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A bacterial pathogenicity determinant associated with necrotizing enterocolitis

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Predominant enterobacteria from infants with necrotizing enterocolitis (NEC) were examined for an unusual ability to ferment lactose. One such isolate, a *Klebsiella pneumoniae* strain, was partially induced for lactose operon expression in tryptone containing media, and was also pathogenic in a rabbit ileal loop model for NEC. A spontaneous segregant of this strain was no longer partially induced for lactose operon expression, and was no longer pathogenic in the rabbit model. The gene responsible for this phenotype was cloned. The resulting plasmid was shown to cause both partially induced lactose operon expression and pathogenicity when introduced into a laboratory *K. pneumoniae* strain. A *K. pneumoniae* mutant deficient in lactose repressor synthesis was also pathogenic in the rabbit model. These results and previous studies on the intraluminal biochemistry of infants with NEC support the hypothesis that an increased ability for lactose fermentation may be a bacterial pathogenic trait with respect to NEC.

Key words: necrotizing enterocolitis; bacterial pathogenicity determinant; lactose operon.

Introduction

Necrotizing enterocolitis (NEC) is a devastating neonatal disease with rapid onset which affects 6% of all premature infants.[1] Twenty to forty per cent of these cases are fatal, representing a major contribution to the mortality of premature infants who survive early respiratory distress. In addition, surviving babies with post-operative short bowel syndrome or malabsorption frequently suffer prolonged hospitalization and costly care.[2,3]

No single theory for the pathogenesis of NEC has been proven. While numerous epidemiological associations have been suggested, only a few consistent underlying factors have been identified. These include prematurity,[2,4] enteral feeding[5,6] and enteric bacteria.[7,8] There is no good experimental evidence to suggest that injury to the intestinal mucosa results primarily from a vascular insult.[2,9,10] There is also no compelling clinical evidence that the initial intestinal injury results from ischemia, as postulated by several investigators in earlier studies.[2,9,11–13] It also has become clear that NEC is not a single disease entity, but rather a pathophysiological syndrome with many precipitants.

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Numerous outbreaks of NEC have been detected in recent years. The fact that instituting infection control measures is always effective in terminating such outbreaks strongly suggests the involvement of an infectious agent under certain conditions.[14] Bacteria most often associated with the disease are *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae* and Clostridia species.[14-17] Evidence for such association is strongest for *K. pneumonia*,[18] and weakest for Clostridia species. A possible role for *Clostridium difficile* toxin in NEC has been disproven.[19-22] Other epidemiological studies have implicated viruses (e.g. Rotavirus, Coronavirus and Coxsackie B2 virus).[14,23,24]

These epidemiological associations are suggestive but do not prove that the implicated organism really causes the disease. The situation is further complicated by the fact that all these organisms can be part of the normal flora in neonates. A solid theory for infectious pathogenicity of NEC therefore awaits the identification of those special pathogenic traits which distinguish bacterial strains isolated from NEC patients from those of normal flora.

In a recent study on the intraluminal biochemistry of neonates with NEC, the occurrence of this disease was associated with low intraluminal pH (<5) and with the presence of large amounts of undigested protein (usually casein) and carbon source (usually lactose) in the bowel.[25] It was further shown[25] that inoculation of a rabbit ileal loop with casein and organic acids at pH 4.0 resulted in hemorrhagic necrotic intestine. This observation led to the conclusion that low pH, in combination with undigested casein—a known chemotactic agent for polymorphonuclear leukocytes[26]—may be responsible for the initial intestinal injury which triggers the disease. Following the initial intestinal injury, a variety of bacteria present in normal flora may contribute to the subsequent necrosis of the intestine.

