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Mouse Paneth cell antimicrobial function is independent of Nod2

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ABSTRACT

Objective Although polymorphisms of the *NOD2* gene predispose to the development of ileal Crohn's disease, the precise mechanisms of this increased susceptibility remain unclear. Previous work has shown that transcript expression of the Paneth cell (PC) antimicrobial peptides (AMPs) α -defensin 4 and α -defensin-related sequence 10 are selectively decreased in *Nod2*^{-/-} mice. However, the specific mouse background used in this previous study is unclear. In light of recent evidence suggesting that mouse strain strongly influences PC antimicrobial activity, we sought to characterise PC AMP function in commercially available *Nod2*^{-/-} mice on a C57BL/6 (B6) background. Specifically, we hypothesised that *Nod2*^{-/-} B6 mice would display reduced AMP expression and activity.

Design Wild-type (WT) and *Nod2*^{-/-} B6 ileal AMP expression was assessed via real-time PCR, acid urea polyacrylamide gel electrophoresis and mass spectrometry. PCs were enumerated using flow cytometry. Functionally, α -defensin bactericidal activity was evaluated using a gel-overlay antimicrobial assay. Faecal microbial composition was determined using 454-sequencing of the bacterial 16S gene in cohoused WT and *Nod2*^{-/-} littermates.

Results WT and *Nod2*^{-/-} B6 mice displayed similar PC AMP expression patterns, equivalent α -defensin profiles, and identical antimicrobial activity against commensal and pathogenic bacterial strains. Furthermore, minimal differences in gut microbial composition were detected between the two cohoused, littermate mouse groups.

Conclusions Our data reveal that *Nod2* does not directly regulate PC antimicrobial activity in B6 mice. Moreover, we demonstrate that previously reported *Nod2*-dependent influences on gut microbial composition may be overcome by environmental factors, such as cohousing with WT littermates.

INTRODUCTION

Crohn's disease (CD) is a chronic intestinal inflammatory disorder that results from the interaction of numerous genetic and environmental factors. Of the genetic loci associated with CD, single nucleotide polymorphisms (SNPs) of the *NOD2* gene display the strongest correlation with disease development.¹ Although *NOD2* is found primarily in monocytes and dendritic cells, it is also constitutively expressed in small intestine epithelial cells known as Paneth cells (PC).² PCs contribute to mucosal enteric immunity through the production of antimicrobial

Significance of this study

What is already known on this subject?

- Polymorphisms of nucleotide-binding, oligomerisation domain 2 (*NOD2*) are the most frequently replicated disease alleles associated with Crohn's disease (CD).
- *NOD2* is highly expressed in Paneth cells (PC), which are small intestinal epithelial cells that secrete a multitude of antimicrobial molecules into the gut lumen. The most potent class of these antimicrobial peptides (AMP) is the α -defensins.
- Previous work using *Nod2*-deficient mice suggests that *Nod2* regulates PC α -defensin expression, which in turn modulates the composition of the gut microbiota.

What are the new findings?

- When *Nod2*-deficient mice are examined on pure genetic background (C57BL/6), they show no differences in α -defensin levels relative to wild-type (WT) mice.
- With the exception of the molecule cryptdin-related sequence 1C, expression of all major PC AMP classes are unaffected by *Nod2* status in C57BL/6 mice.
- The composition of the mouse faecal microbiota is independent of *Nod2* status when WT and *Nod2*^{-/-} littermates are housed in the same cages.

How might it impact on clinical practice in the foreseeable future?

- This work guides future studies of CD pathogenesis by underscoring the importance of controlling for mouse genetic background and housing conditions when studying PCs in mouse models of CD risk alleles.
- The finding that environmental influences can overcome the impact of host genetics on gut microbial composition suggests that microbial modulation strategies (such as probiotics) are feasible, even in the face of strong genetic influences on the intestinal microbiota.

peptides (AMPs). The most potent class of PC-derived AMPs are the α -defensins (cryptdins).³ Genetic alterations that diminish PC α -defensin activity are associated with compositional changes

in the intestinal microbiota.⁴ Because components of the gut microbiota are believed to drive intestinal inflammation in CD, dysregulation of α -defensin function may be an important factor in CD pathogenesis.⁵

Although the precise regulation of α -defensin activity remains unclear, various studies have suggested a role for NOD2 in modulating the function of these molecules. Previous reports have demonstrated that ileal CD patients possess attenuated expression of the two human PC α -defensins (HD-5 and HD-6),⁶ with the greatest reduction in patients with a specific NOD2 polymorphism.⁷ Subsequent work using a mouse model of Nod2-deficiency revealed that *Nod2*^{-/-} mice also display reduced mRNA expression of particular α -defensins.⁸ Specifically, *Nod2*^{-/-} mice exhibit ~100-fold less transcript expression of the molecules α -defensin 4 (*Defa4*) and α -defensin-related sequence 10 (*Defa-rs10*) relative to wild-type (WT) mice.

Despite the evidence supporting the role of NOD2 in α -defensin regulation, these findings remain subject to a degree of uncertainty. First, a follow-up study in patients with ileal CD demonstrated that reduced α -defensin expression is associated with inflammation, as opposed to NOD2 status.⁹ Second, the precise background of the *Nod2*^{-/-} mice used to implicate Nod2 as a regulator of α -defensin expression is unclear. This is critical, because recent work has demonstrated that mouse background strain plays a key role in defining α -defensin expression patterns.¹⁰ The *Nod2*^{-/-} mice used in the original study were constructed using 129S1/Sv-derived W9.5 embryonic stem (ES) cells injected into C57BL/6 (B6) blastocysts, leaving open the possibility that these animals were on a mixed background at the time of analysis. Therefore, in light of the confounding variable of a mixed genetic background, the precise role of Nod2 in regulating mouse α -defensin expression remains unclear. Further characterisation of the impact of Nod2 on AMP expression is essential to define its precise function in PC biology, as well as its role in CD pathogenesis.

In the present study, we sought to determine the regulatory effects of Nod2 on mouse PC-derived AMP expression, controlling for background strain. This was accomplished by evaluating the PC AMP repertoire of *Nod2*^{-/-} versus WT littermates on a pure B6 background. These animals are available commercially through Jackson Labs, and represent the original *Nod2*^{-/-} strain⁸ that has since been backcrossed to completion on the B6 background. We hypothesized that these *Nod2*^{-/-} B6 mice would display diminished AMP expression and attenuated PC function relative to their WT counterparts. Unexpectedly, we found that the majority of mouse PC-derived AMP classes showed equivalent expression in *Nod2*^{-/-} and WT B6 mice. Furthermore, α -defensin antimicrobial activity and global faecal microbial composition were also strikingly similar in both *Nod2*^{-/-} and WT groups. These findings suggest that PC antimicrobial function is independent of Nod2 status in this mouse strain.

MATERIALS AND METHODS

Mice

Nod2^{+/+} (WT) and *Nod2*^{-/-} B6 mice were obtained from Jackson Laboratories (Bar Harbor, ME), and were housed in specific pathogen-free conditions consistent with guidelines established by the American Association for Laboratory Animal Care and Research. WT and *Nod2*^{-/-} mice were crossed to generate *Nod2*^{+/-} heterozygous animals, which were then bred to produce WT and *Nod2*^{-/-} littermates that were housed together for comparison in our studies (see online supplementary figure

S1). Importantly, *Nod2*^{+/-} offspring were recrossed to generate a continuous pool of WT and *Nod2*^{-/-} littermates, all derived ultimately from the same founders. This is in contrast with previous work, which used littermates from a single *Nod2*^{+/-} heterozygous cross to start homozygous *Nod2*^{+/+} and *Nod2*^{-/-} lines that were housed separately for the duration of the study.¹¹ This distinction is critical, as the mice in our study were never housed separately based on Nod2 status alone, allowing for more consistent environmental exposure to both experimental groups. All mice were housed in the same animal room and sacrificed within 8–12-weeks of age. *Nod2* genotyping was performed on genomic DNA extracted from mouse-tail clippings (see online supplementary methods). Identification of WT and *Nod2*-null mice was confirmed by PCR product sequencing (see online supplementary figures S2 and S3).

Quantitative reverse-transcriptase PCR

Quantitative RT-PCR was performed using TaqMan or SYBR Green assays (Applied Biosystems, Foster City, California, USA) per manufacturer's instructions. Details are described in the online supplementary methods. β -Actin was used as an internal control, and $\Delta\Delta C_t$ values were calculated to obtain fold changes relative to the baseline group.

Acid urea polyacrylamide gel electrophoresis

Ileal tissue protein was extracted as previously described.¹⁰ Protein extracts were analysed by acid urea-polyacrylamide gel electrophoresis (AU-PAGE) followed by mass spectrometric analysis using matrix assisted laser desorption ionisation-time of flight tandem mass spectrometry (MALDI-TOF/TOF MS). Details are provided in the online supplementary methods.

Immunohistochemistry

Primary staining was accomplished using a rabbit polyclonal antilysozyme (Lyz) antibody (1:1500, Diagnostic BioSystems, Pleasanton, California, USA). Biotinylated antirabbit IgG was used as a secondary antibody (1:200, Vector Laboratories, Burlingame, California, USA). Details are provided in the online supplementary methods.

Flow cytometry

Ileal epithelial cells were isolated using EDTA/dispase digestion as previously described.¹² Cells were fixed in 4% paraformaldehyde for 15 min and resuspended in saponin permeabilisation buffer (Invitrogen, Carlsbad, California, USA) with Lyz-FITC antibody (1:10, Dako, Carpinteria, California, USA) and CD45-A647 antibody (1:1000, BD Biosciences, San Jose, California, USA) for 30 min. Flow analysis was performed per established protocols.¹³

Bactericidal gel overlay assay

Bacterial strains were grown to mid-log phase in trypticase soy broth media and resuspended in warm 1% low-melt agarose as previously described.¹⁰ Ileal protein samples were prepared by electrophoresis on a small-scale AU-PAGE gel. Gel overlay plates were incubated overnight at 37°C and imaged for band-associated zones of bacterial clearance. Details are provided in the online supplementary methods.

Bacterial composition analyses

Total DNA was extracted from faecal samples as previously described.¹⁴ Bacterial composition of isolated DNA samples was characterised by PCR amplification of the V1-3 variable region of the 16S rRNA gene.¹⁵ Taxonomic and phylogenetic analyses

of 16S rRNA sequence data are detailed in the online supplementary methods.^{16 17}

Statistics

AMP expression and PC number comparisons were performed using GraphPad Prism 5 (GraphPad, San Diego, California, USA). All variables were found to have a normal distribution. Means were compared using Student's *t* test (2-tailed), and are expressed as mean ± SEM.

RESULTS

Mouse PC AMP expression is predominantly independent of Nod2

To determine the effects of Nod2 deletion on PC AMP gene expression, qRT-PCR was used to quantitate ileal AMP levels in WT and *Nod2*^{-/-} B6 mice. The major PC AMP classes expressed in B6 mice include: α -defensins (cryptidins), cryptdin-related sequence peptides (CRS1C and CRS4C), lysozyme-P (*Lyz*), angiogenin-4 (*Ang4*) and regenerating islet-derived protein 3 γ (*Reg3 γ*).¹⁸ Consistent with earlier reports in CD patients⁶ and *Nod2*^{-/-} mice,¹⁹ we observed no differences in *Lyz* or *Reg3 γ* expression between *Nod2*^{-/-} and WT mice (figure 1A,B). Furthermore, Nod2-deficiency did not alter transcription of the *Ang4* gene (figure 1C). Interestingly, Nod2 deletion also did not affect global levels of α -defensin transcripts (figure 1D), using primers that detect all known PC α -defensin genes.²⁰

By contrast with the AMPs described above, mRNA levels of the CRS peptides analysed in this study did display a unique expression pattern in *Nod2*^{-/-} mice. Specifically, transcript levels of the CRS1C subgroup were significantly decreased in

Nod2^{-/-} mice relative to WT controls (~25% reduction, $p < 0.05$) (figure 1E). Furthermore, we were unable to detect transcripts of the second CRS peptide subgroup, CRS4C, in either WT or *Nod2*^{-/-} B6 mice (figure 1F), though this subgroup was prominently expressed in the ilea of 129/SvEv mice (see online supplementary figure S4A). This is particularly important because a member of this subgroup, *Defa-rs10*, has been reported to be reduced in *Nod2*^{-/-} mice relative to WT controls.⁸ Despite multiple attempts, we were unable to detect *Defa-rs10* transcripts in either WT or *Nod2*^{-/-} B6 mice, though this transcript was also highly expressed in 129/SvEv animals (see online supplementary figure S4A).

In summary, with the exception of the CRS1C group, we failed to reject the null hypothesis that the major mouse AMP classes had equal mRNA expression. Moreover, the 95% CIs of the mean AMP levels for the WT and *Nod2*^{-/-} groups were highly overlapping (see online supplementary table S1), suggesting that if there is a real difference in AMP expression between WT and *Nod2*^{-/-} mice, it is small in magnitude.

Mouse PC α -defensin expression is independent of Nod2

Because previous work has demonstrated reduced expression of the α -defensin *Defa4* in *Nod2*^{-/-} mice,⁸ it was surprising to find equivalent global α -defensin levels in *Nod2*^{-/-} and WT mice. To determine if a compensatory induction of specific α -defensin isoforms could explain these findings, we next measured mRNA levels of selected α -defensin AMPs from 3 different phylogenetic groups (*Defa4*, *Defa3* and *Defa5*).²¹ As shown in figure 2A, *Defa4* transcripts were undetected in both WT and *Nod2*^{-/-} mice, though were present in 129/SvEv animals (see online supplementary figure S4B). Because *Defa4* is expressed exclusively in non-B6 mouse strains,^{10 21} we also quantified transcript expression of *Defa20*, which is the B6 homologue of *Defa4*. Transcripts levels of *Defa20* were equivalent in WT and *Nod2*^{-/-} mice (figure 2B). Finally, *Defa3* and *Defa5* mRNA levels also showed no statistical differences between experimental groups (figures 2C,D).

At the protein level, PC α -defensins were assessed using AU-PAGE, which localises α -defensins to a series of bands at the cathodal end of the gel.²² Figure 2E shows that α -defensin banding patterns were identical between WT and *Nod2*^{-/-} mice. Subsequent MALDI-TOF/TOF MS analysis demonstrated that the identities of the α -defensin bands in WT and *Nod2*^{-/-} mice were also indistinguishable (see online supplementary table S2). This is further highlighted in online supplementary figure S4C, which reveals that pooled samples of WT B6 and *Nod2*^{-/-} B6 ileal protein extracts have a distinct banding pattern from those of WT 129 mice. This supports the premise that mouse strain profoundly influences the ileal α -defensin profile, while Nod2-deficiency does not result in measurable changes in the expression of these molecules.

Nod2 does not regulate PC numbers

Our findings thus far demonstrate minimal differences in PC AMP expression in ileal tissue from WT and *Nod2*^{-/-} mice. However, if Nod2-deficiency results in decreased AMP expression at a cellular level, it is possible that a compensatory increase in PC numbers could result in similar total AMP levels between WT and *Nod2*^{-/-} groups. To ensure that alterations in PC number were not masking some degree of Nod2-mediated AMP regulation, we enumerated PCs in WT and *Nod2*^{-/-} mice. H&E staining of ileal sections from both experimental groups showed that WT and *Nod2*^{-/-} mice have normal tissue architecture, and display no signs of inflammation (figure 3A). Quantification of

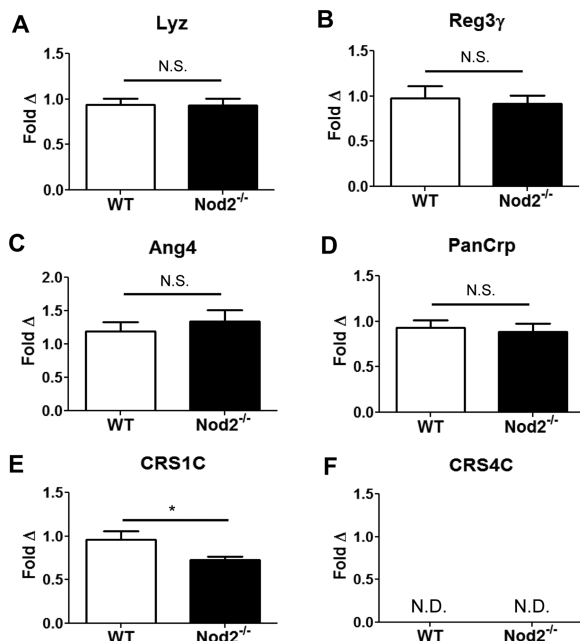


Figure 1 Paneth cell (PC) antimicrobial peptide transcript expression is primarily unaffected by Nod2. Ileal transcript levels of (A) lysozyme-P (*Lyz*); (B) regenerating islet-derived protein 3 gamma (*Reg3 γ*); (C) angiogenin 4 (*Ang4*); (D) total PC α -defensins (*PanCrp*); (E) cryptdin-related sequence (CRS)1C and (F) CRS4C are shown for wild-type (WT) and *Nod2*^{-/-} mice (n=8–10 mice/group). CRS4C was not detected (ND) in any animal. Copy number is normalised to β -actin and expressed as a fold Δ relative to the WT group. Data are shown as means with SEM. * $p < 0.05$; N.S.—not significant ($p > 0.6$), based on Student's *t* test.

