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Expression of the Eukaryotic *Trypanosoma cruzi CRA* Gene in *Yersinia enterocolitica* and Induction of an Immune Response against CRA in Mice

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The 70-kb plasmid pYV of *Yersinia enterocolitica* directs the secretion of a set of proteins, called Yops, that are produced during infection of humans and animals. *Trypanosoma cruzi*, the agent of American trypanosomiasis, synthesizes a cytoplasmic protein termed CRA that is considered to be *T. cruzi* specific. To produce CRA in *Y. enterocolitica*, we constructed a hybrid *yopE-CRA* gene that we integrated into plasmid pYV by homologous recombination. Recombinant *Y. enterocolitica* produced a chimeric Yop-CRA protein that was secreted in large amounts in the surrounding medium. This protein reacted with sera directed against either CRA or YopE. To test the ability of the recombinant strain to induce an immune response against CRA, we inoculated C57BL/6J mice by gastric intubation with live recombinant bacteria. A clear antibody response directed against CRA was detected in the mouse serum. The CRA-presenting *Y. enterocolitica* strain also carried a bioluminescence detection marker, which allowed us to monitor colonization of the intestinal lumen of infected mice. No significant differences were observed between the infectivity of the CRA antigen-producing and -nonproducing *Y. enterocolitica* strains, despite the fact that one of them no longer produced YopE.

Yersinia enterocolitica strains of serotypes O:3 and O:9 are invasive pathogens responsible for gastroenteric syndromes in humans and animals (35). Full virulence expression of *Y. enterocolitica* requires a plasmid 70 kb in size (12, 30, 36). This plasmid, called pYV, encodes an original system leading to the synthesis and secretion of large amounts of proteins termed Yops (10 to 20% of total cell proteins) (6, 13, 14, 27). This system is composed of several loci called *vir*, encompassing a region of about 20 kb (6). One of these loci, *virF*, encodes a transcriptional regulator that activates *yop* genes and some other *vir* loci when bacteria are incubated at 37°C (5, 20). The large *virC* operon is involved in Yop secretion (26). The synthesis and secretion of Yops are optimized when bacteria are incubated in the absence of Ca^{2+}. However, the relationships between the bivalent cations, the expression of *yop* genes or *vir* loci, and the export process have not yet been explained. The *yop* genes encoding the Yops are scattered all over plasmid pYV, either alone or arranged in operons (3, 7, 29). Several of them have been cloned and sequenced (24, 27).

The Yop-specific secretion mechanism does not involve the removal of an N-terminal signal sequence (27). The information required for export is nevertheless localized in the N-terminal domain (24, 27). In the case of YopH, the first 48 amino acids of the protein are sufficient to ensure secretion. Chimeric proteins obtained by fusion of the 5′-terminal part of the *yopH* gene with DNA fragments encoding foreign proteins can be secreted by the Yop secretion machinery. So far, three bacterial heterologous proteins have been secreted by means of this system: the cholera toxin B subunit (33), the alkaline phosphatase, and the α peptide of β-galactosidase (25).

By inserting foreign genes downstream from *yop* promoters, production of heterologous antigens can be obtained in laboratory animals infected with recombinant *Y. enterocolitica* (32, 33). Inoculation of mice with a *Y. enterocolitica* strain expressing the cholera toxin B (CT-B) gene from the *yopH* promoter elicits not only a serum immunoglobulin G (IgG) and IgA response and mucosal secretory IgA production in the intestines of laboratory animals (33) but also protection against the action of the cholera toxin (34). This immunization, which was better than that observed after inoculation of CT-B itself, required the use of a live recombinant *Yersinia* carrier.