Taken together, these findings suggest that bacteria with an unusual ability to rapidly ferment undigested lactose may contribute to the initial intestinal injury in NEC, by causing an abnormally low pH in the ileum and the colon.[25,27,28] This idea is already supported by a variety of observations: 90 to 95% of neonates developing NEC have been fed prior to the onset of the disease;[2,9,10,29] there is an increased lactose concentration in stools of infants prior to the onset of NEC;[30] intraluminal gas typical of NEC is 30% hydrogen, and enteric bacteria from these patients make hydrogen only in the presence of milk;[31] increased D-lactate found in the urine of NEC patients is also evidence of vigorous bacterial fermentation;[32] and excess infant formula—a breast milk substitute—is maladsorbed, passes to the colon and serves as substrate for colonic bacterial flora.[2,9,15,29]

To test this hypothesis predominant enteric bacteria from infants with NEC were examined for increased ability to ferment lactose, compared to normal flora controls. Here we report that this phenotype correlated with pathogenicity with respect to NEC, using a recently developed rabbit ileal loop model.[25] A preliminary report of our findings was presented at the 1987 Meeting of the Society for Pediatric Research (Anaheim, CA).

Results

Tryptone mediated induction of the lactose operon

Strains DC1 to DC6, from patients with NEC, and strains DC7 to DC12, normal flora controls (see Materials and methods), were assayed for β-galactosidase specific activity following growth in tryptone broth in the presence and absence of inducer. Results are shown in Table 1. All strains, except DC4, a *K. pneumoniae* strain from an

Table 1 β-galactosidase specific activities following growth in tryptone

Name	Strain	Source	Induced	Uninduced	Ratio[a]
DC1	*K. pneumoniae*	NEC patient	1.24	0.0023	539
DC2	*E. coli*	NEC patient	4.56	0.0194	235
DC3	*K. pneumoniae*	NEC patient	2.55	0.019	134
DC4	*K. pneumoniae*	NEC patient	6.49	0.59	11
DC5	*E. cloacae*	NEC patient	4.21	0.044	96
DC6	*E. cloacae*	NEC patient	3.88	0.0027	1437
DC7	*K. pneumoniae*	Normal flora	3.76	0.047	80
DC8	*E. coli*	Normal flora	2.35	0.0062	379
DC9	*E. aerogenes*	Normal flora	0.74	0.0023	322
DC10	*E. aerogenes*	Normal flora	0.82	0.0024	342
DC11	*E. cloacae*	Normal flora	6.38	0.0712	90
DC12	*K. oxytoca*	Normal flora	2.96	0.0098	302
HB101	*E. coli*	Lab strain	4.54	0.021	216
MR1	*K. pneumoniae*	M. Riley	1.27	0.0048	264
DC17	*K. pneumoniae*	This work	1.80	0.96	1.9
DC18	*HB101/pCC1*	This work	7.41	1.98	3.7

See Materials and methods for details. Results shown are the average of three independent determinations. Reproducibility was always within 10%.

[a] Ratio of induced to uninduced β-galactosidase specific activities.

Table 2 Lactose enzyme specific activities in different growth media

Name	Growth medium	β-galactosidase			Lactose permease		
		Induced	Uninduced	Ratio	Induced	Uninduced	Ratio[a]
DC4R[b]	Tryptone	6.49	0.59	11	0.123	0.077	1.6
	Minimal	4.83	0.028	173	0.127	0.0075	17
DC4W[c]	Tryptone	6.83	0.057	120	0.045	0.0035	14
	Minimal	5.38	0.029	185	0.040	0.0015	27
DC4R*[d]	Tryptone	6.61	0.61	11	0.129	0.081	1.6
	Minimal	5.01	0.027	186	0.124	0.0073	17

See Materials and methods for details. Results shown are the average of three independent determinations. Reproducibility was always within 10%.

[a] Ratio of induced to uninduced specific activities.

[b] Original DC4 strain.

[c] White segregant derived from DC4R.

[d] Back segregant of DC4W.

NEC patient, had normal β-galactosidase specific activities when compared to normal flora isolates of the same species.

