

The role of cathelicidin in control of *Helicobacter pylori* colonisation in the stomach

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KEY MESSAGES

1. A new animal model for *Helicobacter pylori* infection in the stomach was established.
2. A probiotic was transformed with a host defence peptide cathelicidin. This preparation can be used for the treatment and prevention of *H pylori* infection and gastritis.
3. This oral biological preparation can also be used for the treatment of ulcerative colitis. It has multiple actions including anti-inflammatory, anti-bacterial, and maintenance of the defence

mechanisms in the gastrointestinal mucosa.

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Introduction

The effect of host factors on colonisation with *Helicobacter pylori* remains poorly understood.¹ Cathelicidin is a pleiotropic host defence peptide responsible for the maintenance of innate immunity. In the stomach, the mature peptide is actively produced by surface epithelial cells, as well as chief and parietal cells, and is present in the gastric secretion. Consistent with its function as a host defence peptide, cathelicidin possesses microbicidal activity against a broad spectrum of microorganisms, such as bacteria and parasites. Mice that are deficient in cathelicidin are known to be more susceptible to necrotic skin infection caused by Group A *Streptococcus*.²

Cathelicidin is up-regulated in gastric secretion and epithelium inflamed by *H pylori* infection. In vitro, cathelicidin is bactericidal for several strains of *H pylori*, including SD4, SD14, and Sydney strain 1 (SS1), suggesting that it may play a role in protection against *H pylori* infection.³ Nevertheless, in vivo evidence of the effect of cathelicidin on *H pylori* colonisation is lacking. Study of *H pylori* infection in cathelicidin-knockout mice can help elucidate the role of cathelicidin in the control of *H pylori* colonisation in the stomach and protection against *H pylori*-induced chronic gastritis. The therapeutic potential of a bioengineered probiotic with the ability to actively secrete cathelicidin can be explored for its effect on bacterial colonisation and gastritis.

Methods

This study was conducted from December 2008 to June 2011. The *H pylori* standard strain SS1 was used. The *Lactococcus lactis* NZ3900 and plasmid pNZ8149 were purchased. *L lactis* was transformed

with pNZ8149-usp-Cath plasmid by electroporation as described previously.⁴ This plasmid contains secretion signal peptide usp45 and the nine-residue propeptides LEISSTCDA immediately upstream of mouse cathelicidin (mCRAMP). In the presence of the inducer nisin, mCRAMP is produced and secreted under the control of *nisA* promoter.

mCRAMP knockout (*Cnlp*^{-/-}) and 129/SVJ wild-type (*Cnlp*^{+/+}) mice were used. *Cnlp*^{-/-} and *Cnlp*^{+/+} mice were gavaged with either 10⁸ colony forming units (CFU) of *H pylori* SS1 or a sterile brain heart infusion broth (as a control) using gastric intubation needles every other day for a total of three doses.

Yellow colonies that formed on the Elliker agar were selected for PCR analysis. Positive clones containing recombinant plasmid gave the product size 712 bp, whereas the self-circulating vector had a band at 479 bp. Western blot revealed no bands in the *L lactis* (N0) and *L lactis* transformant without nisin induction (N4) groups. A clear band was noted at 4.7 kDa after nisin induction (N4I) [Fig 1].

In the treatment experiment, *L lactis* transformed with control plasmid (N) and mCRAMP-encoded *L lactis* (N4) were incubated in M17 broth with 0.5% lactose. Nisin (250 ng/mL) was added and further incubated for 3 hours (N4I). After *H pylori* infection for 1 month, the successfully infected *Cnlp*^{+/+} and *Cnlp*^{-/-} mice were randomised to one of three groups to receive (1) distilled water, (2) 10 log CFU of *L lactis* transformed with control plasmid (N), or (3) 10 log CFU of mCRAMP-encoded *L lactis* with the addition of inducer nisin (N4I). Oral administration was given every other day for 2 months.

In the pre-treatment experiment, both *Cnlp*^{-/-} and *Cnlp*^{+/+} mice were pre-treated with mCRAMP-

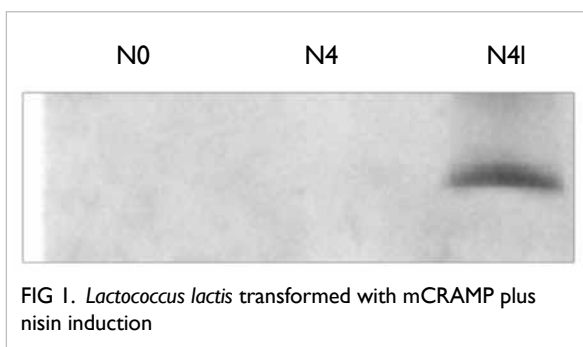


FIG 1. *Lactococcus lactis* transformed with mCRAMP plus nisin induction

encoded *L lactis* and its control plasmid given once every 2 days for 2 weeks before *H pylori* challenge. They were then gavaged with either a 10^8 CFU of *H pylori* SS1 or a sterile brain heart infusion broth (as a control) every other day for a total of three doses. The *H pylori* infected mice continued to receive either distilled water, 10^{10} CFU *L lactis* transformed with control plasmid, or 10^{10} CFU of mCRAMP-encoded *L lactis* with the inducer nisin for 3 hours. These animals were treated with these preparations every other day for 2 months.