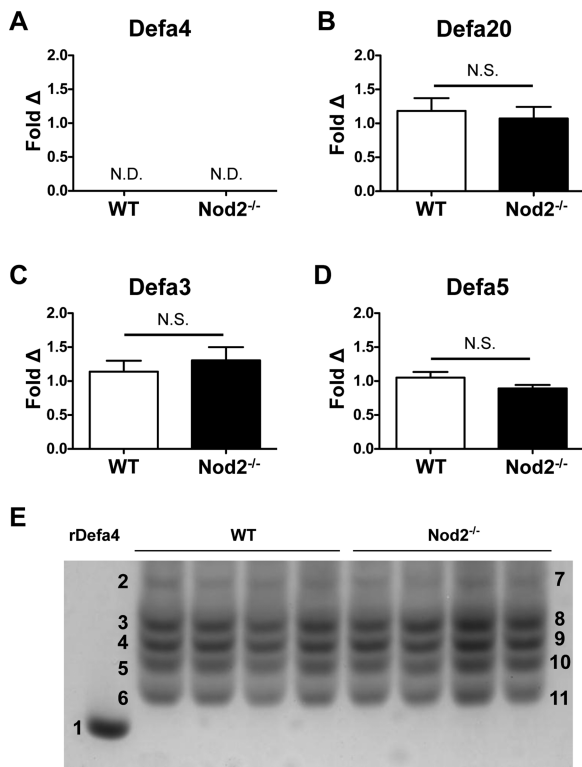


Figure 2 Paneth cell α -defensin expression is independent of Nod2. mRNA expression of α -defensin isoforms (A) Defa4; (B) Defa20; (C) Defa3; and (D) Defa5 in the ileum of wild-type (WT) and *Nod2*^{-/-} mice (n=8–10 mice/group). Copy number is normalised to β -actin and expressed as a fold Δ relative to the WT group. Data are shown as means with SEM. ND— not detected; NS— not significant (p>0.1), based on Student's t test. (E) acid urea-polyacrylamide gel electrophoresis demonstrates peptide expression patterns of α -defensin isoforms in the ileum of WT and *Nod2*^{-/-} mice. First lane is recombinant Defa4 control; each additional lane represents an individual mouse. Individual bands (based on calculated mass determined via mass spectrometry): 1-Defa4 (recombinant); 2,7-Defa5; 3,8-Defa24; 4,9-Defa20/Defa21; 5,10-Defa2; and 6,11-Defa22.

intestinal crypts revealed no differences between the two groups of mice, and therefore, no effect of Nod2 on crypt development (figure 3B). Using anti-Lyz staining as a PC marker, immunohistochemistry displayed neither ectopic placement of PCs, nor hyperplastic growth of these cells (figure 3C). Furthermore, there was no significant difference in the number of Lyz⁺ cells per crypt between WT and *Nod2*^{-/-} mice (figure 3D). Finally, to minimise the bias of counting PCs from selected high-power fields, flow cytometry of epithelial cell preparations from the entire ileum of WT and *Nod2*^{-/-} mice was performed. Using CD45 as a haematopoietic cell maker, PCs were identified as the Lyz⁺CD45⁻ cell fraction (figure 3E). Quantification from three independent experiments confirmed that WT and *Nod2*^{-/-} ilea contained an equivalent percentage of PCs within their epithelia (figure 3F). Total numbers of epithelial cells from each group were not significantly different. In summary, this analysis demonstrates that Nod2 does not regulate α -defensin expression on a per-cell basis.

PC α -defensin antimicrobial activity is unaffected by Nod2

The similarity in the AU-PAGE banding pattern of the α -defensin region between WT and *Nod2*^{-/-} mice indicates that Nod2 does not regulate post-translational processing or

conformational folding of PC α -defensins. To confirm that Nod2-deficiency does not affect the functional microbicidal activity of PC α -defensins, we tested the ability of ileal protein extracts from WT and *Nod2*^{-/-} mice to inhibit the growth of commensal and pathogenic bacterial strains. Specifically, we assessed α -defensin antimicrobial activity against the commensal bacterium *Escherichia coli* strain NC101 (which has been shown to induce intestinal inflammation in genetically engineered mice),²³ as well as the intestinal pathogens *Listeria monocytogenes* and *Salmonella enterica*. To test α -defensin antimicrobial activity against these organisms, we excised the α -defensin zone of an AU-PAGE minigel (figure 4A), overlaid this onto agarose plates of confluent bacteria, and observed for zones of bacterial inhibition. Figure 4B–D demonstrates comparable zones of α -defensin-mediated bacterial inhibition for all tested bacteria, regardless of host Nod2 status. Therefore, Nod2 does not influence the bacteriostatic activity of PC α -defensins against relevant proinflammatory commensal and pathogenic bacterial strains. It is interesting to note that *Nod2*^{-/-} mice are reported to have increased susceptibility to oral *L. monocytogenes* infection.⁸ The present data suggest this is unlikely due to defective α -defensin antimicrobial function.

Nod2 has minimal effects on global faecal microbial composition

Given the similarities in PC AMP function between WT and *Nod2*^{-/-} mice, we next sought to determine if there were differences in the global microbial composition of our experimental groups. Previous studies have demonstrated that *Nod2*^{-/-} mice have alterations of their intestinal microbiota relative to WT animals.^{11–24} However, as described in our methods, the breeding strategy used in the present study allows for more rigorous control of environmental influences through cohousing of *Nod2*^{-/-} and WT littermates. By principal coordinates analysis of samples based on deep sequencing of the 16S gene, it appeared that the cage the mice were housed in had a stronger effect on microbial community composition than did the WT or *Nod2*^{-/-} genotype (figure 5). Indeed, the null hypothesis that the cage has no effect on gut microbial structure was rejected for five of the first eight coordinates at a 10% false discovery rate (table 1). By contrast, we failed to reject the null hypothesis that genotype has no effect on microbial composition for any of the first 15 coordinates at a p<0.05 threshold (table 2). This was also true at the individual taxa level, where we failed to reject the null hypothesis that individual taxa were not significantly different between WT and *Nod2*^{-/-} at a 10% false discovery rate (see online supplementary table S3). These data suggest that the cage environment plays a stronger role in structuring the microbial community than the absence of Nod2 expression.

DISCUSSION

Polymorphisms of the *NOD2* gene remain the most replicated risk alleles for the development of CD.²⁵ Despite this high reproducibility, the precise mechanisms by which *NOD2* dysfunction increases CD susceptibility are unclear. Previous work using *Nod2*^{-/-} mice suggests a key role for Nod2 in the transcriptional regulation of specific PC α -defensins.⁸ However, in the present study, we demonstrate that there are no profound effects of Nod2-deficiency on PC α -defensin mRNA expression, protein levels, or antimicrobial activity when mouse background is strictly controlled. Moreover, using cohoused WT and *Nod2*^{-/-} littermates, we reveal that local cage conditions outweigh Nod2-mediated effects on faecal microbial composition.

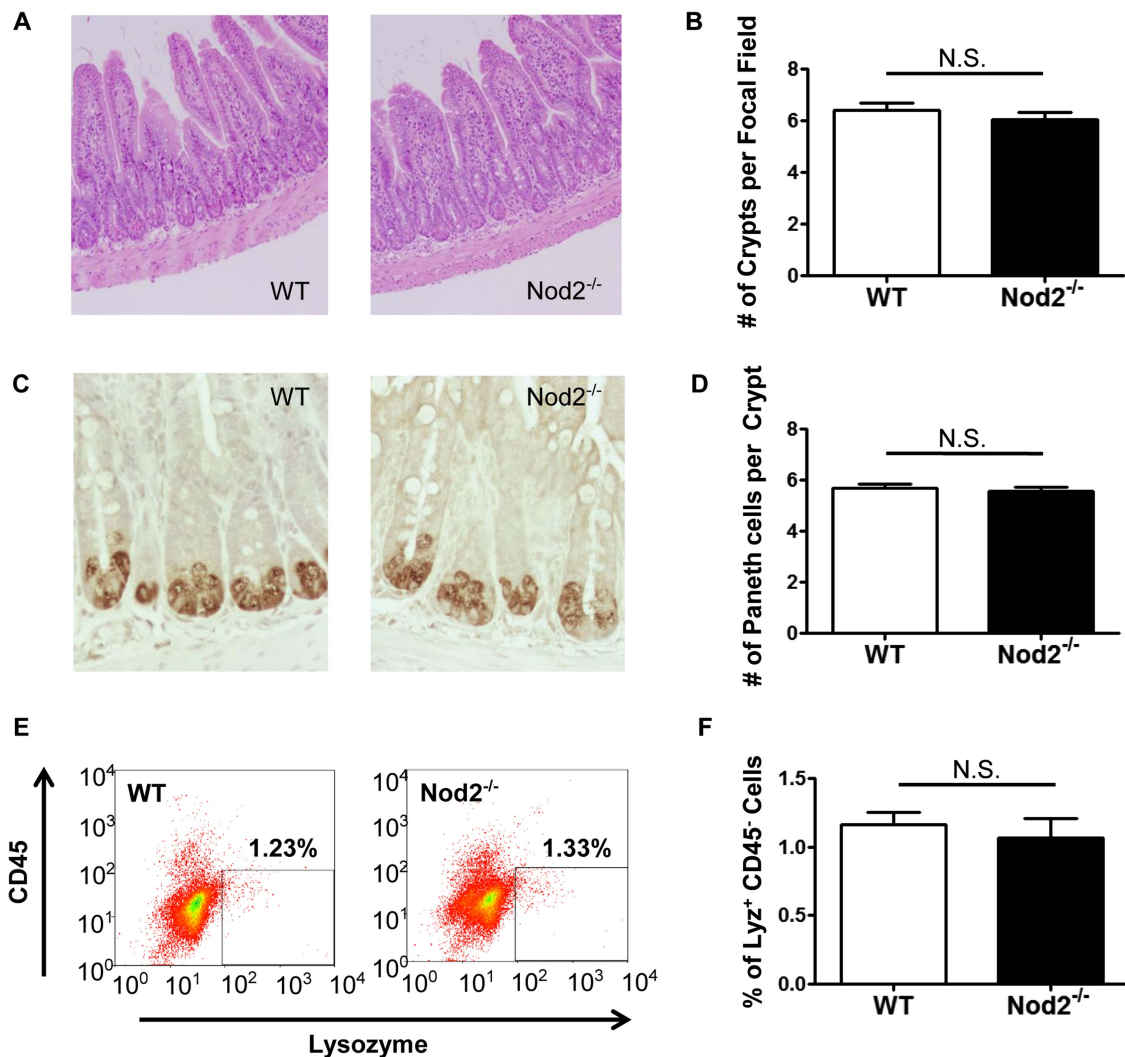


Figure 3 *Nod2* does not influence Paneth cell (PC) development. (A) H&E staining of ileal tissue shown at 400 × magnification from wild-type (WT) and *Nod2*^{-/-} mice was used to quantify intestinal crypts. Panel (B) represents average data from 18–20 high power fields/group (3–4 mice/group). Panel (C) shows immunohistochemical staining of ileal tissue for lysozyme (Lyz), allowing for the quantification of PCs per crypt. Panel (D) represents average data from 40–45 crypts/group (5–6 mice/group). (E,F) Flow cytometry was used to measure the percentage of Lyz⁺ CD45⁻ PCs from the ileal epithelium of WT and *Nod2*^{-/-} mice. Data are representative of three independent experiments (NS— not significant, $p > 0.1$, based on Student's *t* test).

The differing α -defensin expression results demonstrated by our analysis versus those previously reported⁸ likely stem from the variability of mouse background used in the former study. Numerous investigations demonstrate that B6 and 129 mouse strains possess distinct α -defensin profiles and antimicrobial properties.^{10 21 26} Such findings underscore the importance of clearly defining mouse background strain when performing α -defensin studies. The original *Nod2*-null mice used to demonstrate *Nod2*-dependent α -defensin regulation were constructed by injecting genetically manipulated 129S1/Sv-derived W9.5 ES cells into B6 blastocysts. However, no mention of backcrossing to a pure background strain was indicated, and hence, the precise background of mice used in the final analysis remains unclear. Indeed, the detection of *Defa4* and *Defa-rs10* transcripts in their experimental groups suggests that the mice previously studied possessed at least a component of the original 129S1/Sv genetic background, as these genes are not found in B6 strains.^{10 21} The background ambiguity of these mice leaves open the possibility that formerly observed differences in α -defensin expression may be due to variations in mouse strain

between experimental groups, as opposed to a true effect of *Nod2* deficiency.

In the present study, *Nod2*^{-/-} mice were obtained commercially through Jackson Labs, and represent the original strain generated by Kobayashi *et al* that has since been backcrossed to completion on the B6 background. Using B6 *Nod2*^{-/-} and WT littermates, we found no differences in α -defensin expression between the two groups. The lack of *Defa4* and *Defa-rs10* expression in both experimental groups is consistent with the absence of these genes in the B6 mouse strain.^{10 21} The lack of *Nod2*-mediated α -defensin regulation was also true at the protein level, and could not be explained by differences in PC number between WT and *Nod2*^{-/-} mice. Similar to the α -defensins, there was no evidence of *Nod2*-driven transcriptional regulation of other AMP classes, including *Lyz*, *Reg3 γ* and *Ang4*, consistent with previous reports.¹⁹

Interestingly, the CRS AMP subclasses did display a unique expression pattern in our experimental groups. CRS peptides comprise a prominent family of AMPs, which were first described as PC-specific transcripts that are highly similar to the

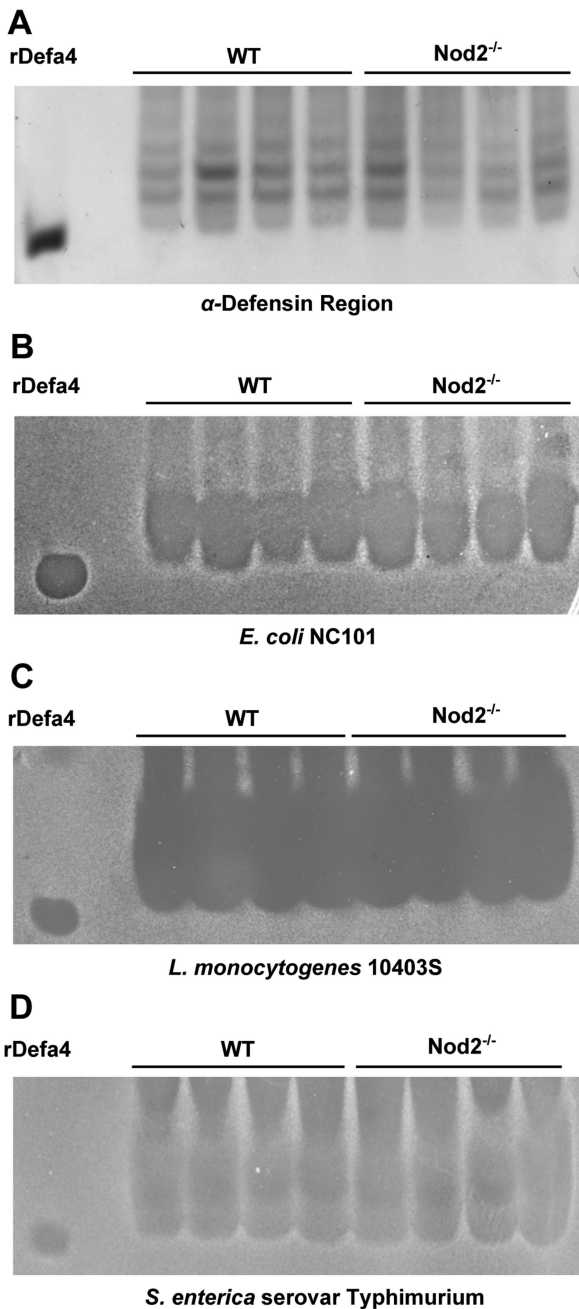


Figure 4 Antimicrobial activity of Paneth cell α -defensins is unaffected by Nod2. (A) wild-type (WT) and *Nod2*^{-/-} mouse-derived ileal protein extracts were resolved by acid urea-polyacrylamide gel electrophoresis. An excised gel strip containing the α -defensins was placed onto bacteria-laden agarose. Bacterial clearance zones are shown for (B) *Escherichia coli* NC101, (C) *Listeria monocytogenes* 10403S, and (D) *Salmonella enterica* serovar Typhimurium. Similarly sized zones of bacterial growth inhibition are seen for all bacterial strains, regardless of Nod2 status.

α -defensins.²⁷ These molecules are divided into two primary groups, CRS1C and CRS4C, based on their C-terminal peptide sequence.²⁸ CRS4C peptides form homodimers and heterodimers that expand the diversity of AMPs within PC secretions.²⁹ They include Defa-rs10, which was previously reported to be decreased in *Nod2*^{-/-} mice relative to WT controls.⁸ However, our evaluation of the NIH B6 mouse genome assembly demonstrated no evidence of the *Defa-rs10* gene in B6 animals.¹⁰ This again implies that the background of mice used previously

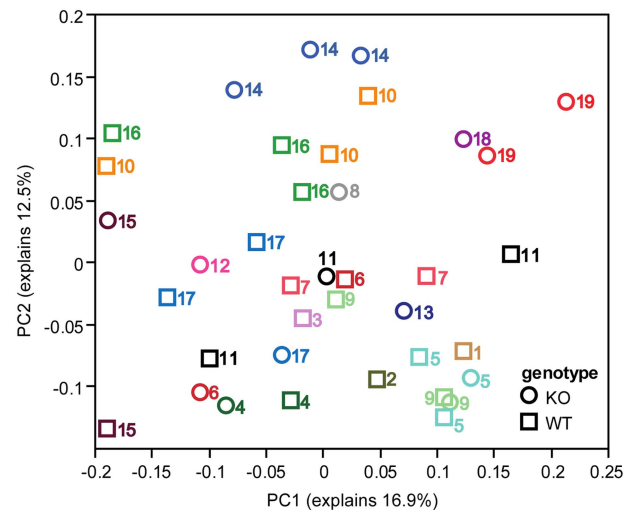


Figure 5 Local cage environment overrides *Nod2*-mediated influences on the intestinal microbiota. Principal coordinates analysis (PcoA) using the first two coordinates of a PcoA based on Bray-Curtis dissimilarity of 454 sequences of 16S rRNA from stool samples. *Nod2*^{-/-} mice (KO) are indicated by circles, while wild-type (WT) mice are shown with squares. The numbers by each symbol indicate the cage of the animal, and each cage is depicted in a unique colour. PC, principal coordinate.

possessed a non-B6 component. Indeed, we found no transcript expression of the CRS4C class in either WT or *Nod2*^{-/-} mice on a B6 background. Similarly, no mRNA expression of *Defa-rs10* was detected in our experimental groups. This was confirmed using TaqMan quantitative RT-PCR primers, as well as the SYBR green primer sequences published originally by Kobayashi *et al.*⁸

By contrast with CRS4C, mRNAs for CRS1C have been detected in the B6 mouse small intestine.³⁰ Accordingly, we found a modest ~25% reduction of CRS1C expression in *Nod2*^{-/-} mice relative to WT controls. Although previous work has suggested a role of Wnt/Tcf-4 signalling in the regulation of CRS1C expression,³¹ there have been no reports of Nod2 influencing transcript levels of these molecules. However, despite finding slightly reduced CRS1C expression in *Nod2*^{-/-} mice, our examination of the promoter region of the CRS peptide genes revealed no canonical NF- κ B binding sites (data not shown). Therefore, the direct effect of Nod2 on CRS1C mRNA expression remains unclear. Moreover, to our knowledge, CRS1C peptides have not been characterised or detected at the protein level. Therefore, it is unclear what effect this reduction in CRS1C gene expression may have on the bactericidal activity of PC secretions.