Partially induced synthesis of β-galactosidase was observed in strain DC4 when grown in tryptone broth, but not when grown in minimal medium (see Table 2, lines 1 and 2. It should be noted that strain DC4 was renamed DC4R for reasons explained below). A similar tryptone mediated induction was observed for lactose permease synthesis (Table 2). By adding back individual components of minimal medium to cultures grown in tryptone broth, and *vice versa*, it was shown that this is an induction effect, caused by tryptone, a pancreatic digest of casein USP (Difco) (see Table 3). Using dialysis it was shown that a tryptone component with a molecular weight of less than 3000 has a pronounced dose-dependent stimulatory effect (see Fig. 1). Further characterization of the compound responsible for this effect is in progress.

Furthermore, it was shown that β-galactosidase induction starts approximately 4 min after addition of tryptone, whereas induction by the direct inducer isopropyl-β-

Table 3 Effect of growth media components on β-galactosidase induction in DC4R

Medium	Component added	β-galactosidase activity
Tryptone	None	0.61
	Glycerol	0.65
	Minimal salts	0.59
Minimal salts	Glycerol	0.026
	Casamino acids	0.030
	Glycerol+casamino acids	0.034
	Tryptone	0.55

See Materials and methods for details. Casamino acids was added to a final concentration of 1%. All media were prepared from concentrated stocks so that both tryptone and minimal salts were always 1x strength where present. Results shown are the average of 3 independent determinations. Reproducibility was always within 10%.

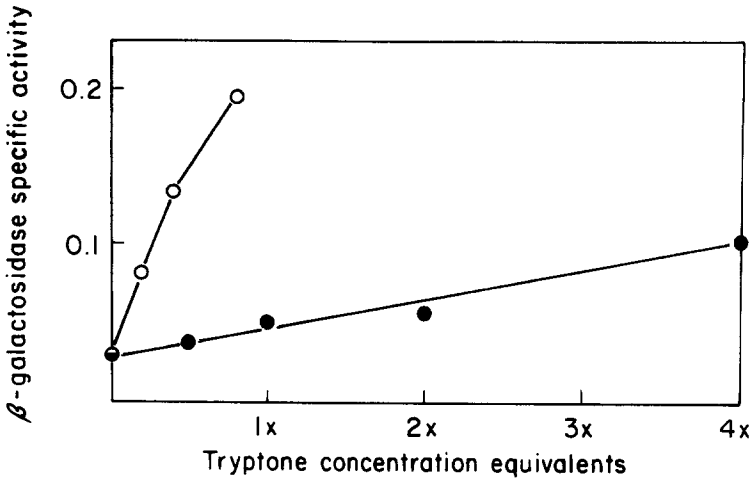

Fig. 1. β-galactosidase induction in DC4R by different tryptone fractions. Tryptone was fractionated as described in Material and methods. To measure the stimulatory effect of each fraction, strain DC4R was grown at 37°C in minimal medium and dialyzed tryptone fractions were added as indicated. β-galactosidase assays were performed as described in Materials and methods. ○: <3000 M, tryptone fraction; ●: >3000 M, tryptone fraction.

D-thiogalactoside (IPTG) takes approximately 2 min under the same conditions[40] (data not shown).

Segregation of the lactose phenotype

Strain DC4 grows as deep red colonies on MacConkey lactose agar, but at a low frequency throws off white segregants. Numerous sectored colonies typical of a segregation event were observed. The parental and segregant strains were renamed DC4R (red) and DC4W (white) respectively. DC4W in turn segregated back to red on MacConkey lactose plates at low frequency. One such isolate was designated DC4R*. This observation suggests that the initial segregation event was not due to the irreversible loss of a plasmid.

β-galactosidase assays on DC4W revealed that lactose operon expression in this strain is no longer partially induced, and is in fact indistinguishable from that observed in a normal flora *K. pneumoniae* strain. The back segregant, however, was once again partially induced for lactose enzyme synthesis in the presence of tryptone, and behaved