In this work, we constructed recombinant *Y. enterocolitica* strains expressing epitopes of the eukaryotic *Trypanosoma cruzi* CRA antigen. *T. cruzi* is the protozoan agent of Chagas' disease in humans (for a review, see reference 10). It has a complex life cycle involving several transformations and stages in its mammalian and insect hosts. By analysis of a genomic bank from the metacyclic trypomastigote form of *T. cruzi*, Lafaille et al. (19) isolated a fragment containing 23 repeats of 42 nucleotides. This fragment is part of a gene encoding a cytoplasmic protein of 225 kDa that is called CRA, for cytoplasmic repetitive antigen. Homologous fragments were also isolated by others (15, 16). The function of CRA in the pathogenesis of *T. cruzi* is unknown. Whether or not CRA is a stage-specific antigen is still debated. According to Lafaille et al. (19), only the epimastigote form produces CRA. However, mRNA of CRA has also been detected in the metacyclic trypomastigote form by Hoft et al. (15). Sera of Chagas' disease patients contain antibodies directed against CRA (15, 16, 19). CRA appears to be specific to *T. cruzi*, since sera from patients infected with other parasites do not react with this antigen (15, 16, 19).

To express *CRA* in *Y. enterocolitica*, the *T. cruzi* gene was

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TABLE 1. Strains and plasmids used

Strains and plasmids	Characteristics	Reference or origin
Strains (serotype)		
W22703 (O:9)		4
439-80 (O:9)		1
W1024 (O:9)		9
KNG1024	W1024 Lux$^+$	17
Plasmids		
pIC20H		23
7/2	pBluescript II KS carrying the *T. cruzi CRA'* gene (orientation 1)	Gift of E. Saman
pKNG101	*oriR6K mobRK2 strAB sacBR*	17
pKNG108	pKNG101 with the *Xba*I-*Sal*I fragment of pMSH20	This study
pMS3	pTM100 with an *Xho*I fragment of pYVe439-80 containing *yopE* and *yerA*	This study
pMS100	pBluescript II KS carrying only *yopE*	32a
pMSH18	pMS3 with the *Pst*I-*Hin*dIII fragment of clone 7/2 containing *CRA'*	This study
pMSH19	pIC20H with a *Pst*I-*Xho*I fragment of pYV439-80 containing the last 276 bp of *yopE*	This study
pMSH20	pMSH18 with the *Pst*I-*Xho*I fragment of pYV439-80 containing the last 276 bp of *yopE*	This study
pMSH21	pTZ18R with the fusion *yopE'-CRA'-yopE"* from pMSH20 (orientation 1)	This study
pMSH22	pTZ18R with the fusion *yopE'-CRA'-yopE"* from pMSH20 (orientation 2)	This study
pSD2	pUC19 with the *Xho*I fragment of pYVe439-80 containing *yopE* and *yerA*	10a
pTM100	pACYC184 with *oriT* of RK2	25
pKNG1024	pYVe1024 with *yopE'-CRA'-yopE"*	This study
pYVe227	Virulence plasmid of *Y. enterocolitica* W227 serotype O:9	4
pYVe1024	Virulence plasmid of *Y. enterocolitica* W1024	9
pYVe439-80	Virulence plasmid of *Y. enterocolitica* 439-80 serotype O:9, undistinguishable by restriction from pYVe227	1

placed under the control of the *yop* regulon by fusion with *yopE*. This led to the secretion of a YopE-CRA chimeric protein by the bacteria. Integration of the *yop-CRA* gene fusion into plasmid pYV itself ensured its stable maintenance in the host strain during infection of laboratory animals. Inoculation of mice with this strain elicited an antibody response against CRA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *Y. enterocolitica* strains used in this work belong to serotype O:9. Strain 439-80 is wild type, and W227 and W1024 are nalidixic acid-resistant mutants. Strain W22703 is a restriction mutant (restriction negative, modification positive) of W227 isolated earlier in this laboratory (4). Strain KNG1024 is a derivative of strain W1024 constructed by replacing the *blaA* gene (encoding β-lactamase A) by the *luxAB* genes (coding for bacterial luciferase) (17). In this strain, the *luxAB* genes are expressed constitutively from the *Escherichia coli lac* promoter.