It is important to underscore that the present study focuses on Nod2-mediated regulation of PC AMP production, and does not examine the effects of Nod2-deficiency on PC secretion. It has been established that the ligand for Nod2, muramyl dipeptide (MDP), is a PC secretagogue.³ Subsequent work has supported this premise, demonstrating decreased bactericidal activity of Nod2-deficient intestinal crypt secretions stimulated by MDP.³² Furthermore, Nod2-deficiency also interfered with carbamyl-choline (CCh)-mediated PC secretion, suggesting that Nod2 may affect a common inductive signalling pathway for PC secretion. A presumptive link between Nod2 and global PC secretion has been made via the molecule KCNN4, which is an established risk allele for CD.³³ Specifically, the mouse homologue of KCNN4 (*Kcnn4*) is a K⁺ channel that regulates Ca²⁺ flux within PCs.³⁴ Patients with NOD2 risk polymorphisms display reduced levels of KCNN4 mRNA expression, leading to the hypothesis that Nod2-dysfunction may depress PC secretion

Table 1 Effects of mouse cage on gut bacterial composition

Component	Cumulative % explained	p Value	Multiple testing correction
1	16.89	0.10	0.22
2	29.38	1.57E-06	2.35E-05
3	36.01	2.49E-05	1.87E-04
4	40.87	4.83E-05	2.41E-04
5	45.47	0.15	0.29
6	50.00	1.98E-04	7.42E-04
7	54.07	0.15	0.26
8	57.98	0.0012	0.0035
9	61.16	0.84	0.90
10	64.19	0.61	0.76
11	67.05	0.23	0.33
12	69.87	0.28	0.39
13	72.40	0.63	0.73
14	74.84	0.91	0.91
15	77.04	0.02	0.06

p Values are calculated using a one-way analysis of variance in which the null hypothesis is that cage has no effect on the distribution of the coordinate. The results for the first 15 coordinates of the Bray–Curtis analysis shown in figure 5 are depicted. Only cages that had at least three animals were included in the analysis (n=7 cages). The 'Multiple Testing Correction' column shows correction for multiple hypothesis testing (in this case testing 15 coordinates) using the Benjamini and Hochberg procedure. For coordinates reaching statistical significance by multiple testing (p<0.05), the null hypothesis is rejected, indicating that the mouse cage appears to have an effect on gut bacterial composition.

by downregulating the expression of this potassium channel.³³ Interestingly, we found no differences in *Kcnn4* transcript levels in WT and *Nod2*^{-/-} mice (data not shown). Nevertheless, a regulatory effect of *Nod2* on PC secretion cannot be ruled out by the present study.

Even if we stipulate that *Nod2*-deficiency may result in some degree of defective PC secretion, the present study raises questions as to the biological relevance of such impairments.

Table 2 Effects of *Nod2* genotype on gut bacterial composition

Component	Cumulative % explained	p Value	Multiple testing correction
1	16.89	0.77	0.89
2	29.38	0.26	0.99
3	36.01	0.11	1.62
4	40.87	0.92	0.99
5	45.47	0.68	0.85
6	50.00	0.97	0.97
7	54.07	0.56	0.94
8	57.98	0.31	0.78
9	61.16	0.31	0.92
10	64.19	0.24	1.20
11	67.05	0.58	0.87
12	69.87	0.34	0.74
13	72.40	0.12	0.93
14	74.84	0.44	0.83
15	77.04	0.58	0.79

p Values are calculated using the median values for WT (n=13) and KO (n=14) for animals in each cage (n=1–3 animals per cage) for a t test in which the null hypothesis is that host genotype has no effect on the distribution of the coordinate. The results for the first 15 coordinates of the Bray–Curtis analysis shown in figure 5 are depicted. The 'Multiple Testing Correction' column shows correction for multiple hypothesis testing (in this case testing 15 coordinates) using the Benjamini and Hochberg procedure. No components reach statistical significance (p<0.05) indicating that the null hypothesis is accepted for all components, suggesting that *Nod2* genotype has minimal effect on gut bacterial composition.

Previous work using *Mmp7*^{-/-} mice that possess reduced α -defensin activity demonstrated profound shifts in the gut microbiota of these animals.⁴ Similar alterations of the intestinal microbiota would be expected in *Nod2*^{-/-} mice, if these mice indeed possess impaired PC function. Previous studies have supported this concept, demonstrating increased levels of *Bacteroides* and Firmicutes bacteria in the ilea of *Nod2*^{-/-} mice relative to WT controls,³² as well as global alterations of *Nod2*^{-/-} gut microbial communities analysed by high-throughput pyrosequencing.^{11 24} While these studies did control for background strain by using pure B6 mice, the precise breeding and husbandry conditions were often unclear. When such conditions were clearly described (as in the case for Rehman *et al*),¹¹ *Nod2*^{-/-} and WT colonies were housed independently through the course of the study. While this approach can offer some insight into the effects of *Nod2* on gut microbial composition, it is limited in its ability to detect confounding cage or maternally transmitted effects on the structure of gut bacterial communities. This is elegantly demonstrated in a recent study by Ubeda *et al*,³⁵ which examined gut microbial composition in MyD88- and TLR-deficient mice. Importantly, this study analysed the intestinal microbiota of knockout (KO) mouse colonies that had been housed and bred in isolation, but also evaluated WT and KO littermates which were housed together prior to necropsy (similar to the strategy used in the present study). Their analysis revealed marked differences in the microbiota of mice from the independent mouse colonies, but minimal alterations in cohoused littermates. This supports the results of our study, which shows no apparent differences in gut microbial composition between cohoused *Nod2*^{-/-} and WT littermates.

The lack of observed *Nod2*-dependent intestinal microbial alterations in the present study may be explained by two possible causes. First, local cage environment (including coprophagia) may be a strong influence on faecal microbial composition, able to suppress differences in the microbial community caused by genotype. Second, there may be a strong maternal influence on the faecal microbiota. This would lead to similar microbial profiles within individual mice of the same litter, regardless of genotype. Such effects have also been extensively described in the literature.^{35 36} Because our study design involved housing mice of the same litter within the same cage, we were unable to distinguish cage and litter effects in this study. In either case, there appears to be a strong cage and/or litter effect that supercedes the influences of *Nod2* on faecal microbial composition.

The findings described in the present study have important clinical implications. First, numerous human CD risk alleles have been associated with PC abnormalities,^{6 37–39} suggesting an important role for these cells in CD pathogenesis.⁴⁰ However, to translate such findings to clinical practice, the mechanisms of these gene-mediated PC impairments must be elucidated. Mouse models provide an opportunity to study such mechanisms.⁴¹ In this study, we have established a paradigm for studying PCs in mouse models of CD risk allele dysfunction, highlighting the importance of controlling for mouse background and housing conditions in such investigations. Second, our data raise concerns with the commonly accepted hypothesis that NOD2 dysfunction leads to attenuated PC microbicidal activity, which in turn, alters the composition of the gut microbiota, thereby predisposing to the development of CD. This highlights the importance of identifying alternative roles of NOD2 in the control of intestinal inflammation, both within PCs as well as other components of the innate immune system. An elegant approach to characterising such functions will be to examine PC function in knock-in mice carrying the human

NOD2 SNP13 polymorphism, which have been described previously.⁴² Finally, we demonstrate that environmental factors may overcome genetic influences on gut microbial structure. Ultimately, if we hope to modulate intestinal microbial communities as a treatment strategy for CD, it will be imperative to override the impact of host genetics on the gut microbiota. The homogenisation of the intestinal microbiota of *Nod2*^{-/-} mice and their WT littermates suggests that strong environmental pressures may supersede gene-based influences on gut microbial composition. Future studies should focus on identifying specific environmental factors that can overcome the microbial effects of distinct CD risk alleles. This may lead to novel, patient-specific treatment strategies for this disease.

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ATTTGGAGACATCTCCGGGTTGTCAAAGGAGTGATCCGGCAGAGGATGGACATGCGGGCGC
ATTAGCTAGTCGGCGGGGTAACGGCCACCGAGGCTACGATGCGTAGGGGTTCTGAGAGG
AAGGTCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGG
AATATTGGTCAATGGGCGGAAGCCTGAACCAGCCAAGTCGCGTGAGGGAAAGACGGTCCCTA
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ACCCTGAGAAAAAGCAT

>Consensus18 len=447 tot-seq=1418

AGAGTTTGATCATGGCTCAGGATGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAAC
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CCGCATAAGCGCACACTGTTGCATGACAGAGTGTGAAAAATTCGGGAGGTATACGATGGA
CCCGCGTCTGATTAGCCAGTTGGCAGGGTAACGGCCTACCAAAGCGACGATCAGTAGCCG
GCCTGAGAGGGTGGACGGCCACATTGGGACTGAGACACGGCCAACTCCTACGGGAGGC
AGCAGTGGGGGATATTGCACAATGGGGGAAACCCTGATGCAGCGACCGCCGCGTGAGTGAA
GAAGCATTTCGGTGTGTAAAGCTCTAT

>Consensus19 len=509 tot-seq=1377

AGAGTTTGATCCTGGCTCAGGATGAACGCTAGCGACAGGCTTAACACATGCAAGTCGAGG
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GTATGCAACCTGTCCGGTACAGGGGGATAAGCGGCGGAAACGCCGTCTAATACCGCGTGA
CAACCCGAGGGGGCATCCCCTTGGGTTCAAAGGAGAGATCCGGTACCGGCTGGGCATGCG
GCGCATTAGCTAGTTGGCGGGGCAACGGCCACCAAGGCGACGATGCGTAGGGGTTCTGA
GAGGAAGGTCCCCCACACTGGAAGTGAACACGGTCCAGACTCCTACGGGAGGCAGCAG
TGAGGAATATTGGTCAATGGGCGCAAGCCTGAACCAGCCAAGTCGCGTGAGGGAAGACGG
CCCTACGGGTTGTAAACCTCTTTTGGCGGGGAGCAAGCCTGAGCACGTGTGCCAGGCGG
AGAGTACCCGGAGAAAAAGCATCGGCTAA

>Consensus20 len=377 tot-seq=1234

AGAGTTTGATCATGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGC
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ACGGGGACAACGATTGGAACGATCGCTAATACCGGATAGGACGAAAGTTTAAAGATGCT
CCTGGCATCACTGATGGATGAGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCCTA
CCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACTGGGACTGAGACAC
GGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGCGAAAGCCTGAC
CGAGCAACGCCGCGTGA

>Consensus21 len=469 tot-seq=1202

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCATGCCTAATACATGCAAGTCGAAC
GAGGTCCTTCGGGACTCGAGTGGCGAACGGGTGAGTAACACGTAGGGAACCTGCCCGTG
CGACCGGGGAGAAGTTTCGAAACGGAAGCTGATACCGGATAGGGACAGAGACGGCATCGA
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TTGGTGAAGTAACGGCCACCAAGGCGACGATGCATAGCCGGCCTGAGAGGGCGGACGGC
CACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATTTTCGT
CAATGGGCGCAAGCCTGAACGAGCAATGCCGCGTGAACGAGGAAGGTCTTCGGATCGTAA
AGTTCTGTTGAAGGGGAAAAAGAGCGGAGAGAGGAAATGGTCTCCGATT

>Consensus22 len=479 tot-seq=1130

AGAGTTTGATCCTGGCTCAGGATGAACGCTAGCGACAGGCTTAACACATGCAAGTCGAGG
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GTATGCAACCTGCCCCCTGCAGGAGGATAAACC GGAGAAAATCCCGACTAATACTGCATAA
CACCTGGGGCCACATGGCTTCGGGGTCAAAGGAAGCAATTCGGGCAGGGGATGGGCATG
CGGCGCATTAGCCAGTTGGCGGGGTAACGGCCACCAAAGCGACGATGCGTAGGGGTTCT
GAGAGGAAGGTCCCCCACATTGGTACTGAGACACGGACCAAACCTCCTACGGGAGGCAGCA
GTGAGGAATATTGGTCAATGGGCGCAAGCCTGAACCAGCCAAGTCGCGTGAGGGATGACC
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>Consensus23 len=500 tot-seq=1026

AGAGTTTGATCCTGGCTCAGGATGAACGCTAGCGACAGGCTTAACACATGCAAGTCGAGG
GGCAGCGGGGAGTGAGGCTTGCTTACTTTGCCGGCGACCGGCGCACGGGTGAGTAACAC
GTATGCAACCTACCCTTTACAGCGGGATAAACC GGAAAGAAAATTCGCCTAATACCGCATAT
ACTCCTTGGGAGGCATCTTCCGAGGGGGGAAGAATTTCCGGTGAAGGATGGGCATGCGTCC
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GAAGTCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGA
GGAATATTGGTCAATGGCCGAGAGGCTGAACCAGCCAAGTCGCGTGAGGGAAGAATGTCC
TATGGATTGTAAACCTCTTTTGTGTCAGGGAGCAAAGTCAACCACGTGTGGGTGTTGGAGAG
TACCTGAAGAAAAAGCATCG

>Consensus24 len=440 tot-seq=1024

AGAGTTTGATCATGGCTCAGGATGAATGCTGGCGGCGTGCCTAATACATGCAAGTCGAGC
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GAGGGAACAGTGAAGCTGCTGACGAGCGGCGGACGGCTGAGTAACGCGTGGGAACATAAC
CCAAAGTGAGGGATAACTACTCGAAAGAGTGGCTAATACCGCATATGATCTTCGGATTAA
AGCATTATGCGCTTTGGGAATGGCCTGCGTACGATTAGATAGTTGGTGAAGTAAAGGCT
CACCAAGTCGACGATCGTTAGATGGTTTGAAGGATGATCATCCAGACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTTCACAATGGGCGAAAGCCTG
ATGGAGCAACGCCGCGTGA

>Consensus25 len=481 tot-seq=1015

AGAGTTTGCATCATGGCTCAGGATGAACGCTAGCGACAGGCCTAACACATGCAAGTCGAGG
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ATGCGACCTGCCCCGTTGCAGGGGATAATCGGGAGAAAATCCCGTCTAATACCGCGTAACG
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TGACATTAGCTAGTTGGCGGGGCAACGGCCACCAAGGCGACGATGTCTAGGGGTTCTGA
GAGGAAGTCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGT
GAGGAATATTGGTCAATGGGCGGAGCCTGAACCAGCCAAGTCGCGTGAGGGATGACGGC
CCTACGGGTTGTAAACCTCTTTTGTGCGGGAGCAAATTCGCCACGTGTGGCGGAGTCGA
G

>Consensus26 len=479 tot-seq=1006

AGAGTTTGCATCATGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAAC
GAAGCACTCTTTTAGATTTCTTTCGGGGAAGAAGAAATTTGTGACTGAGTGGCGGACGGGT
GAGTAACGCGTGGGCAACCTGCCCCATACAGGGGGATAACAGCCGGAACGGCTGCTAAA
ACCGCATAAGCGCACAAAGGCCGCATGGCCTGGTGTGAAAACTCCGGTGGTATGGGATG
GGCCCGCGTCTGATTAGCTAGTTGGCGGGGAGAGGCCACCAAGGCGACGATCAGTAGC
CGACCTGAGAGGGTGGCCGGCCACATTGGGACTGAGACACGGCCAAACTCCTACGGGAG
GCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGACGCCGCGTGAGCG
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>Consensus27 len=446 tot-seq=925

AGAGTTTGCATCATGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAAC
GAAGCATGTAAAACAATCCTTTCGGGTGCGTTTTATATGACTGAGTGGCGGACGGGTGAGT
AACGCGTGGGGAACCTGCCGTATGCAGGGGGACAACAGTCAGAAATGACTGCTAATACCG
CATAAGCACACAATGCTGCATGGCATGGTGTGAAAAGATTTATCGGCATACGATGGCCCC
GCGTCTGATTAGCCAGTTGGCAGGGTAACGGCCTACCAAAGCAACGATCAGTAGCCGGCT
TGAGAGAGTGACCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGACGCCGCGTGAGTGATGAA
GTATTTCCGGTATGTAAAGCTCTATCA

>Consensus28 len=436 tot-seq=754

AGAGTTTGCATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCCTAACACATGCAAGTCGAAC
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GAGTAACGCGTGGGCAACCTGCCCTGCACTGGGGGACAACAGCCGGAACGGCTGCTAAT
ACCGCATATGCCCCGACCCCCGCATGGGGCGGCGGGGGAAGCTCCGGCGGTGCAGGATGG
GCCCCGCTCTGATTAGCTGGTTGGCGGGGCAACGGCCCCACCAAGGCGACGATCAGTAGCC
GGCCTGAGAGGGTGGGCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGGATATTGCACAATGGGGGAAACCCTGATGCAGCGACGCCGCGTGAGTGAA
TGGAGTGCTTCGGCAT

>Consensus29 len=507 tot-seq=717

AGAGTTTGCATCCTGGCTCAGGATGAACGCTAGCTACAGGCTTAACACATGCAAGTCGAGG
GGCAGCATGGCCTATCTTTTCGGGATGGGCCGATGGCGACCGGCGCACGGGTGAGTAACGC
GTATCCAACCTTCCCTTTACTGGGGTCCAGCCCGTCGAAAAGGCGGATTAATCCCCCATGT
TCTCCGTCCCGGACATCTGTGTGCGGAGCAAAGATTTATCGGTAAAGGATGGGGATGCGTC
CGATTAGCTTGTGGCGGGGTAACGGCCACCAAGGCATCGATCGGTAGGGGTTCTGAGA
GGAAGTCCCCCACACTGGAACCTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
AGGAATATTGGTCAATGGCCGTAAGGCTGAACCAGCCAAGTAGCCTGAGGGAAGACTGCC
CTATGGGTTGTAAACCTCTTTTATGCGGGGATAAAGGTGTCCACGTGTGGATGTTTGCAG
GTACCGCATGAATAAGGACCGGCTAAT

>Consensus30 len=479 tot-seq=686

AGAGTTTGCATCCTGGCTCAGGACGAACGCTGGCGGCACGCCTAACACATGCAAGTCGAAC
GGTGAAGGGGAGCTTGCTCCTCCGGAACAGTGGCGGACGGGTGAGTAACACGTGAGCAA
CCTGCCTTTTCAGAGGGGGACAACAGTTGGAAACGACTGCTAATACCGCATAACGTATTTT
GGCGGCATCGCCGGAATACCAAAGGAGCAATCCGCTGAAAAGATGGGCTCGCGTCTGATTA
GATAGTTGGTGAAGGTAACGGCTCACCAAGTCGACGATCAGTAGCCGACTGAGAGGTTGA
ACGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGGATA

TTGGACAATGGGGGAAACCCTGATCCAGCGATGCCGCGTGAGGGAAGAAGGTTTTTCGGAT
TGTA AACCTCTGTGGAGGGGACGATAATGACGGTACCCCTTTAGGAAGCCACGGCTAA
>Consensus31 len=493 tot-seq=667

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GGCAGCGGGGGCGCAGCAATGCGCCTGCCGGCGACCGGCGCACGGGTGAGTAACACGTAT
GCAACCTGCCCCGCCGAGGGGTATAACCGGGGGAAACCCCGACTAATCCCGCATGACACC
CCGTGGAGGCATCTCCTCGGGGTCAAAGGAGCGATCCGGCGGCGGATGGGCATGCGTCGC
ATTAGCTAGTCGGCGGGGTAACGGCCACCAGGGCGACGATGCGTAGGGGTTCTGAGAGG
AAGGCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGG
AATATTGGTCAATGGGCGAAGCCTGAACCAGCCAAGTCGCGTGAGGGAAGAAGGCCCTG
CGGGTCGCAGACCTCTTTGGACGGGGAGCAAGGCCGCCGACGTGTCGGCGGAAGGAGAGT
ACCCGTAGAACAA

>Consensus32 len=474 tot-seq=648

AGAGTTTGATCATGGCTCAGGATGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAAC
GGGTTTTATATAACAGA ACTCTCCGGAGGGAAGATATATAAACCTAGTGGCGGACGGGTG
AGTAACGCGTGGGCAACCTGCCCTGTACCGGGGGACAACAGCCAGAAATGGCTGCTAATA
CCGCATAAGCCTGCTGTGCCGCATGGCACAGCAGGGAAAACACGGTGGTACAGGATGGG
CCCGCTCTGATTAGTTAGTTGGCAGGGTAACGGCCTACCAAGACAACGATCAGTAGCCG
GCTTGAGAGAGTGGACGGCCACATTGGGACTGAGACACGGCCAGACTCCTGCGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGGGAAACCTGATGCAGCAATGCCGCGTGGGTGAA
GAAGCACTCCGGTGCCTAAAGCCCTGTCAGCAGGGAAGAAAATGACAGTACCTG

>Consensus33 len=515 tot-seq=630

AGAGTTTGATCATGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAAC
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GTAACGCGTGGGCAACCTGCCCGTACCGGGGGATAACAGCCGGAACGGCTGCTAATAC
CGCATATGCTTTGCGGGCCGCATGGCCCGCAAAGCAAACTCCGGTGGTACGGGATGGGC
CCGCGTCTGATTAGCTGGTTGGCGGGGTAACGGCCACCAAGGCGACGATCAGTAGCCGG
CCTGAGAGGGCGGCCGGCCACATTGGGACTGAGACACGGCCAGACTCCTGCGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGGGAAACCTGATGCAGCAGCGCCGCGTGGGCGAGG
AAGTACCTCGGTATGTAAAGCCCTATCAGCAGGGAAGAAAACAGGACAGTACCTGACTAAG
AAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTA

>Consensus34 len=505 tot-seq=574

AGAGTTTGATCATGGCTCAGGATGAACGCTAGCGGCAGGCTTAACACATGCAAGTCGAGG
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ATGCAACCTACCCATTACAGGGGGATAACACTGAGAAAATCGGTACTAATACCCCATAA
TCCAGGGAGGCATCTTCTTGGGTTGAAAACCTCCGGTGGTAATGGATGGGCATGCGTTGTA
TTAGTTAGTTGGTGGGGTAACGGCTCACCAAGACAACGATACATAGGGGGACTGAGAGGT
TAACCCCCACATTGGTACTGAGACACGGACCAAACCTCCTACGGGAGGCAGCAGTGAGGA
ATATTGGTCAATGGACGCAAGTCTGAACCAGCCATGCCGCGTGCAGGAAGACGGCTCTAT
GAGTTGTAAACTGCTTTTGTACGAGGGTAAACGCAGATACGTGTATCTGTCTGAAAGTAT
CGTACGAATAAGGATCGGCTAACTC

>Consensus35 len=509 tot-seq=545

AGAGTTTGATCATGGCTCAGGATGAACGCTAGCTACAGGCTTAACACATGCAAGTCGAGG
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GTATCCAACCTGCCTCATACTCGGGGATAGCCTTTTCGAAAAGAAAGATTAATACCCGATGT
CATAGTCCTACCGCATGATGGGATTATTAAGAATTTCCGGTATGGGATGGGGATGCGTTC
CATTAGTTAGTTGGCGGGGTAACGGCCACCAAGACAACGATGGATAGGGGTTCTGAGAG
GAAGGTCCCCACATTGGAACCTGAGACACGGTCCAAACCTCCTACGGGAGGCAGCAGTGAG
GAATATTGGTCAATGGACGAGAGTCTGAACCAGCCAAGTAGCGTGAAGGATGACTGCCCT
ATGGGTTGTAAACTTCTTTTATATGGGAATAAAATGTTCCACGTGTGGGATTTTGTATGT
ACCATATGAATAAGGATCGGCTAACTCCG

>Consensus36 len=493 tot-seq=544

AGAGTTTGATCCTGGCTCAGGATGAACGCTAGCGACAGGCTTAACACATGCAAGTCGAGG
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CTTGCCTATCAGAGGGGAACAGCCCGGCGAAAGTCCGATTAATGCCCCATAAAACAGGGA
TCCCGCATGGGGTTATTTGTTAAAGATTTCATCGCTGATAGATAGGCATGCGTTCCATTAG
GCAGTTGGCGGGGTAACGGCCACCAAACCGACGATGGATAGGGGTTCTGAGAGGAAGGT
CCCCACATTGGAAGTGAACACGGTCCAAACTCCTACGGGAGGCAGCAGTGAGGAATAT
TGGTCAATGGCCGTAAGGCTGAACCAGCCAAGTCCGCTGAAGGAAGAAGGATCTATGGTT
TGTAAACTTCTTTTATAGGGGAATAAAGGTATCCACGTGTGGATATTTGCATGTACCCTA
TGAATAAGCATCG

>Consensus37 len=517 tot-seq=528

AGAGTTTGTATCCTGGCTCAGGATGAACGCTAGCGGCAGGCTTAACACATGCAAGTCCGGC
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ATGCAACCTGCCCCCTGTCAGGGGACAACCCGCCGAAAGGCGGGCTAAGCCCGGTATAT
CGCCCCGGGGCATCCCCGGGGGAGGAAAGGAGCGATCCGGACAGGGATGGGCATGCGGCG
CATTAGCTGGTCCGGCGGGTAACGGCCCACCGAGGCGACGATGCGTAGGGGTTCTGAGAG
GAAGGCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAG
GAATATTGGTCAATGGGCGGGAGCCTGAACCAGCCAAGCCGCTGAGGGAAGAAGGTACA
GCGTATCGTAAACCTCTTTTGTGTCAGGGAACAAAGGCGGGGACTAGTCCCCGGATGAGGT
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>Consensus38 len=515 tot-seq=526

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CCCCGGGGGCATCCCCGGGGGAGGAAAGCCCTTCTGGGCGGGCGGGGATGGGCATGCGG
CGCATTAGGAAGACGGCGGTGTGACGGACCACCGTTCCGACGATGCGTAGGGGTTCTGAG
AGGAAGGCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTG
AGGAATATTGGTCAATGGCCGGAAGGCTGAACCAGCCAAGCCGCTGAGGGAGGAAGGCG
CAGAGCGTCGCAGACCTCTTTTGCCGGGGGACAAAAGGCCGACTCGTCCGGTCTCTGAGG
GTACCCGGAGAAAAAGCATCGGCTAACTCCGTGCC

>Consensus39 len=481 tot-seq=493

AGAGTTTGTATCATGGCTCAGGATGAACGCTGGCGGCGTGCCTAACACATGCAAGTCCGAGC
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GTAACGCGTGGGCAACCTGCCTTACACAGGGGGATAACAGTTAGAAATGACTGCTAATAC
CGCATAAGACCGCAGTACCGCATGGTACAGCGGTAACAACTCCGGTGGTGTAAAGATGGGC
CCGCGTCTGATTAGGTAGTTGGCGGGGTAACGGCCCACCAAGCCGACGATCAGTAGCCGA
CCTGAGAGGGTGACCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGGGAAACCTGATGCAGCGACGCCGCTGAGCGATG
AAGTATTTCCGGTATGTAAAGCTCTATCAGCAGGGAAGAAAATGACGGTACCTGACTAAGA
A

>Consensus40 len=512 tot-seq=491

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AGTAACGCGTGAGCAACCTGCCCTTCGGAGGGGGATAGTGTCTGGAAACGGACAGTAATA
CCGCATAACGTATTTTTACCGCATGATAGAAATACCAAACTGAGGTGCCGAAGGATGGG
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GACTGAGAGGTTGAACGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGGGAAACCTGATGCAGCAACGCCGCTGAAGGAA
GACGGTTTTTCGGATTGTAAACTTCTGTTCTTAGTGAAGAAGAATGACGGTAGCTAAGGAG
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>Consensus41 len=513 tot-seq=484

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GGCCTGAGAGGGTGGACGGCCACATTGGGACTGAGACACGGCCAACTCCTACGGGAGG

CAGCAGTGGGGATATTGGACAATGGGGGAAACCCTGATCCAGCGACGCCGCGTGAGTGA
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CAAGAAGCCCCGGCTAACTACGTGCCAGCAGCC

>Consensus42 len=481 tot-seq=481

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAAC
GAAGCGCTCGGACGGAGTCTCTTCGGAGAGGAAGGCCGGGCGACTGAGTGGCGGACGGGT
GAGTAACGCGTGGGCAACCTGCCCTGCACAGGGGGACAACAGCCGAAACGGCTGCTAAT
ACCGCATAAGCGCACAGCTTCGCATGAAGCGGTGCGAAAAGCTTTGGCGGTGCAGGATGG
GCCCCGCTCTGATTAGCTTGTGGCGGGGCAACGGCCACCAAGGCGACGATCAGTAGCC
GGCCTGAGAGGGTGGACGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGACGCCGCGTGAGCGA
AGAAGTATTTTCGGTATGTAAAGCTCTATCAGCAGGGAAGAAATGACGGTACCTGAGTAA
G

>Consensus43 len=482 tot-seq=469

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ATGCAACCTACCCTTGTGTCAGGGGGATAAGCCGGAGAAATCCGGTCTAATACCGCGTGACA
TCGGGATTCCGCATGGGATTCGGATCAAAGGATTTATCCGGACAAGGATGGGCATGCGGC
GCATTAGCTAGTTGGCGGGGTAACGGCCACCAAGGCGACGATGCGTAGGGGTTCTGAGA
GGAAGGTCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGA
GGAATATTGGTCAATGGGCGAGAGCCTGAACCAGCCAAGTCGCGTGAGGGAATAAGGTCC
TATGGATTGTAAACCTCTTTTGTGGGGAGCAATGAGCGTCACGTGTGATGCGTTGAGAG
TA

>Consensus44 len=509 tot-seq=465

AGAGTTTGATCATGGCTCAGGATGAACGCTGGCGGCGTGCCTAACACATGCAAGTTCGAGC
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GTAACGCGTGGACAACCTGCCCTATGCAGGGGGATAACATCCGAAACGGGTGCTAATAC
CGCATAAGCGCACAGTACCGCATGGTACGGTGTGAAAAGCTGAGGCGGCATAGGATGGGT
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CCTGAGAGGGTGGACGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGACGCCGCGTGGGTGAGG
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AGCCCCGGCTAACTACGTGCCAGCAGCCG

>Consensus45 len=481 tot-seq=455

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CATGCCCTTTAGTCTAGGATAGCCATTGGAAACGATGATTAATACTGGATACTCCTTACG
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CTGAAGCAGCAACGCCGCGTGGAGGATGAAGGTTTTTCGGATTGTAAACTCCTTTTGTAG
AGAAGATAATGACGGTATCTAACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG
T

>Consensus46 len=515 tot-seq=417

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ACCGCATAGGCGCACAGCTTCGCATGAAGCAGTGTGGAAAACCGAGGTGGTGTGGGATGG
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GGCCTGAGAGGGTGGACGGCCACATTGGGACTGAGACACGGCCAAACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGACGCCGCGTGAGCGA
AGAAGTATTTTCGGTATGTAAAGCTCTATCAGCAGGGAAGAAATGACGGTACCTGACTAA
GAAGCCCCGGCTAAATACGTGCCAGCAGCCGCGGT

>Consensus47 len=486 tot-seq=391

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GAGCAACTTGCCCTCATCAGGGGAATAATCGCTGGAAACGGCGTCTAATGCCCCATGGTG
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AATATTGGTCAATGGCCGGAAGGCTGAACCAGCCATGCCGCGTGAAGGTCAGTGCCCTAT
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CGTACG

>Consensus48 len=483 tot-seq=369

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AGATGCTAATACCGCATAACAACAAAAGCCACATGGCTTTTGTGTTGAAAGATGGCTTTGG
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GGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGAAGTGAACACGGTC
CATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAG
CAACACCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTGGAGAAGAAC
TGC

>Consensus49 len=517 tot-seq=367

AGAGTTTGATCATGGCTCAGGATGAACGCTAGCTACAGGCTTAACACATGCAAGTCGAGG
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>Consensus50 len=522 tot-seq=363

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GAGAGGAAGGTCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCA
GTGAGGAATATTGGTCAATGGGCGGGAGCCTGAACCAGCCAAGTTCGCGTGAGGGACGACG
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>Consensus51 len=519 tot-seq=355

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>Consensus52 len=517 tot-seq=349

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>Consensus53 len=508 tot-seq=348

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>Consensus54 len=521 tot-seq=321

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ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGACGCCGC
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>Consensus55 len=512 tot-seq=320

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GCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGACGCCGCGTGAGTGAGG
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>Consensus56 len=488 tot-seq=319

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GAGGAATATTGGTCAATGGGCTTACGCCTGAACCAGCCAAGTCGCGTGAGGGAAGACTGC
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GAGTACCT

>Consensus57 len=510 tot-seq=310

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AGTAACGCGTGGGGAACCTGCCCTGTACCGGGGACAACAGCCAGAAATGGCTGCTAATA
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GCTTGAGAGAGCGGACGGCCACATTGGGACTGAGACACGGCCAGACTCCTGCGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAATGCCGCGTGGGTGAA
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>Consensus58 len=530 tot-seq=309

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GAGGAAGCCCCCCCCACACTGGAAGTGAACACGGTCCAGACTCCTACGGGAGGCAGCAG
TGAGGAATATTGGTCAATGGGCGGAAGCCTGAACCAGCCAAGTCGCGTGAGGGAATAAGG
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>Consensus59 len=517 tot-seq=303

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GAGTAACGCGTGGGCAACCTGCCCCATACCGGGGGATAACAGCCGGAACGGCTGCTAAT
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GGCCTGAGAGGGCGGCCCGCCACATTGGGACTGAGACACGGCCAGACTCCTGCGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGGGAAACCCCTGATGCAGCGACGCCGCGTGAGCGA
TGAAGTATCTCGGTATGTAAAGCTCTATCAGCAGGGAAGAAAGTGACAGTACCTGACTAA
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>Consensus60 len=502 tot-seq=301

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CCGCGTCTGATTAGCTGGTTGGTGAGGTAACGGCCACCAAGGCGACGATCAGTAGCCGA
CCTGAGAGGGTGGCCGGCCACATTGGGACTGAGACACGGCCAAACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGGGAAACCCCTGATGCAGCAACGCCGCGTGAGTGAAG
AAGTATTTCCGTATGTAAAGCTCTATCAGCAGGGAAGAAAGACGGTACCTGACTAAG
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>Consensus61 len=521 tot-seq=297

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CACCATTTGGAGACATCTCCAGGTGGTCAAAGGAGGCGACTCCGGCATAGGATGGCCATG
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GAGAGGAAGGTCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCA
GTGAGGAATATTGGTCAATGGGCGCAAGCCTGAACCAGCCAAGTCGCGTGAGGGAAAGACG
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>Consensus62 len=494 tot-seq=294

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TTCTACGGAAGAAAGAGGGGGACCTGCTTGACGGCCTCTCGCGAGCGGAGCGGCCGATGA
CTGATTAGCCGGTTGGTGAGGTAACGGCTCACAAAGCAACGATCAGTAGCTGGTCTGAG
AGGACGACCAGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCTATTCGCGGTGTGGGATGACGGCC
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TGACGGTACTCTAA

>Consensus63 len=498 tot-seq=291

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CTCGCGTCTGATTAGCTTGTGGCGGGGCAACGGCTCACCAAGGCGACGATCAGTAGCCG
GCCTGAGAGGGTGGACGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGGC
AGCAGTGGGGGATATTGCACAATGGGGGGAACCCTGATGCAGCGACGCCGCGTGGGCGAT
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>Consensus64 len=499 tot-seq=289

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CACAATGGGGGGAACCCTGATGCAGCGATGCCGCGTGGAGGAAGAAGTTTTTCGGATTGT
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>Consensus65 len=503 tot-seq=288

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ACCCGCGTCTGATTAGCTGGTTGGTGGAGTAACGGCTCACCAAGGCGACGATCAGTAGCC
GGCTTGAGAGAGTGGACGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGGGGAACCCTGATGCAGCGACGCCGCGTGGAGTA
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GAAGCCCCGGCTAACTACGTGCC

>Consensus66 len=519 tot-seq=279

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ACCCGCGTCTGATTAGCTTGTGGCGGGGTAACGGCCACCAAGGCGACGATCAGTAGCC
GGCCTGAGAGGGTGGACGGCCACATTGGGACTGAGACACGGCCAAACTCCTACGGGAGG
CAGCAGTGGGGGATATTGGACAATGGGGGGAACCCTGATCCAGCGACGCCGCGTGGAGTA
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>Consensus67 len=517 tot-seq=277

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GGTCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAA
TATTGGTCAATGGCCGGGAGGCTGAACCAGCCAAGTCGCGTGAGGGAAGACGGCCCTACG
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>Consensus68 len=510 tot-seq=258

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CGGACTGAGAGGTTGATCGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAG

GCAGCAGTGGGGGATATTGCACAATGGAGGAACTCTGATGCAGCGACGCCGCGTGAGGG
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>Consensus69 len=522 tot-seq=255