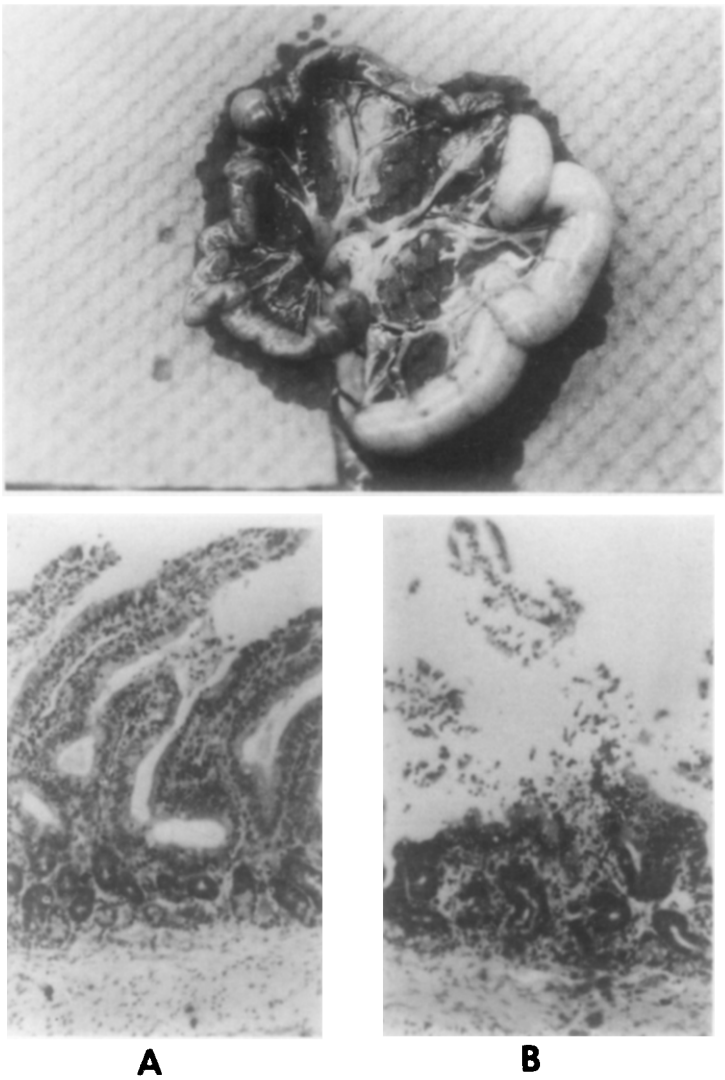

Fig. 2. Pathogenicity of bacterial strains in the rabbit ileal loop model for NEC. Pathogenicity was assessed as described in Materials and methods (top). Shown on the left is rabbit ileal loop following incubation with the non-pathogenic strain DC4W; on the right, a similar loop following incubation with the pathogenic strain, DC4R. Representative histologic sections (100×) are also shown. A: DC4W; B: DC4R.

in all respects like the original DC4R strain. Lactose permease assays showed that expression of the lactose permease gene is similarly affected (see Table 2).

Partially induced synthesis of lactose enzymes and pathogenicity

The three *K. pneumoniae* strains DC4R, DC4W and DC4R* were tested for pathogenicity in the ileal loop rabbit model for NEC. The original DC4R strain was pathogenic, whereas the DC4W segregant had lost pathogenicity (see Fig. 2 and Table 4, lines 1–3). The back segregant DC4R* was as pathogenic as DC4R. These results were reproducible and consistent in 3 independent determinations.

The observation that DC4W is neither pathogenic nor partially induced in lactose operon expression is consistent with the hypothesis that an increased ability for lactose fermentation may be a pathogenic trait with respect to NEC.[25]

To determine whether increased synthesis of lactose enzymes is in itself a sufficient

Table 4 Pathogenicity of various isolates in the rabbit ileal loop model

Strain	Pathogenicity Grade	β-galactosidase specific activity (uninduced)
DC4R	3.0	0.59
DC4W	0.0	0.057
DC4R*	2.75	0.61
MR1	0.25	0.0048
MR1/pCC1	2.0	0.46
DC17	1.75	0.96

See Materials and methods for details. The pathogenicity grades shown are the average of at least 3 independent determinations. Results were always reproducible within 1 grade. See text for a description of the strains. Uninduced β-galactosidase specific activities after growth in tryptone are also shown for comparison.

condition for pathogenicity, we tested a wild type *K. pneumoniae* laboratory strain (MR1) and a mutant (DC17), derived from MR1, that synthesizes lactose enzymes at a high constitutive rate in the rabbit ileal loop assay (see Table 1 and Materials and methods). Results in Table 4 show that high constitutive synthesis of lactose enzyme causes a definite increase in pathogenicity grade (compare lines 4 and 6).