E. coli SM10 lambda *pir*$^+$ (28, 31) was used for its capacity to replicate *oriR6K*-containing plasmids and to mobilize *mob*-containing plasmids. The plasmids used are listed in Table 1.

Y. enterocolitica was routinely grown in brain heart infusion broth (BHI; Difco, Detroit, Mich.) or in BHI supplemented with 4% (wt/vol) glucose, 20 mM $MgCl_2$, and 20 mM sodium oxalate. Sucrose sensitivity of recombinant strains, mediated by the presence of the *sacB* gene, was controlled by plating on tryptic soy agar (Gibco, Paisley, Scotland) supplemented with 5% sucrose (17). For inoculation of mice, the bacteria were grown as follows. A 100-μl sample of an overnight culture grown at room temperature (RT) was inoculated in 100 ml of BHI contained in a 500-ml conical flask with four baffles and inoculated overnight with shaking (100 rpm) at room temperature. Bioluminescence measurements were done in tryptic soy broth (Gibco) (18). The antibiotics used were ampicillin (50 or 200 μg ml^{-1}), gentamicin (5 or 10 μg ml^{-1}), kanamycin (25 μg ml^{-1}), nalidixic acid (35 μg ml^{-1}), and streptomycin (25 or 100 μg ml^{-1}).

Induction of the *yop* regulon and protein analysis. Induction of the *yop* regulon was done as described by Cornelis et al. (7). Analysis of supernatant proteins and whole cell lysates by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were carried out as outlined by Sory and Cornelis (32). Monoclonal antibody 6G1 is directed against YopE (2). Purified CRA, rabbit antiserum against CRA, and human Chagas' disease serum were provided by E. Saman (Innogenetics, Ghent, Belgium).

DNA manipulations and analysis. DNA was prepared and analyzed by standard methods (22). DNA restriction enzymes were from Pharmacia Inc. (Uppsala, Sweden), Boehringer (Mannheim, Germany), or New England Biolabs (Beverly, Mass.). The probe used to detect bacteria containing *CRA* by colony hybridization was the 960-bp *Eco*RI fragment of plasmid 7/2. The probe used to check the restriction pattern of pKNG1024 after integration of *CRA* into pYV was the entire plasmid 7/2, the entire *yopE*-containing plasmid pMS100, or the *Xba*I-*Sal*I fragment of pMSH20.

Animal infection. Animals were infected as described by Sory et al. (33). Briefly, *Y. enterocolitica* strains were inoculated to specific-pathogen-free 10-week-old female C57BL/6J mice (bred at the animal house of the medical faculty of the University of Louvain, Brussels, Belgium). Mice were inoculated by gastric intubation with about 10^{10} live bacteria grown as described above, washed in phosphate-buffered saline (PBS; 50 mM sodium phosphate, 150 mM NaCl [pH 7.4]), and resuspended in 300 μl of PBS.

Recovery of bacteria from infected mice and study of plasmid content. For each mouse, three Peyer's patches were excised from the ileal tissue, washed in PBS, and homogenized together in 4 ml of tryptic soy broth supple-

mented with yeast extract during 20 s by using an Ultra-Turrax TP18/10 mixer (Janke and Kunkel, Stauffen, Germany). They were then immediately analyzed in the luminometer as described below.

For recovery of bacteria from mouse stools, each mouse was placed for 15 to 30 min on a wire mesh resting on a petri dish. For each mouse, about 100 mg of stools was collected, resuspended in 4 ml of tryptic soy agar supplemented with yeast extract, homogenized as described for the Peyer's patches, and allowed to decant on ice. The upper phase was then removed and analyzed in the luminometer as described below.