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CACTCCTGAGGGACATCCTTCGGGGGTCAAAGGAGGCGACTCCGGTGACGGATGGGCATG
CGGCGCATTAGGTAGTTGGTAGGGTAACGGCCTACCAAGCCGACGATGCGTAGGGGTCT
GAGAGGAAGGTCCCCACATTGGAAGTGAACACGGTCCAACTCCTACGGGAGGCAGCA
GTGAGGAATATTGGTCAATGGCCGGAAGGCTGAACCAGCCAAGTCGCGTGAGGGAATAAG
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>Consensus70 len=525 tot-seq=254

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>Consensus71 len=512 tot-seq=239

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AATATTGGTCAATGGCCGAGAGGCTGAACCAGCCATGCCGCGTGAAGGCGAGTGCCCTAC
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>Consensus72 len=508 tot-seq=227

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GCAGTGGGGAATATTGGGCAATGGGCGCAAGCCTGACCCAGCAACGCCGCGTGAAGGAAAG
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>Consensus73 len=520 tot-seq=225

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CACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATTTTCGGCA
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>Consensus74 len=513 tot-seq=221

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>Consensus75 len=515 tot-seq=221

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GCAGTGGGGAATATTGGGCAATGGGCGCAAGCCTGACCCAGCAACGCCGCGTGAAGGAAG
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>Consensus76 len=514 tot-seq=212

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ACGGGAGGCAGCAGTGAGGGATATTGGTCAATGGGGGAAACCCTGAACCAGCAACGCCGC
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>Consensus77 len=516 tot-seq=210

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GCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCGACGCCGCGTGAAGTAAG
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>Consensus78 len=514 tot-seq=209

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>Consensus79 len=527 tot-seq=204

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>Consensus80 len=526 tot-seq=204

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>Consensus81 len=528 tot-seq=201

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>Consensus82 len=517 tot-seq=196

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>Consensus83 len=519 tot-seq=194

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>Consensus84 len=495 tot-seq=192

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>Consensus85 len=518 tot-seq=187

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>Consensus86 len=522 tot-seq=181

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>Consensus87 len=516 tot-seq=177

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>Consensus88 len=508 tot-seq=174

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>Consensus89 len=516 tot-seq=174

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>Consensus90 len=522 tot-seq=171

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>Consensus91 len=519 tot-seq=171

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>Consensus92 len=510 tot-seq=167

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>Consensus93 len=531 tot-seq=167

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>Consensus94 len=521 tot-seq=165

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>Consensus95 len=518 tot-seq=162

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>Consensus96 len=492 tot-seq=156

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>Consensus97 len=540 tot-seq=154

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>Consensus98 len=488 tot-seq=150

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>Consensus99 len=506 tot-seq=145

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>Consensus100 len=506 tot-seq=144

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>Consensus101 len=512 tot-seq=142

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>Consensus102 len=522 tot-seq=141

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>Consensus103 len=490 tot-seq=136

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>Consensus104 len=520 tot-seq=136

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>Consensus105 len=507 tot-seq=132

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>Consensus106 len=517 tot-seq=132

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>Consensus107 len=530 tot-seq=132

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>Consensus108 len=520 tot-seq=131

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>Consensus109 len=531 tot-seq=129

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>Consensus110 len=526 tot-seq=125

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>Consensus111 len=508 tot-seq=123

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>Consensus112 len=527 tot-seq=122

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>Consensus113 len=516 tot-seq=121

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>Consensus114 len=518 tot-seq=120

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>Consensus115 len=517 tot-seq=120

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>Consensus116 len=519 tot-seq=117

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>Consensus117 len=520 tot-seq=116

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>Consensus118 len=506 tot-seq=115

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>Consensus119 len=501 tot-seq=114

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>Consensus120 len=444 tot-seq=113

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>Consensus121 len=466 tot-seq=109

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>Consensus122 len=511 tot-seq=107

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>Consensus124 len=498 tot-seq=106
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>Consensus125 len=523 tot-seq=106
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>Consensus126 len=510 tot-seq=105
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>Consensus127 len=510 tot-seq=104
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>Consensus128 len=471 tot-seq=101
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>Consensus129 len=499 tot-seq=101

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>Consensus130 len=503 tot-seq=99

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>Consensus131 len=497 tot-seq=94

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>Consensus132 len=515 tot-seq=93

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>Consensus133 len=515 tot-seq=93

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>Consensus134 len=507 tot-seq=92

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>Consensus135 len=510 tot-seq=89

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>Consensus136 len=508 tot-seq=88

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>Consensus137 len=517 tot-seq=88

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>Consensus138 len=521 tot-seq=87

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>Consensus139 len=520 tot-seq=86

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>Consensus140 len=481 tot-seq=85

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GGGAAAGATTTATCGGTGGTGGATGTGCCCGCGTTGGATTAGCTTGTGGTGGGGTAACG
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GACACGGTCCAGACTCCTACGGGAGGCAGCAGCTAAGAATATTGGGCAATGGAGGAAACT
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>Consensus141 len=515 tot-seq=84

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>Consensus142 len=505 tot-seq=84

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>Consensus143 len=501 tot-seq=83

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>Consensus144 len=508 tot-seq=83

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>Consensus145 len=503 tot-seq=82

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>Consensus146 len=505 tot-seq=82

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>Consensus147 len=517 tot-seq=81

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>Consensus148 len=511 tot-seq=80

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>Consensus149 len=504 tot-seq=77

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>Consensus150 len=502 tot-seq=76

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CGGCCTGAGAGGGTGACGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGAGGAACTCTGATGCAGCGACGCCGCGTGAGTGA
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>Consensus151 len=519 tot-seq=74

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>Consensus152 len=507 tot-seq=74

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ATTAGCTGGACGGCGGGGTAACGGCCACCGTGGCGACGATGTCTAGGGGTTCTGAGAGG
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AATATTGGTCAATGGGCGGGAGCCTGAACCAGCCATGCCGCGTGAAGGCGAGTGCCCTAC
GGCGTTAAACTTCTTTTGTGCGGGAGCAATAAGGCCACGTGTGGCCCGATGAGAGTACC
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>Consensus153 len=500 tot-seq=74

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CCACATTGGGACTGAGATACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
GCAATGGGGGCAACCCCTGACGCAGCAACGCCGCGTGCGGGACGAAGGCCTTCGGGTTGTA
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>Consensus154 len=510 tot-seq=74

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CGGCCTGAGAGGGTGGACGGCCACATTGGGACTGAGACACGGCCCAAACCTCCTACGGGAG
GCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCCTGATGCAGCGACGCCGCGTGAGTG
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>Consensus155 len=516 tot-seq=74

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CAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGGGTGAAGG
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>Consensus156 len=503 tot-seq=71

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CGCATAAGCGCACAGTACCGCATGGTACAGTGTGAAAACTCCGGTGGTATAAGATGGAC
CCGCGTCTGATTAGCCAGTTGGCGGGGTAACGGCCACCAAAGCGACGATCAGTAGCCGA
CCTGAGAGGGTGACCGGCCACATTGGGACTGAGACACGGCCAAACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAAGTGAAG
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AGCCCCGGCTAACTACGTGCCAG

>Consensus157 len=522 tot-seq=70

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GAAGGTCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGA
GGAATATTGGTCAATGGGCGGGAGCCTGAACCAGCCAAGCCGCGTATGGAGGAAGGCGC
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>Consensus158 len=424 tot-seq=69

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TCGCATGGCACTGATATCAAAGGAGCAATCCGCTGAAAAGATGGGCTCGCGTCCGATTAGG
CAGTTGGCGGGGTAACGGCCACCAAACCGACAATCCGGTAGCCGGACTGAGAGGTTGAAC
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>Consensus159 len=498 tot-seq=68

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GAATATTGCGCAATGGAGGAAACTCTGACGCAGTGACGCCGCGTATAGGAAGAAGGTTTT
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>Consensus160 len=524 tot-seq=66

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GAGGAAGGCCCCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGT
GAGGAATATTGGTCAATGGGCGGAAGCCTGAACCAGCCAAGTCCGCGTGAAGGACTAAGGC
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>Consensus161 len=505 tot-seq=66

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TATTGCACAATGGGGGAACCTGATGCAGCGACGCCGCGTGAGTGAAGAAGTATTTTCGG
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>Consensus162 len=508 tot-seq=63

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CGCATAAGACCCGCGTACCGCATGGTACAGCGGTAAAACTCCGGTGGTATGAGATGGGC
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CCTGAGAGGGTACCAGCCACATTGGGACTGAGACACGGCCAACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGGGAACCTGATGCAGCGACGCCGCGTGAGCGATG
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>Consensus163 len=528 tot-seq=62

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GAAGGTCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGA
GGAATATTGGTCAATGGTTCGGGAGACTGAACCAGCCAAGCCGCGTGAGGGAGGAAGGTAC
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>Consensus164 len=502 tot-seq=61

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>Consensus165 len=510 tot-seq=59

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>Consensus166 len=413 tot-seq=57

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GTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGG
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>Consensus167 len=514 tot-seq=56

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>Consensus168 len=516 tot-seq=56

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>Consensus169 len=509 tot-seq=56

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>Consensus170 len=528 tot-seq=55

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>Consensus171 len=503 tot-seq=55

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>Consensus172 len=519 tot-seq=54

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>Consensus173 len=516 tot-seq=54

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>Consensus174 len=530 tot-seq=53

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>Consensus175 len=495 tot-seq=51

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>Consensus176 len=515 tot-seq=51

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>Consensus177 len=501 tot-seq=50

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>Consensus178 len=518 tot-seq=50

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>Consensus179 len=516 tot-seq=49

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>Consensus180 len=512 tot-seq=49

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>Consensus181 len=508 tot-seq=48

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>Consensus182 len=505 tot-seq=48

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>Consensus183 len=487 tot-seq=47

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>Consensus184 len=507 tot-seq=47

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>Consensus185 len=514 tot-seq=45

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>Consensus186 len=515 tot-seq=45

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>Consensus187 len=508 tot-seq=44

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>Consensus188 len=517 tot-seq=44

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>Consensus189 len=513 tot-seq=43

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>Consensus190 len=514 tot-seq=43

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>Consensus191 len=453 tot-seq=40

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>Consensus192 len=508 tot-seq=40

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>Consensus193 len=510 tot-seq=39

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>Consensus194 len=517 tot-seq=39

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>Consensus195 len=503 tot-seq=38

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>Consensus196 len=529 tot-seq=37

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>Consensus197 len=513 tot-seq=37

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>Consensus198 len=530 tot-seq=36

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>Consensus199 len=499 tot-seq=33

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>Consensus200 len=518 tot-seq=32

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>Consensus201 len=527 tot-seq=32

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>Consensus202 len=512 tot-seq=32

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>Consensus203 len=513 tot-seq=32

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>Consensus204 len=510 tot-seq=32

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>Consensus205 len=532 tot-seq=31

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>Consensus206 len=495 tot-seq=30

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>Consensus207 len=512 tot-seq=29

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>Consensus208 len=530 tot-seq=28

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>Consensus209 len=516 tot-seq=26

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>Consensus210 len=503 tot-seq=26

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>Consensus211 len=512 tot-seq=25

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>Consensus212 len=506 tot-seq=25

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>Consensus213 len=493 tot-seq=25

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>Consensus214 len=528 tot-seq=25

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>Consensus215 len=521 tot-seq=25

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>Consensus216 len=527 tot-seq=24

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>Consensus217 len=526 tot-seq=23

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>Consensus218 len=519 tot-seq=23

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>Consensus219 len=531 tot-seq=23

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>Consensus220 len=519 tot-seq=22

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>Consensus221 len=530 tot-seq=22

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>Consensus222 len=500 tot-seq=20

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>Consensus223 len=499 tot-seq=20

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>Consensus224 len=506 tot-seq=20

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>Consensus225 len=505 tot-seq=19

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>Consensus226 len=529 tot-seq=19

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>Consensus227 len=531 tot-seq=19

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>Consensus228 len=512 tot-seq=19

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>Consensus229 len=515 tot-seq=19

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>Consensus230 len=512 tot-seq=19

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>Consensus231 len=481 tot-seq=19

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SUPPLEMENTAL MATERIALS

Methods

Mouse genotyping

Genotyping for the Nod2 knockout allele was performed on mouse DNA extracted from tail clippings. Analysis of the Nod2 knockout allele was performed on genomic DNA extracted with REExtract-N-Amp extraction buffer (Sigma-Aldrich, St. Louis, MO), while genomic DNA extracted with 50 mM sodium hydroxide was used to determine the presence of the WT allele. PCR analysis identified *Nod2*^{-/-} mice based on the increased size of exon 3 in the disrupted *Nod2* gene (Suppl. Fig. 2). Specific primer sequences used were as follows: 1) WT allele: forward, 5'-ACAGAGATGCCGACACCATACTG-3'; reverse, 5'-TGGAGAAGGTTGAAGAGCAGAGTC-3'; and 2) Nod2 knockout allele: forward, 5'-TGACTGTGGCTAATGTCCTTTGTG-3'; reverse, 5'-TTCTATCGCCTTCTTGACGAGTTC-3'. Confirmation of the knockout allele was accomplished by sequencing the PCR genotyping products, which showed that the larger amplicon includes a thymidine kinase gene that is part of the engineered construct that was originally used to disrupt the Nod2 gene (Suppl. Figs. 2-3).[1]

Quantitative reverse-transcriptase polymerase chain reaction

Total RNA was extracted from ileal tissue using an RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's instructions. Complementary DNA was generated using SuperScript II reverse transcriptase (Invitrogen, Carlsbad,

CA). Quantitative RT-PCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) per manufacturer's instructions. Specific primer/probe sets were obtained from Applied Biosystems as follows: *Actb* (Mm00607939_s1), *Lyz* (Mm00727183_s1), *Ang4* (Mm03647554_g1), *Reg3 γ* (Mm00441127_m1), *Defa3* (Mm04205962_gH), *Defa5* (Mm00651548_g1), *Defa4* (Mm00651736_g1), *Defa20* (Mm00842045g1), and *Defa-rs10* (Mm00833275_g1). The following primer sets were utilized with SYBR Green PCR Master Mix (Applied Biosystems), per manufacturer's protocols: 1) Global α -defensins: forward, 5'-GGTGATCATCAGACCCCAGCATCAGT-3'; reverse, 5'-AAGAGACTAAACTGAGGA GCAGC-3'; 2) CRS1C: forward, 5'-TGCTCTTCAAGATGTAGCCCAACG-3'; reverse, 5'-TGGAGCTTGGGTGGTGATTGCA-3'; and 3) CRS4C: forward, 5'-GCATGGAATCTGGGTCAAGATAAC-3'; reverse, 5'-AGAAGGAAGAGCAATCAAGGCTAAG-3'.

Acid urea polyacrylamide gel electrophoresis

Samples were subject to 2 extraction steps using 60% acetonitrile, 1% trifluoroacetic acid (TFA), incubated at 4°C with rotation and clarification by centrifugation. Resulting supernatants were lyophilized, re-suspended and dialyzed in 5% acetic acid. Dialysates were then lyophilized, and 300 μ g protein aliquots were solubilized in 30 μ l of AU-PAGE loading solution (3 M urea, 5% acetic acid). These were next electrophoresed on a 12.5% AU-PAGE gel for 1 h

at 150 V and 4 h at 400 V, alongside a sample of recombinant α -defensin 4 provided by Dr. André Ouellette (University of Southern California). Resolved proteins were then visualized by staining with 0.05% Coomassie Brilliant Blue in 30% methanol and 15% formalin, followed by destaining in 25% methanol and 1% formalin.

Individual Coomassie-stained AU-PAGE bands were excised and destained twice in 50% acetonitrile, 25 mM ammonium bicarbonate at room temperature for 10 min. Lyophilized gel slices were extracted for protein with 0.5% TFA, 50% acetonitrile in two steps at room temperature for 10 min. Extracted protein was subsequently lyophilized and re-suspended in 0.1% TFA for analysis. Samples were submitted to the Proteomics Core Facility of the University of North Carolina at Chapel Hill School of Medicine for mass spectrometric analysis via matrix assisted laser desorption ionization-time of flight tandem mass spectrometry in the linear mode using α -cyano-4-hydrocinnamic acid for the matrix.

Immunohistochemistry

2 cm segments of terminal ileum were fixed in 10% phosphate-buffered formalin for 12 hr. The tissue segments were longitudinally embedded in paraffin and cut in 5 μ m sections for histological analysis. Sections were de-paraffinized in separate containers of fresh xylene for a total of 8 min. Rehydration was accomplished in a series of ethanol dilutions. Sections were then treated with 3% hydrogen peroxide to inhibit the action of endogenous peroxidase, blocked

with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h at 25°C, and incubated with 1:1500 rabbit polyclonal antibody to human lysozyme (Diagnostic BioSystems, Pleasanton, CA) in 3% BSA overnight at 4°C. Sections were subsequently incubated with 1:200 biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) in 1% BSA for 30 min at 25°C, and a 1:50 dilution of Avidin DH and biotinylated enzyme (Vector Laboratories, Inc.) in PBS for 30 min at 25°C. The sections were visualized with DAB chromogen reagent (Dako, Carpinteria, CA).

Bactericidal gel overlay assay

Bacterial strains *E. coli* NC101[2], *Listeria monocytogenes* 10403S, and *Salmonella enterica* serovar Typhimurium were grown to mid-log phase in trypticase soy broth (TSB) media, washed with 10 mM sodium citrate-phosphate buffer, re-suspended in warm 0.03% TSB, 1% low-melt agarose, 10 mM sodium citrate-phosphate buffer and 0.02% Tween 20 at 4×10^5 CFU/mL, and plated onto a Petri dish as a 1 mm deep undergel. Ileal protein samples (100 µg) were prepared by electrophoresis on a small-scale 12.5% AU-PAGE gel for 1 h at 150V. Resolved gels were washed with ice-cold 10 mM sodium phosphate buffer for 15 min, placed atop the bacteria-laden solid agarose layer, and incubated at 37°C for 3 h. Subsequently, the gel was removed and replaced with a layer of warm 6% TSB, 0.8% low-melt agarose to form a nutrient-rich overgel. Gel overlay plates were incubated overnight at 20°C and then imaged for band-associated zones of bacterial clearance.