Cloning of the gene responsible for tryptone-mediated induction and pathogenicity

The genetic determinant responsible for the tryptone-mediated induction of β-galactosidase and lactose permease in DC4R, the pathogenic *K. pneumoniae* strain, was cloned as follows. First, a library was constructed consisting of *Bam*H1 digested chromosomal DNA from DC4R, ligated into the *Bam*H1 site of the plasmid pBR322.[39,41] The restriction enzyme *Bam*H1 was chosen because there are no *Bam*H1 sites in the entire *K. pneumoniae* lactose operon.[42]

To select the clones of interest this library was transformed[39] into the *E. coli* strain HB101 (*hsdS20, recA13, lacY1, rpsL20*),[43] deficient in host-restriction and containing a mutation conferring a deficiency in the lactose permease. Plasmids endowing this strain with the ability to grow on lactose as a sole carbon source were selected. All recombinants found contained a hybrid plasmid with a common 2.3 kb insert in the *Bam*H1 site of pBR322. One such plasmid was designated pCC1 and was retained for further study. Strain HB101 harboring the plasmid pCC1 was named DC18. It was further shown that this plasmid confers the phenotype of interest, i.e. tryptone-mediated partially induced synthesis of β-galactosidase (see Table 1). This result strongly suggests that it has been possible to transfer this trait from *K. pneumoniae* to an *E. coli* background.

For southern hybridization[39] our insert was labeled with biotin-11-dUTP, using appropriate kits from Bethesda Research Labs, and hybridized to pCR100, a plasmid containing the entire *K. pneumoniae* lactose operon.[34] This experiment showed that there is no homology between the cloned fragment and any of the genes in the lactose operon (data not shown). There was no homology between the cloned insert and plasmid DNA prepared from strain DC4R,[38] suggesting that the cloned fragment is of chromosomal origin.

The plasmid pCC1 was introduced into the wild type *K. pneumoniae* laboratory strain MR1 by transformation and the resulting strain was examined for pathogenicity in the rabbit ileal loop assay. Results show (Table 4, line 5) that the cloned fragment contains a pathogenicity determinant able to dramatically increase the pathogenic potential of strain MR1 with respect to NEC.

Discussion

A recent study of intraluminal biochemistry of NEC cases[25] and results presented here support the hypothesis that an increased ability for lactose fermentation in certain strains of intestinal bacteria is a pathogenic trait with respect to NEC. Previously it was shown[25] that rabbit loops inoculated with organic acid and protein (mimicking the intestinal content of neonates with NEC) develop all the gross and histological symptoms associated with the disease[25] (see Materials and methods). In this work we show that inoculating rabbit loops with infant formula and appropriate bacterial strains can do the same. This further strengthens our belief that the rabbit model is a valid model for NEC, and lends support to the idea that rapid bacterial fermentation of undigested carbohydrate leads to the appearance of organic acids in the intestine.

The proposed model for infectious pathogenicity may not explain every case of NEC, since direct evidence for increased lactose fermentation was obtained only in one out of six isolates of predominant *Enterobacteriaceae* studied here. On the other hand, nothing in the model requires that increased lactose fermentation be limited to the *Enterobacteriaceae*. It is still possible that a number of other cases are due to increased lactose fermentation by more fastiduous bacterial species such as Clostridia or other lactose fermenting anaerobes.