The presence of *H pylori* and *L lactis* was shown by immunohistochemical staining. Rabbit anti-*H pylori* polyclonal and goat anti-*L lactis* polyclonal were used as primary antibodies,

respectively. Streptavidin-HRP conjugate was used as the secondary antibody for staining *L lactis*. The bacteria were visualised by diaminobenzidine chromogen. Goat antibody to mCRAMP was used as primary antibody. Alexa Fluor anti-goat 568 was used as secondary antibody. Sections were evaluated with a laser confocal microscope.

The relative density of *H pylori* was quantified by semi-quantitative PCR, detecting *H pylori*-specific 16S rDNA as previously described using specific primer HP5.⁵ The amount of mouse GAPDH DNA in the same specimen was measured for normalisation. The relative density of *H pylori* in the samples was expressed as the ratio of expression of *H pylori*-specific 16S rDNA to GAPDH DNA.

The RNA was used to generate the first strand of cDNA by reverse transcription. Specific primers were used to screen the expression of mCRAMP, TNF- α , IL-1 β , and IL-6.

The mucus layer was identified by periodic acid-Schiff staining. The mucus-containing cells were stained purple-red. The thickness of the mucus-secreting layer was measured perpendicularly to the mucosal surface from the edge of the epithelium to the outermost part of the mucus-secreting layer under microscopy. All analyses were performed blind.

Cultured 10^8 CFU SS1 and 10783 *H pylori* were treated with 0.5, 1, 8, 64, and 128 μ g/mL mCRAMP,

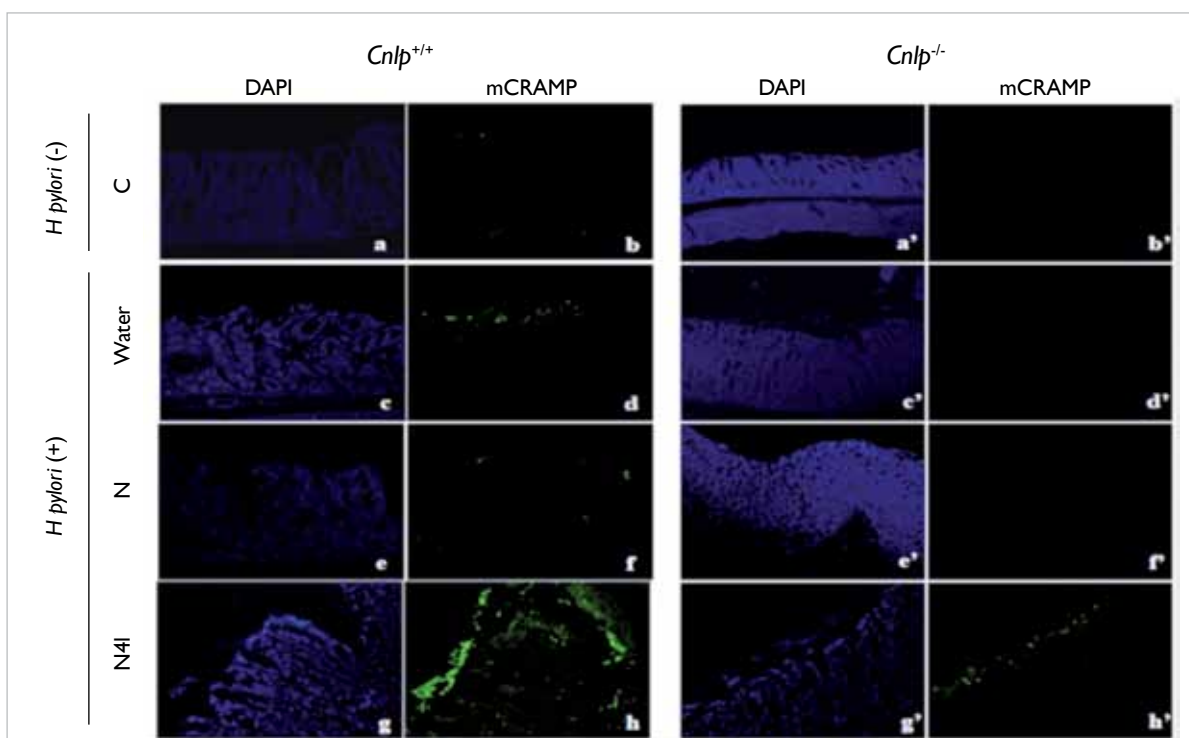


FIG 2. Immunofluorescent staining of mCRAMP in the stomach of *Cnlp*^{+/+} and *Cnlp*^{-/-} mice