Bacterial composition analyses

1) Bacterial DNA extraction – 100 mg of frozen tissue or feces was re-suspended in sterile lysis buffer (200 mM NaCl, 100 mM Tris [pH 8.0], 20 mM EDTA, 20 mg/ml lysozyme [Sigma-Aldrich, St. Louis, MO]) for 30 min at 37°C. This was then supplemented with 40 µl of proteinase K (20 mg/ml) and 85 µl of 10% SDS and incubated for 30 min at 65°C. Homogenization was accomplished by adding 300 mg of 0.1 mm zirconium beads (BioSpec Products, Bartlesville, OK) and bead beating for 2 min (BioSpec). Supernatants were then isolated and DNA extracted using phenol/chloroform/iso-amyl alcohol (25:24:1), followed by precipitation with absolute ethanol for 1 hr at -20°C. Finally, the precipitated DNA was cleaned up using a DNeasy Blood and Tissue extraction kit (Qiagen) per manufacturer's instructions.

2) 16S rRNA gene sequencing – Forward primers were tagged with 10 bp unique barcode labels at the 5' end along with the adaptor sequence to allow multiple samples to be included in a single 454 GS FLX Titanium sequencing plate.[3, 4] 16S rRNA PCR products were quantified, pooled, and purified for the sequencing reaction. 454 GS FLX Titanium sequencing was performed on a 454 Life Sciences Genome Sequencer FLX machine (Roche, Florence, SC) at the microbiome core at UNC-Chapel Hill.

3) Analysis of 16S rRNA sequences – Taxonomic and phylogenetic analyses of 16S rRNA sequence data generated by the 454 GS FLX Titanium sequencer were processed as follows. Sequences were removed if (i) they were less than 300 base-pairs, (ii) there was not an exact match to a 5' primer, or (iii)

there were any N's in the sequences. 135,982 sequences met all QC criteria. These sequences were clustered with the program AbundantOTU[5] which produced 257 OTUs representing 126,526 (93%) of the sequences. Consensus sequences were checked for chimeras with UCHIME[6] using the gold database (http://greengenes.lbl.gov/Download/Sequence_Data/Fasta_data_files/gold_strains_gg16S_aligned.fasta.gz) as reference. UCHIME detected 5 chimeras in the consensus sequences, for which the corresponding OTUs were removed from downstream analysis. To check for non-microbial contamination, consensus sequences were mapped with blastn to version 108 of the Silva 16S rRNA database (<http://www.arb-silva.de/>). All consensus sequences had a match of ≥ 295 basepairs with a percent identity $\geq 91\%$. We conclude that non-microbial contamination was not a significant problem in our dataset. OTUs were assigned to a taxonomy using the java-based stand-alone version of the RDP classifier (v. 2.4). Consensus sequences representing each OTU are given in supplemental File 1.

To correct for variation in total sequence counts between samples, the abundance of each OTU in a given sample was standardized by calculating the logged sequence abundance using the following formula:

$$\text{LOG}_{10} [(\text{Frequency} / \# \text{ sequences in sample}) * \text{Average \# of sequences per sample} + 1]$$

The logarithm was used to lessen the influence of more dominant OTUs. In order to take the log of zero, a pseudo-count (one sequence) is added to each sample for each taxa. In order to minimize the effect of this pseudo-count, all

samples are normalized to the average number of sequences per sample before the addition of the pseudo-count.

PCoAs were performed in the package Mothur 1.24.1 (using the program “pcoa”) based on Bray-Curtis dissimilarity defined as:

$$\frac{\sum_{k=1}^{k=n} |y_{ik} - y_{jk}|}{\sum_{k=1}^{k=n} (y_{ik} + y_{jk})}$$

where y_{ik} and y_{jk} are the log-transformed and normalized values for taxa k in samples i and j respectively and n is the number of taxa in all samples.

Supplemental Figure Legends

Supplemental Figure 1. *Breeding strategy used to ensure WT and Nod2^{-/-} littermates were reliably generated and utilized throughout the course of the study.* Littermates were generated from Nod2^{+/-} (purple) mice on a pure C57BL/6 background. WT (blue) or Nod2^{-/-} (red) mice were sacrificed, while Nod2^{+/-} (purple) mice were re-bred to produce additional littermates. The dashed line highlights the strategy of previous studies, in which littermates were used to start independently housed homozygous WT and Nod2^{-/-} lines.

Supplemental Figure 2. *WT, Nod2^{+/-}, and Nod2^{-/-} mice can be distinguished by PCR.* Agarose gel electrophoresis of PCR reactions of genomic DNA from eight mice: three WT (lanes 2-4), three Nod2^{+/-} (lanes 5-7), and two Nod2^{-/-} mice (lanes 8-9). The PCR reaction analyzed in lane 10 contained no template and therefore serves as a negative control.

Supplemental Figure 3. *Confirmation of WT and Nod2^{-/-} PCR products.* Above: DNA sequence alignment of the 348 bp WT allele amplicon (Suppl. Fig. 2, top row) with the corresponding sequence of exon 3 of the wild-type Nod2 gene from NCBI's assembly of the C57BL/6 mouse genome. The alignment was generated by the T-COFFEE DNA sequence alignment algorithm.[7] Alignment gaps are indicated by hyphens, indeterminate bases are denoted with "N", and non-identical aligned bases are identified with red textual coloring. Below: Similar DNA sequence alignment of the 776 bp of the 5' end of the 945 bp Nod2 knockout allele amplicon (Suppl. Fig. 2, bottom row). The presence of two canonical sequences for *loxP* sites are indicated by black outlined boxes in the Nod2 knockout allele amplicon sequence. The 3' end and orientation of a thymidine kinase (tk) gene is indicated by a black, bent arrow.

Supplemental Figure 4. *Variations in α -defensin mRNA and protein expression based on mouse background strain.* Transcript expression of total *CRS4C* (A, top) and the *CRS4C* isoform *Defa-rs10* (A, bottom) as well as *Defa4* (B) in WT 129, WT B6 and *Nod2^{-/-}* B6 ileal tissue (n=6-7 mice/group). Copy number is normalized to β -actin and expressed as a fold Δ relative to the WT 129 group. Data are shown as means with SEM. N.D. - not detected. (C) AU-PAGE of ileal protein from WT 129, WT B6 and *Nod2^{-/-}* B6 mice. The Paneth cell α -defensin region is indicated by the black outlined box. First lane is recombinant *Defa4* control; each additional lane represents a pooled sample from three mice.

Supplemental File 1. Consensus sequences representing each OTU in fasta format.

References

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Supplemental Figure 1.

Breeding strategy

Nod2^{+/+} × **Nod2^{-/-}**

Nod2^{+/+} Wild-type
Nod2^{+/-} Nod2-het
Nod2^{-/-} Nod2-null



Nod2^{+/-} **Nod2^{+/-}**
Nod2^{+/-} **Nod2^{+/-}**



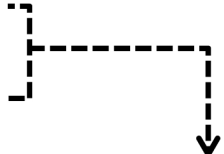
Nod2^{+/-} × **Nod2^{+/-}**

Re-bred to produce additional Nod2^{+/+} and Nod2^{-/-} littermates



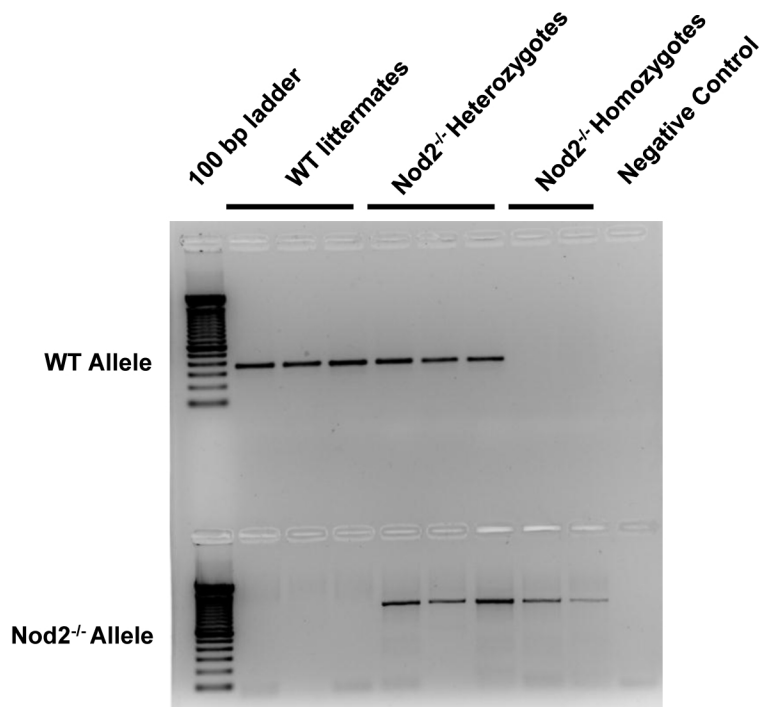
Nod2^{+/-} **Nod2^{+/-}** **Nod2^{+/-}** **Nod2^{+/-}**
Nod2^{+/+} **Nod2^{+/+}** **Nod2^{-/-}** **Nod2^{-/-}**

- weaned to separate cages by sex
- sacrificed at 8-12 wk
- allowed for housing of Nod2^{+/+} and Nod2^{-/-} in identical environmental conditions



- previous work (Rehman *et al.*, 2011) used Nod2^{+/+} and Nod2^{-/-} littermates to start homozygous inter-cross lines
- Nod2^{+/+} and Nod2^{-/-} mice were then housed separately, and hence not exposed to identical environmental conditions

Supplemental Figure 2.



Supplemental Figure 3.

WT PCR Product

WT PCR product 1 GTGGTGGGCGAA-CNGACAGTGGCAGG-NCACTACTTGCTGCAGCGTTTGACCTGCTGTGGGCAACAGGNNAGGAGCTT 78
 Nod2 gene 1 GTGGTGGGCGAAGCAGGCAGTGGCAAGAGCACT-C-TTCTGCAGCGTTTGACCTGCTGTGGGCAACAGGG-AGGAGCTT 77

WT PCR product 79 CCAGGTAGTTTCTCTTCATTTTCCATTCCAGCTGCCGACAGTTGCAATGCGTGGCCAAACCGCTGTCCCTGAGGACGCTG 158
 Nod2 gene 78 CCAGG-AGTTTCTCTTCATTTTCCATTCCAGCTGCCGACAGTTGCAATGCGTGGCCAAACCGCTGTCCCTGAGGACGCTG 156

WT PCR product 159 CTCTTTGAGCACTGCTGCTGGCCTGATGTCGCTCAGGACGATGTCTTCCAGTTCTTCTTGACCATCTGACCGTGTCTT 238
 Nod2 gene 157 CTCTTTGAGCACTGCTGCTGGCCTGATGTCGCTCAGGACGATGTCTTCCAGTTCTTCTTGACCATCTGACCGTGTCTT 236

WT PCR product 239 GTTAAACCTTTGATGGCTTGGACGAGTTCAAGTTCGGTTACCAGCCGGGAGCGCCACTGCTCTCCAATTGACCCACGT 318
 Nod2 gene 237 GTTAAACCTTTGATGGCTTGGACGAGTTCAAGTTCGGTTACCAGCCGGGAGCGCCACTGCTCTCCAATTGACCCACGT 316

WT PCR product 319 CAGTCCAGACTCTGCTCTTCAACCTTCTCC 348
 Nod2 gene 317 CAGTCCAGACTCTGCTCTTCAACCTTCTCC 346

Nod2^{-/-} PCR Product

Nod2^{-/-} PCR product 1 GGNCT-TACTGACT-GGTGGCATGCACCATCCTGTGTTAACCATATCAAAGTCCC GGCTGATTTCTGCTTTGCAG 78
 Nod2 gene 1 GATGCTA TACTGACTAGGTGGCATGCACCATCCTGTGTTAACCATATCAAAGTCCC GGCTATATTCTGCTTTGCAG 80

Nod2^{-/-} PCR product 79 GGAAGAACCAGGTTTAGGAAGCCCTGGGTCTGTCCCCAAACCCCTCAGCTTAGAGTGAAATAAGGCAGGACAGATGC 158
 Nod2 gene 81 GGAAGAACCAGGTTTAGGAAGCCCTGGGTCTGTCCCCAAACCCCTCAGCTTAGAGTGAAATAAGGCAGGACAGATGC 160

Nod2^{-/-} PCR product 159 CCACTAGGACAGATGCCTACTAGCCCTTCCACACATTGCATCCCCATGCAGGCTACTCTCTGTGTAGGTTCCCGTGTGA 238
 Nod2 gene 161 CCACTAGGACAGATGCCTACTAGCCCTTCCACACATTGCATCCCCATGCAGGCTACTCTCTGTGTAGGTTCCCGTGTGA 240

Nod2^{-/-} PCR product 239 CCGTGGTTCACCCCTCATTGTCCTTTGTGAG-GTGGCGGCG---GCCGAAGCTT TAACTTCGTATAGCATACTT 314
 Nod2 gene 241 CCGTGGTTCACCCCTCATTGTCCTTTGTATCCTAGCTGCTGAGTGTGAGAAAGTTCATATCTAAGCTGAGGAC-CATG 314

Nod2^{-/-} PCR product 315 ATACGAAGTTA-TGTCGACTTAG TAACTTCGTATAG---CATA--CATTAT-TCGAAGTTATAGCTTCGTGATGGA- 385
 Nod2 gene 320 GTGTTGACTCAGTCTCGCTTCCCTCAGTACTACGATGGGTCGGGAATCTTGCCTGGAGGATATATACACGGAGAACAT 399

Nod2^{-/-} PCR product 386 -TTGAACTTGGCAAACAATACTGAGAAT-GAAGTGTATGGAACAGAGG-CTGCTGATCTCGTTCTCAGGCTATGA 462
 Nod2 gene 400 CTTGGAGCT--GCAG-ACTGAAGTGGCACAGCCGGGCCCTGCAGAGAGCCCTGCATCTGGCCTGGAGGACCTC- 475

Nod2^{-/-} PCR product 463 AACTGACACATTGGAAACCACAGTCTTA---GAACCACAAGTGGGAATCAAGAGAAAACAAT-GATCCCACGAGAG 538
 Nod2 gene 476 -TTTGATACCCATGGTACCTGAAACAGAGATGCCACCACTAGTGGT---GGGCGAAGCAGGAGTGGCAAGAGCA 551

Nod2^{-/-} PCR product 539 ATCTA-TAGATCTATAGATCATGAGTGGAGGAAATGAGCTGCCCTTAATTTGGTTTGTCTTGTAAATATGATATCC 617
 Nod2 gene 552 CTCTCTGCAGCGTTTGACCTG-CTGTGGCAACAGGGAGGCTTCCAGGAGTTTCTC-----TTCATTTCCCATC 625

Nod2^{-/-} PCR product 618 AACTATGAAACATTTATCATAAAGCAATAGTAAAGGCCCTCAGTAAAGA-GCAGGCAATTTATCTAATCCACCCCA 696
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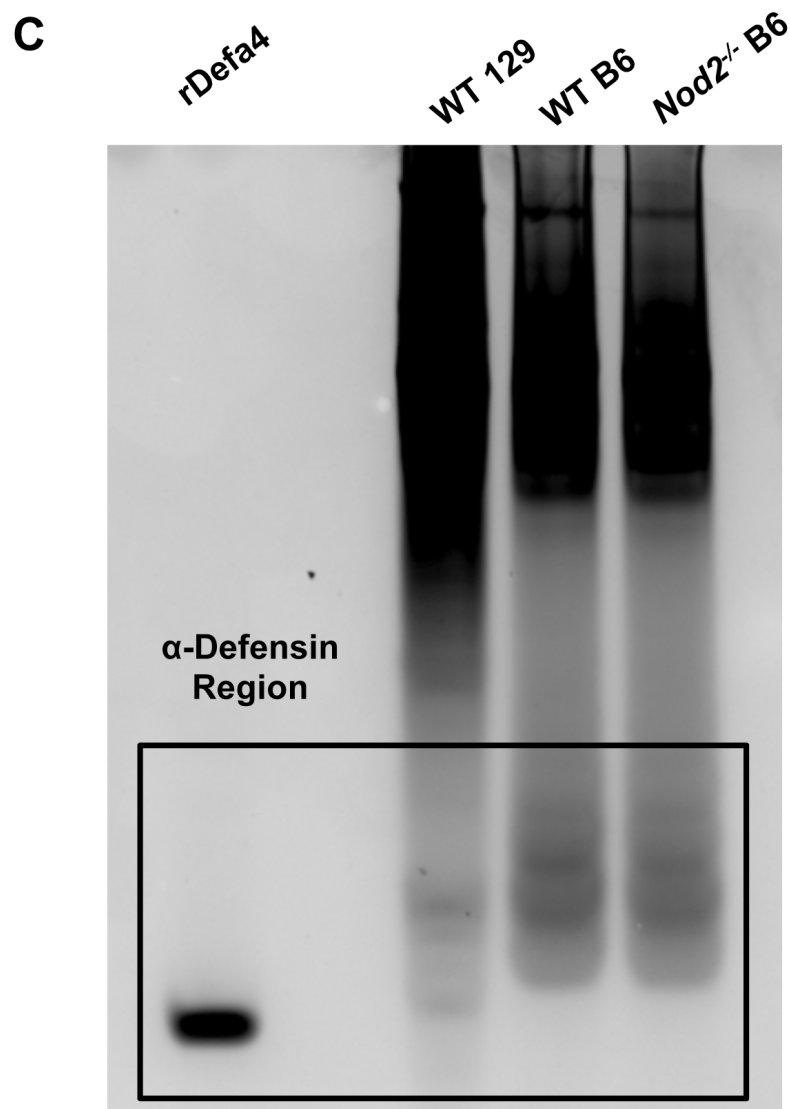
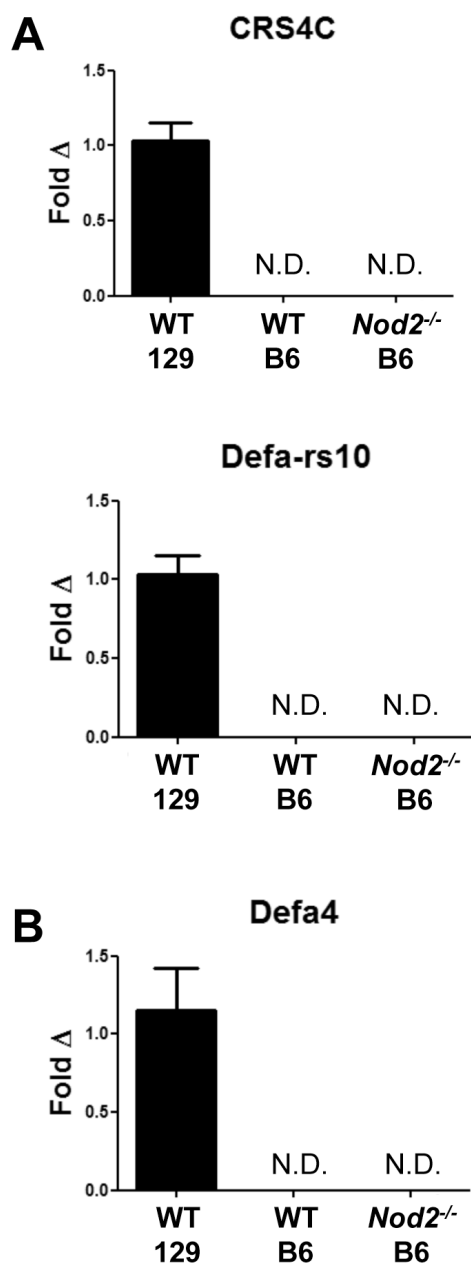
Nod2^{-/-} PCR product 697 CACCCCGTAGCTCCAATCTCCATTCAAATGAGGTACTGTTCTCACCCCTCTTAAACAAGTATGACAGGAAAA 776
 Nod2 gene 702 ATGTGCTCAGGACGATGCTTCCAGTTCCTTCTTACCATCTGACCGTGTCTT-GTTAAACCTTTGATGGCTTGGACGA 780

loxP site

loxP site

tk

Supplemental Figure 4.