Several studies[4,6,44] have shown that NEC is often observed in formula-fed infants. Infant formula is high in lactose and protein content. The protein present in infant formula, casein, is similar to the tryptone-based medium used in our experiments. Recently we have shown that infant formula diluted up to 10^5-fold in minimal medium also causes a 10-fold induction in lactose enzyme synthesis in DC4R but not in DC4W. However, we have as yet no direct proof that β-galactosidase synthesis is also induced in the intestine nor that there is in fact increased acid production with the pathogenic strain *in vivo*.

If fully induced levels of β-galactosidase are the same in strains DC4R and DC4W (see Table 2) why is there a color difference between these two strains on MacConkey lactose agar? Recently we have begun experiments to try to determine the physiological basis for this color difference. So far we have shown (1) that DC4R is inducible by lower concentrations of lactose (0.001%) than DC4W; (2) that induction by 1% lactose (the concentration present in MacConkey lactose agar) is not as efficient as induction by IPTG: DC4R was induced to 27% of maximal β-galactosidase specific activity and DC4W only to 12%; and (3) that there are differences in the kinetics of induction of strains DC4R and DC4W by lactose (data not shown). These recent results suggest that the tryptone-mediated induction of the lactose operon is only one manifestation of the genetic difference between DC4R and DC4W. Yet, all the phenotypic differences between DC4R and DC4W are consistent with the idea that DC4R has an increased ability for lactose fermentation in the gut.

Our working hypothesis is that a strain such as DC4R can begin fermentation of lactose as soon as infant formula from an intermittent feeding reaches the intestine, whereas fermentation by normal inducible strains will be delayed, until the lactose operon is fully induced.[36] We believe that this rapid onset of lactose fermentation and the ensuing immediate production of organic acids may be responsible for the onset of NEC under certain circumstances.

It is interesting to note that strain MR1 containing plasmid pCC1 elicits a slightly more severe pathogenic response than strain DC17, which synthesizes lactose enzymes constitutively, even though β-galactosidase specific activity is higher in strain DC17. The possibility that the genetic change occurring in the segregation from DC4R to DC4W involves a regulatory gene, which affects lactose enzyme expression as well as

expression of other gene products important in pathogenicity has not been ruled out by our results.

In further studies we hope to elucidate the nature of the gene product(s) encoded by plasmid pCC1. We are also fractionating the tryptone broth in order to identify more precisely the chemical nature of the component responsible for the induction phenomenon. In addition we are looking at more predominant *Enterobacteriaceae* in stools of infants diagnosed with NEC, and hope to identify and characterize more isolates of the type described here.

Materials and methods

Media. Tryptone broth contains per l: 10 g Bacto-tryptone (Difco), 5 g NaCl. Minimal growth medium contains Vogel-Bonner minimal salts[33] supplemented with 2 mg ml^{-1} glycerol.

Bacterial strains. Stools were collected from neonates in the Syracuse area at the onset of NEC symptoms. For each patient, the species of *Enterobacteriaceae* isolated on MacConkey lactose agar was either unique or by far the most predominant in the stool. These organisms were purified and designated DC1 to DC6. Purified normal flora *Enterobacteriaceae* controls (DC7–DC12) were obtained from stools of healthy infants at the Westchester County Medical Center nursery, Valhalla, New York. All isolates were identified according to standard procedure using the Microscan system. DC4R is the original DC4 isolate. DC4W is a white segregant isolated upon subculturing of DC4R on MacConkey lactose agar. DC4R* is a red back segregant of DC4W obtained upon subculturing of DC4W on MacConkey lactose agar. Purity of strains DC4R, DC4W, and DC4R* was maintained by repeated subculture on MacConkey lactose agar. MR1, a wild-type *K. pneumoniae* laboratory strain and plasmid pCR100,[34] a pBR322 derivative containing the entire *K. pneumoniae* lactose operon, were obtained from Dr Monica Riley. Strain DC17 was isolated following ethyl methanesulfonate mutagenesis[35] of the wild type strain MR1, as follows. Mutagenized cells were grown overnight in phenyl-β-galactoside to enrich for mutants constitutive in β-galactosidase synthesis,[36] and individual clones with this phenotype were identified by plating on tryptone plates containing XGal (5-bromo-4-chloro-3-indolyl-β-D-galactoside).[36] One clone with high β-galactosidase activity in the presence and absence of inducer, in both tryptone and minimal medium, was designated DC17. Introduction of a wild type lactose operon in *trans* (by transformation of DC17 with plasmid pCR100) restores the normal inducible phenotype, suggesting that DC17 is most likely deficient in repressor synthesis. All strains were maintained in 60% glycerol at −20°C.[39] Prior to their use in the experiments described, freezer stocks were subcultured onto nutrient agar and the phenotype of individual colonies was verified.

