorly defined.

OBJECTIVES
We wanted to assess the contributions of the different enterotoxins of an ETEC strain to the induction of small intestinal secretion and early innate immune responses in weaned piglets. Isogenic mutant strains of an LT+ ST+ ETEC strain were constructed that lack the expression of LT in combination with one or both types of ST enterotoxins (STα and/or STb). The small intestinal segment perfusion technique and microarray analysis were used to probe circadian immune responses induced by these mutant strains 4h after infection in combination with a PBS control. Simultaneously, net fluid absorption of pig small intestinal mucosa was measured 4h after infection.

MATERIALS AND METHODS
Bacterial strain and mutants
Enterotoxin deletion mutants of the hemolytic porcine E. coli strain GS26, serotype O149:K91:F6c were generated using the bacteriophage lambda recombinase system (λ-Red) of Datenko and Wanner [1]. Enterotoxin production was quantified in the different mutant strains using specific enzyme immunoassays. The enterotoxin phenotype of the used strains is shown in Table 1.

Small Intestinal Segment Perfusion
For this study we used 3-5week-old weaned piglets. The abdomen of anesthetized piglets was opened and 6 segments of 20 cm length were made in the mid jejunum. The segments were cannulated with silicone tubes at the proximal and distal ends (Figure 1) to inject and collect fluid respectively. Segments were first injected with 2.5x10\textsuperscript{6} CFU of wild type GS26, one of the four GS26 mutant strains or with PBS only. Each segment was then perfused during 4h by injecting 2ml perfusion buffer every 15 minutes. At the end of the experiment net fluid absorption of each segment was calculated from the difference between inflow and outflow divided by the surface area of each segment. A small piece of tissue from each segment was sampled for RNA isolation.

Microarray Analysis
We used the Porcine Genome Array (Affymetrix) containing 23,937 probe sets, representing 20,201 Sus scrofa genes. The normalized intensity values of the different conditions were compared and differential transcripts were selected based on the more stringent cut-off of the uncorrected P-values, i.e. P<0.001. The cut-off on the P-values was combined with a cut-off on the fold-change of two (i.e. an absolute log2-ratio > 1).

qRT-PCR:
Nine genes from the microarray analysis (IL8, PAP, FABP2, IL1A, IL17A, TLR4, MMP1, MMP3 and CYF1A1) were selected for confirmation by quantitative real-time PCR. The relationship between the levels of gene expression of these genes (qRT-PCR versus microarray data) was determined by linear regression (data not shown).

RESULTS AND DISCUSSION

STb seems to play an important role in the induction of intestinal secretion
Small intestinal segment perfusion with the different strains (Figure 2) showed:
\begin{itemize}
\item no difference in intestinal secretion induced by the wild type strain and the mutant only expressing STb.
\item a significant difference between the STa+ and STb\textsuperscript{low} strain (P<0.01) indicating that the amount of STb produced is important.
\item that the strain secreting only STα induced a significant lower effect on net absorption compared to the wild type (P<0.01), indicating that STα only plays a minor role.
\item that the mutant strain not expressing enterotoxin is no longer able to reduce net absorption.
\end{itemize}

ETEC regulates expression of porcine genes important in inflammation
\begin{itemize}
\item PBS versus ETEC: 153 transcripts down-regulated and 157 transcripts up-regulated
\item 15 transcripts are down-regulated by ETEC with log-ratio < -2 and these transcripts are associated with the intestinal metabolism or with transport of fluids and electrolytes.
\item 23 transcripts are up-regulated by ETEC with log-ratio>2 and the 13 of them represent immunomodulatory genes (Table 2).
\end{itemize}

Microarray results suggest a role for STb in ETEC-induced immune responses
\begin{itemize}
\item Only 2 mutant strains showed differential expression from wild type (Figure 3).
\item 15 transcripts are up- or down-regulated by ETEC but not related to enterotoxin expression. We suggest a regulation of these genes by LPS or other metabolites. Among them are MMP1, PAP, IL8, IL1RN and DUSO2.
\item 26 transcripts are differentially regulated by the 3 strains secreting normal levels of STb and/or STα. Among them are MMP3 and IL1A.
\item 24 transcripts are still differentially regulated by the STb\textsuperscript{low} strain but not by the enterotoxin-negative strain, indicating a role for STb in their regulation. Among them are IL17A and IL1B.
\end{itemize}

CONCLUSION
Microarray analysis showed on the one hand a non-toxin related general antibacterial response comprising genes such as panprotease-associated protein, interleukin 6 and matrix metalloproteinase 1. On the other hand, results demonstrated an important role for STb in small intestinal secretion early after infection as well as in the ETEC induced immune response by the significant differential regulation of immune mediators like matrix metalloproteinase 1, interleukin 1 and interleukin 17.

Table 1 Enterotoxin phenotype of GS26 mutants used in this study

Table 2 Transcripts of immune related genes upregulated by wild type ETEC

Figure 1 Cannulated segment

Figure 2 Effect of wild type and mutant GS26 ETEC strains on net fluid absorption (mg/cm²) in 4h-infected jejunal segments

Figure 3 Number of differential regulated transcripts for the comparisons made