Sections were stained with DAPI (blue) and antibody to mCRAMP (green). The present bioengineered probiotic preparation, the mCRAMP-encoded *L lactis* (N4I) successfully delivered exogenous mCRAMP to the gastric epithelium in the *Cnlp*^{+/+} and *Cnlp*^{-/-} mice. There was no staining of endogenous mCRAMP in *Cnlp*^{-/-} mice.

LL-37, LL17-32, or phosphate buffer saline as negative control. *H. pylori* growth was determined by the OD595nm value of each well.

Acute ulcerative colitis was induced in mice by adding 3% dextran sulfate sodium to drinking water for 7 days.

Results

To assess whether *H. pylori* and *L. lactis* could colonise the surface of gastric epithelium in *Cnlp*^{+/+} and *Cnlp*^{-/-} mice, Giemsa and immunohistochemical stainings and scanning electron microscopy were performed. Both *H. pylori* and *L. lactis* were observed on the gastric mucosa of *Cnlp*^{+/+} and *Cnlp*^{-/-} mice after oral administration.

The mCRAMP level in the stomach was determined by immunofluorescence staining. In uninfected mice, gastric epithelial cells showed no mCRAMP expression. After *H. pylori* infection, gastric epithelial cells stained positively for mCRAMP, suggesting that *H. pylori* infection could induce mCRAMP expression in the gastric mucosa of *Cnlp*^{+/+} mice (Figs 2 d, f, and h). Oral administration of mCRAMP-encoded with *L. lactis* successfully delivered mCRAMP to the gastric epithelium in both *Cnlp*^{+/+} and *Cnlp*^{-/-} mice (Figs 2 h and h').

Lack of endogenous mCRAMP increased *H. pylori* infection and inflammation in the gastric mucosa. These pathological changes were prevented either by treatment or pre-treatment with cathelicidin-encoded *L. lactis*.

The adherent mucus on the gastric mucosa was markedly depleted in gastritis mucosa. The effect was significantly prevented by the mCRAMP-encoded probiotic.

mRNA expressions of TNF- α , IL-1 β , and IL-6 were markedly increased by *H. pylori* infection in both *Cnlp*^{+/+} and *Cnlp*^{-/-} mice. The increased levels were prevented by mCRAMP-secreting *L. lactis*.

Cathelicidin and its analogues inhibited growth of normal and antibiotic-resistant strains of *H. pylori*. Cathelicidin encoded *L. lactis* prevented acute ulcerative colitis through multiple actions in mice.

Discussion

We have successfully established infection with *H. pylori* in a new type of mice with genetic background of 129/SVJ. The infection was confirmed by immunostaining and electron microscopy of the gastric mucosa. This type of mouse model is useful to define the role of endogenous cathelicidin and also the therapeutic action of exogenous cathelicidin in *H. pylori* infection and gastritis. We demonstrated for the first time that deficiency of cathelicidin (mCRAMP) can enhance *H. pylori* infection and inflammation in the gastric mucosa. Supplementation

with a transformed probiotic *L. lactis* with mCRAMP protected against *H. pylori* infection and inflammation in the stomach. This is the first form of a biological preparation that combines a probiotic and a host defence peptide in a single preparation to prevent and treat *H. pylori*. This mCRAMP encoded *L. lactis* was shown to secrete mCRAMP both in vitro and in vivo. We also showed that human and mouse cathelicidin could not only eradicate the normal strain SS1 but also kill the clarithromycin resistant 10783 strain of *H. pylori*. Moreover, the small fragment of human cathelicidin produces the best outcome to reduce bacterial growth. There are fewer side effects for this probiotic preparation, compared with the antibiotics conventionally prescribed for *H. pylori* infection. This new form of preparation may provide a better option for the treatment of *H. pylori* infection and its related diseases in murine animals, perhaps also in humans. To further extend the therapeutic application of mCRAMP-encoded *L. lactis*, it was shown to be equally effective for murine UC, a disease related to bacterial infection. This biological preparation has several advantages over the prototype drug sulfasalazine. In addition to its anti-inflammatory action, it preserves the mucus level and maintains the integrity and function of the gastric mucosa. In conclusion, amalgamation of a probiotic with a host defence peptide is a good therapeutic agent for bacteria-associated diseases in the gastrointestinal tract.

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