	Mean	SD	95% CI	P-value
<i>Lyz</i>				
WT	0.94	0.21	0.78-1.10	0.94
<i>Nod2</i> ^{-/-}	0.93	0.22	0.78-1.09	
<i>Reg3γ</i>				
WT	0.97	0.42	0.65-1.30	0.74
<i>Nod2</i> ^{-/-}	0.92	0.25	0.71-1.12	
<i>Ang4</i>				
WT	1.12	0.43	0.86-1.52	0.49
<i>Nod2</i> ^{-/-}	1.35	0.53	0.96-1.72	
<i>PanCrp</i>				
WT	0.93	0.26	0.73-1.13	0.73
<i>Nod2</i> ^{-/-}	0.88	0.30	0.67-1.10	
<i>CRS1C</i>				
WT	0.96	0.28	0.74-1.18	0.04
<i>Nod2</i> ^{-/-}	0.73	0.12	0.64-0.82	
<i>CRS4C</i>				
WT	N.D.	-	-	-
<i>Nod2</i> ^{-/-}	N.D.	-	-	-
<i>Defa4</i>				
WT	N.D.	-	-	-
<i>Nod2</i> ^{-/-}	N.D.	-	-	-
<i>Defa20</i>				
WT	1.18	0.60	0.76-1.61	0.67
<i>Nod2</i> ^{-/-}	1.07	0.52	0.67-1.47	
<i>Defa3</i>				
WT	1.14	0.45	0.76-1.52	0.53
<i>Nod2</i> ^{-/-}	1.30	0.55	0.84-1.77	
<i>Defa5</i>				
WT	1.05	0.24	0.85-1.25	0.11
<i>Nod2</i> ^{-/-}	0.89	0.16	0.77-1.01	

Supplemental Table 1. *WT and Nod2*^{-/-} antimicrobial peptide mRNA expression levels. This table displays the mean, standard deviation (SD), 95% confidence interval (CI), and p-value (based on Student's t-test) for all antimicrobial peptide transcript levels generated in this study (n = 8-10 mice/group for WT and *Nod2*^{-/-} mice).

AU-PAGE Band	Observed m/z	Identified α -defensin	Theoretical m/z	Percent Identity
rDefa4				
1	3754.9551	Defa4	3756.51	99.96%
WT				
2	4076.2622	vDefa24-N	4079.90	99.91%
	4090.9565	vDefa24	4094.90	99.90%
	4317.0957	Defa5	4316.20	99.98%
3	4077.0854	vDefa24-N	4079.90	99.93%
	4091.0470	vDefa24	4094.90	99.91%
	4128.0332	Defa16	4134.92	99.83%
4	2475.1162	Defa20*	2475.34	99.99%
	4232.8076	vDefa23	4231.04	99.96%
	4248.2339	Defa2	4248.08	99.99%
5	4329.6270	Defa21	4330.11	99.99%
	4228.1084	vDefa23	4231.04	99.93%
	4244.2031	Defa2	4248.08	99.91%
6	4347.9673	Defa22	4344.19	99.91%
	4342.5874	Defa22	4344.19	99.96%
	Nod2^{-/-}			
7	4075.8882	vDefa24-N	4079.90	99.90%
	4092.0063	vDefa24	4094.90	99.93%
	4313.2397	Defa5	4316.20	99.93%
8	4076.0728	vDefa24-N	4079.90	99.91%
	4092.0823	vDefa24	4094.90	99.93%
	4133.0376	Defa16	4134.92	99.95%
9	2483.5461	Defa20*	2475.34	99.67%
	4230.8179	vDefa23	4231.04	99.99%
	4244.3823	Defa2	4248.08	99.91%
10	4330.0146	Defa21	4330.11	99.99%
	4229.1157	vDefa23	4231.04	99.95%
	4246.1221	Defa2	4248.08	99.95%
11	4345.3413	Defa22	4344.19	99.97%
	4346.5649	Defa22	4344.19	99.95%

Supplemental Table 2. *WT and Nod2^{-/-} mice express the same set of Paneth cell α -defensins.* Observed mass-to-charge (m/z) values of prominent, software-indicated mass peaks found within the expected cryptdin mass range (2499.0 to 4518.0 m/z) are tabulated in correspondence to the gel bands labeled in Fig.2E. The observed m/z values are listed in increasing value. Determination of the

cryptdin identity of individual m/z values was assisted by a comparison of the observed m/z values with the calculated m/z values of all purified and transcript- and gene-predicted cryptdin peptides based upon their oxidized, singly-protonated forms. Matches of m/z values with greater than 99.6% identity or with mass differences less than 5 Da were deemed sufficient for individual identification of mass peaks. Percent identity of m/z values was calculated from the quotient of the lesser over the greater m/z values of matched pairs. The labels vDefa23 and vDefa24 are used for various non-identical masses since there are multiple Defa23 and Defa24 variant transcripts that have yet to acquire unambiguous identifiers. Note that the identification of Defa20 was extrapolated by the presence of an m/z value that was more than 98% identical to the predicted m/z value of the doubly-protonated form of oxidized Defa20, since mass spectrometric analysis was performed outside the mass range of its predicted, singly-protonated-based m/z value (4950.69).

OTU #	RDP Output	WT	Nod2 ⁻	P-Value	MTC
Consensus198	Bacteroidetes(100);Bacteroidia(85);Bacteroidales(85);Porphyromonadaceae(78);Butyricimonas(21)	0.33	0.08	5.67E-04	0.12
Consensus106	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Porphyromonadaceae(100);Parabacteroides(100)	0.35	0.73	2.27E-03	0.25
Consensus162	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(98);Syntrophococcus(12)	0.10	0.42	3.88E-02	2.83
Consensus31	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Prevotellaceae(100);Prevotella(58)	1.31	1.09	4.32E-02	2.37
Consensus45	Bacteroidetes(100);Bacteroidia(86);Bacteroidales(86);Porphyromonadaceae(84);Paludibacter(28)	0.01	0.51	4.47E-02	1.96
Consensus163	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Pseudoflavonifractor(6)	0.24	0.47	0.06	2.03
Consensus129	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Pseudoflavonifractor(68)	0.30	0.54	0.07	2.05
Consensus155	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiracea_incertae_sedis(24)	0.14	0.41	0.07	2.00
Consensus200	TM7(100);TM7_genera_incertae_sedis(100)	0.29	0.14	0.08	1.85
Consensus187	Firmicutes(83);Clostridia(83);Clostridiales(83);Ruminococcaceae(53);Papillibacter(15)	0.00	0.20	0.08	1.72
Consensus189	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Flavonifractor(71)	0.11	0.32	0.08	1.56
Consensus53	Bacteroidetes(100);Bacteroidia(94);Bacteroidales(94);Prevotellaceae(92);Paraprevotella(44)	0.00	0.40	0.08	1.51
Consensus108	Bacteroidetes(99);Bacteroidia(93);Bacteroidales(93);Porphyromonadaceae(92);Butyricimonas(5)	0.48	0.63	0.09	1.51
Consensus97	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Rikenellaceae(100);Alistipes(100)	0.64	0.39	0.09	1.43
Consensus70	Bacteroidetes(100);Bacteroidia(98);Bacteroidales(98);Porphyromonadaceae(87);Barnesiella(36)	0.83	0.63	0.10	1.43
Consensus95	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiracea_incertae_sedis(58)	0.71	0.52	0.10	1.39
Consensus44	Proteobacteria(100);Epsilonproteobacteria(100);Campylobacteriales(100);Helicobacteraceae(100);Helicobacter(100)	0.66	0.96	0.10	1.31
Consensus185	Bacteroidetes(100);Bacteroidia(91);Bacteroidales(91);Porphyromonadaceae(86);Paludibacter(17)	0.00	0.16	0.11	1.35
Consensus62	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Clostridium_XIVa(32)	0.54	0.84	0.11	1.31
Consensus220	Bacteroidetes(100);Bacteroidia(86);Bacteroidales(86);Porphyromonadaceae(71);Tannerella(28)	0.20	0.08	0.12	1.32
Consensus194	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(94);Marvinbryantia(33)	0.10	0.29	0.12	1.26
Consensus43	Firmicutes(98);Clostridia(98);Clostridiales(98);Ruminococcaceae(49);Sporobacter(9)	1.02	0.76	0.13	1.31
Consensus13	Bacteroidetes(100);Bacteroidia(89);Bacteroidales(89);Prevotellaceae(76);Hallella(36)	1.72	1.36	0.14	1.30
Consensus9	Bacteroidetes(100);Bacteroidia(91);Bacteroidales(91);Porphyromonadaceae(82);Butyricimonas(41)	2.01	1.86	0.14	1.27
Consensus94	Bacteroidetes(100);Bacteroidia(75);Bacteroidales(75);Porphyromonadaceae(69);Barnesiella(21)	0.71	0.52	0.14	1.22
Consensus145	Actinobacteria(100);Actinobacteria(100);Coriobacteridae(100);Coriobacteriales(100);Coriobacterineae(100);Coriobacteriaceae(100);Slackia(37)	0.33	0.48	0.14	1.17

Consensus142	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(97);Moryella(1)	0.46	0.24	0.14	1.13
Consensus22	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Porphyromonadaceae(99);Barnesiella(59)	1.48	1.39	0.15	1.14
Consensus110	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiraceae_incertae_sedis(38)	0.07	0.28	0.15	1.13
Consensus65	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiraceae_incertae_sedis(37)	0.81	0.57	0.15	1.10
Consensus15	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiraceae_incertae_sedis(41)	1.30	1.58	0.15	1.08
Consensus157	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Oscillibacter(99)	0.28	0.46	0.16	1.07
Consensus144	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(97);Syntrophococcus(69)	0.31	0.49	0.16	1.05
Consensus118	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Coprococcus(14)	0.58	0.35	0.16	1.06
Consensus80	Bacteroidetes(100);Bacteroidia(83);Bacteroidales(83);Porphyromonadaceae(66);Barnesiella(38)	0.74	0.56	0.17	1.05
Consensus4	Root(100);Bacteria(99);Bacteroidetes(73);Sphingobacteria(31);Sphingobacteriales(31);Cyclobacteriaceae(9);Aq uiflexum(7)	2.38	2.29	0.18	1.12
Consensus77	Firmicutes(84);Erysipelotrichia(8);Erysipelotrichales(8);Erysipelotrichaceae(8);Sharpea(33)	0.36	0.59	0.19	1.14
Consensus176	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lactonifactor(15)	0.32	0.15	0.20	1.13
Consensus211	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Syntrophococcus(3)	0.16	0.28	0.20	1.11
Consensus123	Firmicutes(78);Clostridia(72);Clostridiales(68);Veillonellaceae(33);Anaerovibrio(1)	0.34	0.53	0.21	1.14
Consensus41	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Clostridium XIVa(41)	0.80	1.02	0.22	1.17
Consensus16	Bacteroidetes(100);Bacteroidia(79);Bacteroidales(79);Porphyromonadaceae(69);Barnesiella(3)	1.58	1.66	0.22	1.15
Consensus205	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiraceae_incertae_sedis(44)	0.11	0.23	0.23	1.15
Consensus125	Firmicutes(100);Clostridia(99);Clostridiales(99);Lachnospiraceae(97);Robinsoniella(26)	0.33	0.20	0.24	1.19
Consensus127	Firmicutes(99);Clostridia(99);Clostridiales(99);Clostridiales_Incertae Sedis XIII(53);Anaerovorax(38)	0.44	0.30	0.25	1.20
Consensus85	Bacteroidetes(100);Bacteroidia(82);Bacteroidales(82);Porphyromonadaceae(81);Tannerella(35)	0.65	0.44	0.25	1.18
Consensus88	Bacteroidetes(98);Bacteroidia(9);Bacteroidales(9);Porphyromonadaceae(79);Barnesiella(33)	0.68	0.56	0.25	1.17
Consensus178	Actinobacteria(100);Actinobacteria(100);Coriobacteridae(100);Coriobacteriales(100);Coriobacterineae(100);Cori obacteriaceae(100);Olsenella(75)	0.11	0.24	0.25	1.15
Consensus18	Bacteroidetes(98);Bacteroidia(83);Bacteroidales(83);Porphyromonadaceae(75);Paludibacter(2)	1.57	1.39	0.25	1.12
Consensus202	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Flavonifractor(64)	0.19	0.32	0.25	1.11
Consensus136	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Flavonifractor(59)	0.30	0.43	0.25	1.09
Consensus139	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiraceae_incertae_sedis(33)	0.30	0.45	0.26	1.08
Consensus175	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(96);Lachnospiraceae_incertae_sedis(31)	0.09	0.23	0.27	1.12

Consensus66	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(96);Anaerostipes(71)	0.73	0.86	0.27	1.11
Consensus210	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Bacteroidaceae(100);Bacteroides(100)	0.19	0.29	0.27	1.09
Consensus128	Firmicutes(98);Erysipelotrichia(93);Erysipelotrichales(93);Erysipelotrichaceae(93);Erysipelotrichaceae_incertae_sedis(9)	0.21	0.36	0.28	1.11
Consensus2	Bacteroidetes(100);Bacteroidia(51);Bacteroidales(51);Porphyromonadaceae(35);Proteiniphilum(15)	2.50	2.63	0.29	1.11
Consensus69	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Butyrivibrio(26)	0.76	0.55	0.29	1.10
Consensus102	Proteobacteria(89);Alphaproteobacteria(87);Kiloniellales(16);Kiloniellaceae(16);Kiloniella(16)	0.35	0.50	0.29	1.08
Consensus26	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Roseburia(55)	0.94	1.19	0.29	1.07
Consensus212	Bacteroidetes(100);Bacteroidia(96);Bacteroidales(96);Porphyromonadaceae(85);Tannerella(21)	0.30	0.19	0.30	1.08
Consensus114	Firmicutes(99);Clostridia(99);Clostridiales(99);Lachnospiraceae(97);Robinsoniella(49)	0.43	0.25	0.31	1.09
Consensus133	Bacteroidetes(100);Bacteroidia(72);Bacteroidales(72);Porphyromonadaceae(45);Tannerella(17)	0.37	0.26	0.31	1.07
Consensus170	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Robinsoniella(22)	0.12	0.26	0.32	1.08
Consensus217	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Oscillibacter(98)	0.22	0.12	0.32	1.07
Consensus38	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Bacteroidaceae(100);Bacteroides(100)	1.00	1.12	0.33	1.10
Consensus74	Bacteroidetes(99);Bacteroidia(65);Bacteroidales(65);Porphyromonadaceae(41);Butyricimonas(27)	0.64	0.78	0.33	1.09
Consensus167	Firmicutes(85);Clostridia(85);Clostridiales(85);Peptococcaceae_1(44);Peptococcus(44)	0.12	0.24	0.34	1.09
Consensus33	Firmicutes(98);Clostridia(98);Clostridiales(98);Ruminococcaceae(97);Clostridium_IV(56)	0.83	1.02	0.35	1.10
Consensus124	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Dorea(42)	0.36	0.23	0.35	1.10
Consensus98	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Pseudoflavonifractor(4)	0.58	0.67	0.35	1.09
Consensus93	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Clostridium_XIVa(48)	0.50	0.65	0.35	1.08
Consensus27	TM7(100);TM7_genera_incertae_sedis(100)	1.41	1.32	0.36	1.07
Consensus203	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Hydrogenoanaerobacterium(43)	0.17	0.26	0.36	1.06
Consensus158	Bacteroidetes(99);Bacteroidia(6);Bacteroidales(6);Porphyromonadaceae(36);Tannerella(1)	0.31	0.39	0.36	1.05
Consensus164	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(97);Acetitomaculum(49)	0.40	0.30	0.36	1.05
Consensus140	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Coprococcus(12)	0.26	0.12	0.37	1.05
Consensus83	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiraceae_incertae_sedis(64)	0.30	0.49	0.37	1.04
Consensus61	Bacteroidetes(99);Bacteroidia(71);Bacteroidales(71);Porphyromonadaceae(69);Barnesiella(32)	0.71	0.83	0.37	1.03
Consensus82	Bacteroidetes(99);Flavobacteria(47);Flavobacteriales(47);Cryomorphaceae(47);Fluviicola(46)	0.47	0.63	0.37	1.02