Bioluminescence measurement. The substrate for luciferase detection was a suspension of 0.1% *n*-decanal (Sigma Chemical Co., St. Louis, Mo.) in water made by a 20-s ultrasonic treatment. The samples were diluted in tryptic soy broth, and 250 μl of each dilution was mixed quickly with 50 μl of substrate suspension in a 1-ml glass vial. The vial was immediately placed in the counting chamber of a Pico-Lite luminometer (Packard Instrument Co., Downers Grove, Ill.). The emitted light (in counts per minute) was scored during 10 s before and after addition of *n*-decanal, and the background luminescence was subtracted from the values obtained with the substrate. Conversion of light emission into number of bacterial cells was based on the correlation graph established by Kaniga et al. (18), showing that 1 cpm is equivalent to 16 bacterial cells.

Collection of plasma. Mice were anesthesized by intraperitoneal injection (10 μl/g of body weight) of a solution containing 0.2% (wt/vol) Rompun (Bayer, Leverkusen, Germany) and 0.5% (wt/vol) Imalgène (Rhone Merieux, Lyon, France). Blood was collected from the retro-orbital plexus with a Pasteur pipette wetted with 10 μl of heparin (2,500 IU ml^{-1}; Novo Industry, Brussels, Belgium) and centrifuged at 650 × *g* for 10 min. Final plasmas were stored frozen at −20°C.

Anti-CRA enzyme-linked immunosorbent assay (ELISA). Ninety-six-well polystyrene plates (Greiner, Nurtingen, Germany) were coated overnight at RT with CRA. Each well received 150 ng of CRA in 50 μl of PBS. Between steps of the assay, plates were washed three times with PBS. After saturation of the plates with 3% (wt/vol) bovine serum albumin (BSA) in PBS for 2 h at RT, serial twofold dilutions (in duplicate) in PBS-BSA of mouse plasma were added. As control sera, we used a rabbit anti-CRA serum as well as the preimmune serum. The plates were left for 2 h at RT. Total mouse immunoglobulins against CRA were detected by incubation for 2 h at RT with specific rabbit anti-mouse horseradish peroxidase-conjugated immunoglobulins (Sigma) diluted 1,000-fold. To reveal horseradish peroxidase-labeled antibodies, 100 μl of a substrate solution containing 0.04% (wt/vol) *o*-phenylenediamine (Sigma) and 0.001% H_2O_2 in 0.1 M citrate–0.2 M phosphate buffer (pH 5.0) was added. The reaction was stopped after 3 min with 50 μl of 2 M H_2SO_4. The A_{492} was measured.