Enzyme assays. β-galactosidase assays were performed on toluenized cells harvested during exponential growth at 37°C, as described by Miller.[35] Lactose permease specific activities were determined as described by Carter *et al.*[37] In this assay the rate of hydrolysis of o-nitrophenyl-β-D-galactopyranoside (ONPG) is measured in intact cells poisoned with azide. Under these conditions the rate-limiting step is lactose permease mediated transport of ONPG into the cell. Specific activities are expressed in arbitrary units. Unless otherwise noted, induction of the lactose operon was achieved by growth in the presence of 1 mM IPTG.[35]

Pathogenicity assays. The pathogenicity of bacterial strains was assayed according to Clark *et al.*[28] Weanling New Zealand white male rabbits were fasted for 8 h. Each rabbit was anesthetized with Ketamine (35 mg kg^{-1}) and Xylazine (5 mg kg^{-1}) intramuscularly. The abdomen was shaved and cleansed for surgery. The peritoneum was opened and ligature was placed just distal to the ligament of Treitz. The intestine was then flushed with sterile saline and ligated into approximately 5 cm intestinal loops, with care being taken to preserve blood supply. These loops were then injected randomly using a 26 gauge needle with 1 ml per 2 cm of intestine of bacteria freshly grown in tryptone broth, diluted to 10^6 organisms per ml in proprietary infant formula. Control loops were injected with infant formula or saline alone. The peritoneum was closed and the animal was allowed to recover from anesthesia. 18 h later the animal was sacrificed by Ketamine anesthesia with blood letting, and the intestine was removed. The contents of each loop was drained into a syringe. Tissue and fluid were then

studied histologically. Distention, mucosal thinning and hemorrhage were typical of a pathogenic response, and microscopy revealed mucosal disruption with villus destruction and a fibrino-purulent exudate. Non-pathogenic strains did not cause these symptoms. A typical strong pathogenic response (Grade 3) and a non-pathogenic response (Grade 0) are shown in Fig. 2. Intermediate responses were scored using the following grading system: Grade 0: villi completely intact (non-pathogenic response); Grade 1: villous tip necrosis with preservation of villous crypts; Grade 2: necrosis of villous tip and crypts with loss of mucosal and submucosal architecture; Grade 3: necrosis extending into muscularis; Grade 4: transmural necrosis. The presence and extent of edema, lymphatic dilation, cell infiltration, hemorrhage and inflammation was also noted. Animal care was in accordance with institutional guidelines.

Fractionation of tryptone. A measured volume of 20× sterile tryptone was dialyzed to equilibrium versus sterile distilled water to retain the >3000 molecular weight fraction. In a complementary experiment a measured volume of sterile water was dialyzed to equilibrium versus an excess of 20× sterile tryptone to collect the <3000 molecular weight fraction in the dialysis bag. Volume changes resulting from dialysis were taken in account in expressing the resulting fractions in tryptone concentration equivalents.

Cloning methods. Plasmid DNA was prepared by the rapid alkaline lysis method of Birnboim and Doly.[38] Transformation was performed using the $CaCl_2$ method.[39] Lambda DNA was purchased from BRL. Restriction enzymes were obtained from IBI and restriction reactions were incubated overnight at 37°C in the buffers supplied by the manufacturer. DNA fragments were separated on 0.7% agarose using the standard Tris-borate buffer system.[39] Gels were run at 50 V for 3 h and stained with ethidium bromide.

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