Consensus149	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Syntrophococcus(6)	0.32	0.20	0.38	1.02
Consensus172	Bacteroidetes(100);Bacteroidia(7);Bacteroidales(7);Porphyromonadaceae(62);Tannerella(9)	0.28	0.37	0.38	1.02
Consensus219	Bacteroidetes(100);Bacteroidia(94);Bacteroidales(94);Porphyromonadaceae(87);Butyricimonas(48)	0.12	0.21	0.39	1.02
Consensus195	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Butyrivibrio(5)	0.28	0.19	0.39	1.01
Consensus232	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Oscillibacter(77)	0.17	0.08	0.39	1.01
Consensus51	Bacteroidetes(100);Bacteroidia(77);Bacteroidales(77);Porphyromonadaceae(7);Barnesiella(25)	0.88	0.75	0.39	1.00
Consensus20	Bacteroidetes(100);Bacteroidia(98);Bacteroidales(98);Porphyromonadaceae(86);Tannerella(51)	1.47	1.56	0.39	0.99
Consensus11	Bacteroidetes(100);Bacteroidia(81);Bacteroidales(81);Porphyromonadaceae(81);Paludibacter(31)	1.67	1.59	0.40	0.98
Consensus71	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Anaerotruncus(95)	0.67	0.80	0.40	0.98
Consensus89	Firmicutes(100);Bacilli(100);Bacillales(100);Staphylococcaceae(100);Staphylococcus(100)	0.29	0.16	0.40	0.97
Consensus100	Firmicutes(89);Clostridia(83);Clostridiales(83);Lachnospiraceae(17);Cellulosilyticum(1)	0.43	0.55	0.40	0.97
Consensus1	Firmicutes(100);Bacilli(100);Lactobacillales(100);Lactobacillaceae(100);Lactobacillus(100)	2.60	2.48	0.40	0.96
Consensus207	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Butyricoccus(100)	0.16	0.07	0.40	0.95
Consensus109	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(98);Robinsoniella(39)	0.50	0.37	0.40	0.94
Consensus204	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiraceae_incertae_sedis(71)	0.16	0.26	0.41	0.94
Consensus5	Firmicutes(100);Bacilli(100);Lactobacillales(100);Lactobacillaceae(100);Lactobacillus(100)	1.98	1.81	0.41	0.93
Consensus161	Proteobacteria(79);Deltaproteobacteria(48);Bdellovibrionales(32);Bdellovibrionaceae(32);Vampirovibrio(32)	0.33	0.41	0.42	0.95
Consensus192	Firmicutes(68);Bacilli(39);Bacillales(27);Bacillaceae_2(17);Paucisalibacillus(1)	0.25	0.13	0.42	0.94
Consensus47	Firmicutes(100);Bacilli(100);Lactobacillales(100);Lactobacillaceae(100);Lactobacillus(100)	0.89	0.75	0.42	0.93
Consensus8	Bacteroidetes(100);Bacteroidia(9);Bacteroidales(9);Porphyromonadaceae(76);Butyricimonas(18)	1.97	1.90	0.43	0.93
Consensus76	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Oscillibacter(100)	0.57	0.70	0.43	0.94
Consensus165	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(95);Clostridium_XIVa(33)	0.02	0.13	0.44	0.93
Consensus150	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Syntrophococcus(89)	0.41	0.31	0.45	0.95
Consensus30	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(89);Acetitomaculum(2)	0.85	0.68	0.45	0.96
Consensus36	Root(100);Bacteria(99);Bacteroidetes(96);Bacteroidia(63);Bacteroidales(63);Porphyromonadaceae(48);Proteiniphilum(23)	1.07	1.15	0.46	0.95
Consensus148	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Rikenellaceae(100);Alistipes(100)	0.36	0.27	0.47	0.97
Consensus214	Bacteroidetes(99);Bacteroidia(94);Bacteroidales(94);Porphyromonadaceae(78);Butyricimonas(29)	0.24	0.17	0.48	0.97

Consensus42	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(96);Robinsoniella(12)	0.74	0.58	0.48	0.98
Consensus6	Firmicutes(91);Erysipelotrichia(85);Erysipelotrichales(85);Erysipelotrichaceae(85);Allobaculum(63)	1.53	1.70	0.48	0.97
Consensus143	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Oscillibacter(100)	0.42	0.34	0.48	0.97
Consensus64	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Syntrophococcus(62)	0.15	0.29	0.49	0.97
Consensus215	Firmicutes(99);Clostridia(99);Clostridiales(99);Ruminococcaceae(99);Acetanaerobacterium(49)	0.21	0.14	0.49	0.96
Consensus21	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Butyrivibrio(52)	1.27	1.41	0.49	0.95
Consensus169	Bacteroidetes(100);Bacteroidia(93);Bacteroidales(93);Porphyromonadaceae(87);Tannerella(39)	0.34	0.26	0.49	0.95
Consensus156	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Robinsoniella(29)	0.34	0.25	0.50	0.94
Consensus34	Bacteroidetes(93);Sphingobacteria(47);Sphingobacteriales(47);Cytophagaceae(26);Meniscus(25)	1.03	1.11	0.50	0.95
Consensus87	Bacteroidetes(99);Bacteroidia(97);Bacteroidales(97);Porphyromonadaceae(91);Barnesiella(57)	0.67	0.59	0.51	0.95
Consensus190	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(98);Clostridium XIVb(98)	0.20	0.27	0.51	0.94
Consensus132	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiracea_incertae_sedis(84)	0.47	0.53	0.52	0.96
Consensus216	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Clostridium XIVa(43)	0.18	0.23	0.53	0.96
Consensus226	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Marvinbryantia(46)	0.10	0.16	0.53	0.96
Consensus91	Bacteroidetes(100);Bacteroidia(84);Bacteroidales(84);Porphyromonadaceae(79);Tannerella(28)	0.62	0.70	0.53	0.95
Consensus12	Bacteroidetes(100);Bacteroidia(94);Bacteroidales(94);Porphyromonadaceae(79);Barnesiella(34)	1.69	1.63	0.53	0.95
Consensus37	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Rikenellaceae(100);Alistipes(100)	1.09	1.00	0.54	0.95
Consensus119	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Coprococcus(18)	0.32	0.41	0.54	0.95
Consensus39	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Porphyromonadaceae(100);Parabacteroides(100)	0.98	1.07	0.55	0.95
Consensus113	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Clostridium XIVa(53)	0.23	0.32	0.55	0.94
Consensus179	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Porphyromonadaceae(100);Odoribacter(99)	0.33	0.28	0.55	0.94
Consensus182	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Rikenellaceae(100);Alistipes(100)	0.30	0.24	0.56	0.96
Consensus160	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Robinsoniella(34)	0.38	0.30	0.58	0.97
Consensus84	Bacteroidetes(100);Bacteroidia(93);Bacteroidales(93);Porphyromonadaceae(78);Tannerella(26)	0.65	0.72	0.58	0.97
Consensus181	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Prevotellaceae(91);Paraprevotella(89)	0.19	0.26	0.58	0.96
Consensus28	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Anaerostipes(32)	1.34	1.27	0.59	0.97
Consensus19	Bacteroidetes(100);Bacteroidia(97);Bacteroidales(97);Porphyromonadaceae(86);Barnesiella(52)	1.39	1.47	0.59	0.97

Consensus104	Bacteroidetes(100);Bacteroidia(97);Bacteroidales(97);Porphyromonadaceae(76);Barnesiella(49)	0.31	0.22	0.60	0.97
Consensus58	Firmicutes(100);Clostridia(99);Clostridiales(99);Lachnospiraceae(99);Acetomaculum(4)	0.64	0.74	0.60	0.96
Consensus188	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Robinsoniella(39)	0.19	0.26	0.60	0.96
Consensus49	Bacteroidetes(100);Bacteroidia(86);Bacteroidales(86);Porphyromonadaceae(82);Barnesiella(42)	0.90	0.95	0.61	0.97
Consensus3	Firmicutes(63);Clostridia(57);Clostridiales(56);Clostridiales_Incertae Sedis XI(25);Finegoldia(17)	0.50	0.41	0.62	0.97
Consensus131	Actinobacteria(100);Actinobacteria(100);Coriobacteridae(100);Coriobacteriales(100);Coriobacterineae(100);Coriobacteriaceae(100);Enterorhabdus(100)	0.40	0.44	0.62	0.97
Consensus168	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(98);Anaerotruncus(45)	0.29	0.23	0.63	0.97
Consensus17	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100);Erysipelotrichaceae(100);Turicibacter(100)	0.68	0.84	0.63	0.96
Consensus134	Firmicutes(99);Clostridia(99);Clostridiales(99);Ruminococcaceae(99);Acetanaerobacterium(37)	0.37	0.43	0.63	0.96
Consensus177	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiraceae_incertae_sedis(4)	0.27	0.21	0.63	0.97
Consensus180	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Rikenellaceae(100);Alistipes(99)	0.30	0.35	0.64	0.97
Consensus59	Bacteroidetes(100);Bacteroidia(96);Bacteroidales(96);Porphyromonadaceae(95);Barnesiella(42)	0.84	0.77	0.65	0.97
Consensus184	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Roseburia(79)	0.18	0.22	0.66	0.98
Consensus23	Firmicutes(99);Erysipelotrichia(92);Erysipelotrichales(92);Erysipelotrichaceae(92);Allobaculum(55)	0.90	1.01	0.66	0.98
Consensus92	Bacteroidetes(100);Bacteroidia(94);Bacteroidales(94);Porphyromonadaceae(91);Tannerella(3)	0.57	0.63	0.66	0.97
Consensus126	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(96);Syntrophococcus(28)	0.43	0.37	0.67	0.97
Consensus81	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Anaerotruncus(57)	0.65	0.60	0.67	0.97
Consensus183	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiraceae_incertae_sedis(42)	0.28	0.23	0.67	0.96
Consensus154	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiraceae_incertae_sedis(75)	0.42	0.37	0.67	0.96
Consensus201	Firmicutes(94);Clostridia(83);Clostridiales(83);Peptococcaceae 1(11);Desulfonispota(1)	0.15	0.19	0.68	0.96
Consensus221	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Coprococcus(43)	0.13	0.17	0.68	0.96
Consensus196	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Syntrophococcus(68)	0.19	0.23	0.68	0.96
Consensus152	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Bacteroidaceae(100);Bacteroides(100)	0.29	0.23	0.69	0.96
Consensus137	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Oscillibacter(71)	0.38	0.43	0.69	0.96
Consensus230	Firmicutes(100);Erysipelotrichia(100);Erysipelotrichales(100);Erysipelotrichaceae(100);Coprobacillus(84)	0.09	0.12	0.69	0.95
Consensus78	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Rikenellaceae(100);Alistipes(100)	0.63	0.69	0.71	0.97
Consensus171	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Clostridium XIVa(66)	0.29	0.24	0.72	0.98

Consensus141	Firmicutes(100);Clostridia(100);Clostridiales(100);Clostridiaceae 1(100);Clostridium sensu stricto(100)	0.11	0.15	0.73	0.98
Consensus121	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(98);Robinsoniella(35)	0.33	0.28	0.73	0.98
Consensus147	Bacteroidetes(100);Bacteroidia(99);Bacteroidales(99);Rikenellaceae(99);Alistipes(99)	0.34	0.30	0.74	0.99
Consensus186	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(99);Anaerotruncus(43)	0.23	0.27	0.74	0.98
Consensus101	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Robinsoniella(29)	0.50	0.44	0.74	0.98
Consensus14	Bacteroidetes(100);Bacteroidia(88);Bacteroidales(88);Porphyromonadaceae(77);Tannerella(34)	1.52	1.56	0.75	0.98
Consensus96	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Pseudoflavonifractor(64)	0.55	0.51	0.75	0.98
Consensus25	Bacteroidetes(99);Bacteroidia(9);Bacteroidales(9);Porphyromonadaceae(8);Paludibacter(18)	1.39	1.36	0.75	0.97
Consensus99	Firmicutes(99);Clostridia(99);Clostridiales(99);Lachnospiraceae(91);Syntrophococcus(6)	0.52	0.47	0.77	0.99
Consensus120	Firmicutes(91);Clostridia(91);Clostridiales(91);Peptococcaceae 1(2);Peptococcus(2)	0.27	0.31	0.77	0.99
Consensus35	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Anaerostipes(28)	0.75	0.82	0.77	0.98
Consensus67	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(97);Ruminococcus(96)	0.61	0.65	0.78	0.98
Consensus173	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Butyricoccus(99)	0.22	0.26	0.78	0.98
Consensus63	Firmicutes(100);Clostridia(87);Clostridiales(87);Lachnospiraceae(82);Lachnobacterium(16)	0.68	0.62	0.78	0.98
Consensus159	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Clostridium XIVa(9)	0.37	0.33	0.79	0.99
Consensus10	Bacteroidetes(100);Bacteroidia(96);Bacteroidales(96);Porphyromonadaceae(76);Barnesiella(32)	1.79	1.76	0.81	1.00
Consensus55	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(97);Robinsoniella(47)	0.66	0.62	0.81	1.00
Consensus193	Bacteroidetes(100);Bacteroidia(76);Bacteroidales(76);Rikenellaceae(72);Rikenella(48)	0.25	0.28	0.81	0.99
Consensus52	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiracea_incertae_sedis(56)	0.22	0.16	0.81	0.99
Consensus56	Bacteroidetes(100);Sphingobacteria(34);Sphingobacteriales(34);Cytophagaceae(33);Meniscus(33)	0.73	0.77	0.82	0.99
Consensus166	Bacteroidetes(99);Bacteroidia(88);Bacteroidales(88);Porphyromonadaceae(8);Barnesiella(37)	0.26	0.29	0.82	0.99
Consensus227	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Pseudoflavonifractor(91)	0.15	0.17	0.82	0.98
Consensus29	Firmicutes(92);Clostridia(82);Clostridiales(82);Lachnospiraceae(47);Sporobacterium(34)	0.93	0.88	0.82	0.98
Consensus213	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(99);Anaerotruncus(58)	0.16	0.18	0.84	0.99
Consensus112	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(92);Coprococcus(19)	0.51	0.48	0.84	0.99
Consensus174	Firmicutes(99);Clostridia(99);Clostridiales(99);Lachnospiraceae(99);Lachnospiracea_incertae_sedis(31)	0.28	0.26	0.86	1.00
Consensus75	Bacteroidetes(100);Sphingobacteria(37);Sphingobacteriales(37);Cytophagaceae(32);Meniscus(29)	0.70	0.72	0.87	1.02

Consensus115	Bacteroidetes(100);Bacteroidia(52);Bacteroidales(52);Porphyromonadaceae(35);Tannerella(11)	0.51	0.53	0.88	1.02
Consensus135	Firmicutes(6);Erysipelotrichia(31);Erysipelotrichales(31);Erysipelotrichaceae(31);Allobaculum(26)	0.38	0.40	0.88	1.01
Consensus223	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Butyrivibrio(77)	0.18	0.16	0.88	1.01
Consensus68	Bacteroidetes(100);Bacteroidia(97);Bacteroidales(97);Porphyromonadaceae(85);Barnesiella(44)	0.77	0.79	0.89	1.01
Consensus40	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Lachnospiracea_incertae_sedis(31)	0.62	0.65	0.89	1.01
Consensus146	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Pseudoflavonifractor(77)	0.36	0.38	0.90	1.02
Consensus191	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Acetitomaculum(25)	0.23	0.24	0.90	1.01
Consensus60	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(94);Robinsoniella(27)	0.60	0.62	0.90	1.01
Consensus151	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiracea_incertae_sedis(47)	0.31	0.32	0.92	1.02
Consensus72	Bacteroidetes(100);Bacteroidia(97);Bacteroidales(97);Porphyromonadaceae(97);Barnesiella(46)	0.69	0.67	0.93	1.02
Consensus228	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Clostridium IV(69)	0.12	0.13	0.93	1.03
Consensus111	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Oscillibacter(75)	0.33	0.34	0.94	1.03
Consensus225	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Oscillibacter(82)	0.14	0.15	0.94	1.02
Consensus117	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Clostridium XIVa(38)	0.47	0.47	0.94	1.02
Consensus105	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Anaerotruncus(98)	0.49	0.50	0.95	1.02
Consensus54	Proteobacteria(100);Betaproteobacteria(100);Burkholderiales(100);Sutterellaceae(100);Parasutterella(100)	0.75	0.76	0.95	1.02
Consensus32	Bacteroidetes(100);Bacteroidia(84);Bacteroidales(84);Porphyromonadaceae(77);Tannerella(38)	0.30	0.32	0.95	1.02
Consensus103	Firmicutes(100);Clostridia(100);Clostridiales(99);Lachnospiraceae(81);Lachnobacterium(2)	0.49	0.48	0.95	1.01
Consensus90	Firmicutes(88);Clostridia(88);Clostridiales(88);Ruminococcaceae(46);Anaerotruncus(16)	0.64	0.65	0.97	1.02
Consensus79	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(99);Robinsoniella(76)	0.62	0.62	0.97	1.02
Consensus130	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiracea_incertae_sedis(53)	0.32	0.31	0.98	1.02
Consensus122	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(97);Robinsoniella(48)	0.37	0.37	0.98	1.02
Consensus50	Bacteroidetes(99);Bacteroidia(71);Bacteroidales(71);Rikenellaceae(16);Rikenella(16)	0.88	0.88	0.98	1.01
Consensus24	Bacteroidetes(100);Bacteroidia(72);Bacteroidales(72);Marinilabiaceae(12);Anaerophaga(12)	1.22	1.23	0.98	1.01
Consensus46	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Marvinbryantia(95)	0.82	0.83	0.98	1.00
Consensus116	Bacteroidetes(100);Bacteroidia(100);Bacteroidales(100);Bacteroidaceae(100);Bacteroides(100)	0.50	0.50	0.98	1.01
Consensus48	Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Lachnospiracea_incertae_sedis(53)	0.69	0.70	0.99	1.01

Consensus209	Bacteroidetes(100);Bacteroidia(98);Bacteroidales(98);Porphyromonadaceae(95);Odoribacter(88)	0.13	0.13	0.99	1.01
Consensus206	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Oscillibacter(83)	0.21	0.21	0.99	1.00
Consensus86	Firmicutes(97);Clostridia(95);Clostridiales(95);Lachnospiraceae(85);Sporobacterium(24)	0.33	0.32	0.99	1.00
Consensus73	Firmicutes(100);Clostridia(100);Clostridiales(100);Ruminococcaceae(100);Oscillibacter(99)	0.66	0.66	1.00	1.00

Supplemental Table 3. *OTU distribution in WT and Nod2^{-/-} mice.* For all OTUs with at least 25 sequences observed in stool samples, this table demonstrates the results of a t-test in which the null hypothesis that the distribution of the OTU is the same in WT and *Nod2^{-/-}* animals. The “OTU” column is an identifier which can be used to map the exact sequence of the OTU using the supplemental sequence file available for this manuscript. The “RDP output” shows the results of running the consensus sequence from AbundantOTU for each OTU through the stand-alone RDP classifier version 2.4. Numbers in parentheses represent scores assigned by the classifier to indicate the strength of the match. The “MTC” column shows correction for multiple testing (in this case testing 219 OTUs) using the Benjamini and Hochberg procedure. In this analysis, the average value for WT and KO animals in each cage was used, although very similar results were obtained considering each OTU values for each animal independently of cage (data not shown).