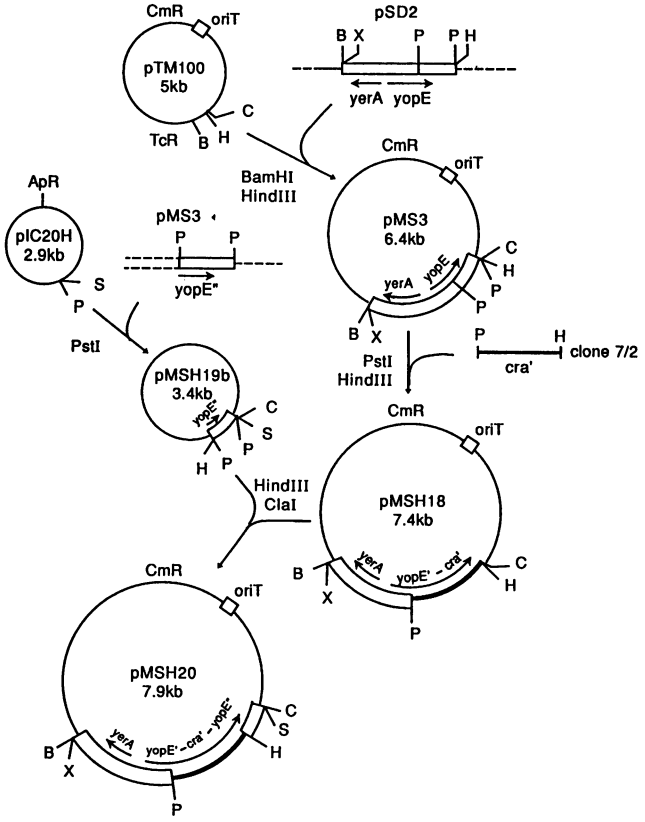

FIG. 1. Construction of pMSH18 and pMSH20. A 1,745-bp *Xho*I fragment of the virulence plasmid pYVe439-80 that contains genes *yopE* and *yerA* was first cloned in pUC19 to yield pSD2. Gene *yopE* encodes the Yop protein of 25 kDa (YopE) (1, 27). Gene *yerA* codes for a protein of 15 kDa that specifically regulates the secretion of large amounts of YopE (11). This *Xho*I fragment was then subcloned in the mobilizable pACYC184-derived plasmid pTM100. The new plasmid, pMS3, was digested by *Pst*I and *Hin*dIII and ligated with a *Pst*I-*Hin*dIII fragment containing a 941-bp fragment of CRA, called *CRA'*. In the first construct, called pMSH18, the fusion *yopE'-CRA'* gene was 1,413 bp in size and contained the first 506 bp of *yopE* (*yopE'*) joined in frame with 941 bp of *CRA*. The last 63 bp of the fusion gene were provided by pACYC184. In the second construct, called pMSH20, the last 276 bp of *yopE* (called *yopE"*) were added at the 3' terminus of *yopE'-CRA'*. To do so, a 460-bp *Pst*I fragment of pMS3 containing the last 276 bp of *yopE* was subcloned in pIC20H to yield pMSH19b, reisolated as a *Hin*dIII-*Cla*I fragment, and inserted in the *Hin*dIII and *Cla*I sites of pMSH18. Plasmid pMSH20 thus contains a fusion *yopE'-CRA'-yopE"* gene 1,641 bp in size. Abbreviations: ApR, ampicillin resistance; CmR, chloramphenicol resistance; TcR, tetracycline resistance; *yopE*, gene encoding the Yop protein of 25 kDa; *yopE'*, the first 506 bp of *yopE*; *yopE"*, the last 276 bp of *yopE*; *CRA'*, a 941-bp fragment of the *CRA* gene of *T. cruzi*; *yerA*, gene encoding the regulator of YopE secretion; *oriT*, origin of transfer; B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xba*I.

RESULTS

Construction of a hybrid *CRA-yopE* gene. The *CRA* gene was expressed in *Y. enterocolitica* by placing the foreign DNA fragment under the control of the *yop* regulon. As a target, we used gene *yopE*, encoding the Yop protein of 25 kDa (YopE). Since we used only a partial *CRA* gene (termed *CRA'*), the gene was directly inserted in frame within *yopE* (Fig. 1). The basis for this construct was a pACYC184-derived plasmid containing the *oriT* site of RK2 to permit its mobilization into *Y. enterocolitica*. Two recombinant plasmids, pMSH18 and pMSH20, were constructed (Fig. 1). To check the hybrid *yop-CRA* gene, it was cloned in both orientations in pTZ18R, giving pMSH21 and pMSH22, and sequenced. In pMSH18, the hybrid gene was composed of the promoter of *yopE* and of its first 506 bp (*yopE'*) joined in frame with 941 bp of *cra*. pMSH20 contained the same hybrid gene followed in frame by the last 276 bp of *yopE* (*yopE"*).

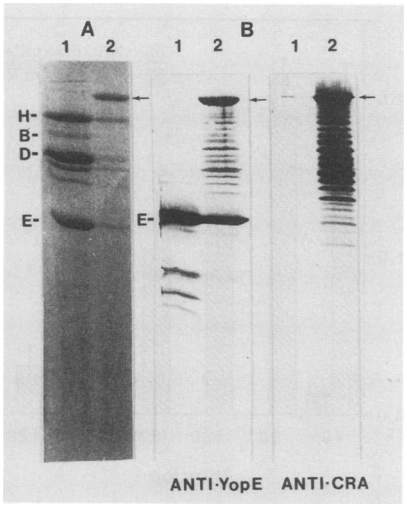

FIG. 2. Coomassie blue-stained SDS-polyacrylamide gel (A) and immunoblots (B) of released proteins of bacteria incubated at 37°C in the absence of Ca^{2+}. Immunoblots were resolved with the anti-YopE monoclonal antibody 6G1 diluted 1/200 or with a rabbit anti-CRA serum diluted 1/200. Lanes: 1, *Y. enterocolitica* W22703 (pYVe227); 2, *Y. enterocolitica* W22703(pYVe227)(pMSH18). Arrows indicate the hybrid YopE'-CRA' protein. Yops are designated according to the conventional nomenclature.

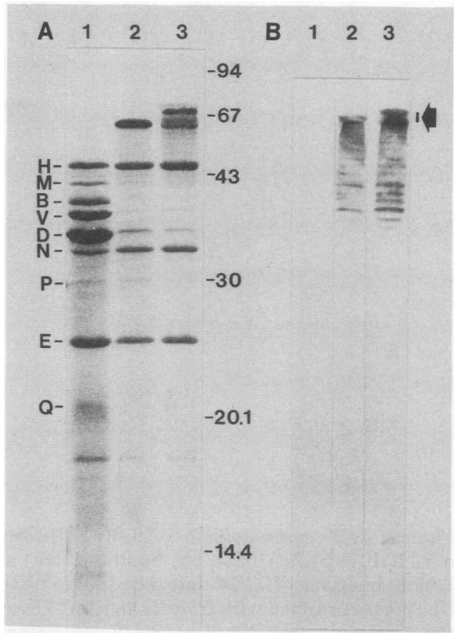

FIG. 3. Coomassie blue-stained SDS-polyacrylamide gel (A) and immunoblot (B) of released proteins of bacteria incubated at 37°C in the absence of Ca^{2+}. The immunoblot was resolved with a pool of human Chagas' disease sera diluted 1/100. Lanes: 1, *Y. enterocolitica* W22703(pYVe227); 2, *Y. enterocolitica* W22703(pYVe227)(pMSH18); 3, *Y. enterocolitica* W22703(pYVe227)(pMSH20). The arrow indicates the hybrid YopE'-CRA' and YopE'-CRA'-YopE" proteins. Yops are designated according to the conventional nomenclature. Molecular masses of size standards are indicated in kilodaltons.

Production of chimeric YopE-CRA proteins in *Y. enterocolitica*. Plasmids pMSH18 and pMSH20 were introduced by mobilization into *Y. enterocolitica* W22703(pYVe227). The recombinant strains were incubated in conditions of Yop production, i.e., at 37°C in a medium deprived of Ca^{2+}. SDS-PAGE analysis (Fig. 2 and 3) showed that Yops as well as new proteins of 65 and 80 kDa were released in the culture supernatant. Immunoblot analysis confirmed that these proteins were the hybrid YopE-CRA proteins. The proteins reacted with the anti-YopE monoclonal antibody 6G1 and with the polyclonal serum directed against CRA. No reaction of YopE with the anti-CRA serum was detected (Fig. 2 and 3).

Two surprising properties of the hybrid Yop-CRA proteins appeared upon analysis by SDS-PAGE and immunoblotting. First, the predicted molecular masses of the chimeric YopE'-CRA' and YopE'-CRA'-YopE" proteins were 49,994 and 57,543 kDa, respectively. The apparent molecular masses of these proteins determined by SDS-PAGE (65 and 80 kDa) were thus greater than expected. Second, immunoblot analysis revealed a set of proteins of molecular mass lower than that of the chimeric proteins. These proteins could be degradation products. They could also derive from an incomplete translation process or from truncated mRNA. Such degradation products, as well as a protein of higher molecular mass than expected, were also observed when a hybrid protein between CRA and the α peptide was expressed in *E. coli* (data not shown).

Integration of the hybrid *yopE'-CRA'-yopE"* gene into pYV. To ensure the stability of *CRA* in *Y. enterocolitica*, the hybrid *yopE'-CRA'-yopE"* gene encoded by pMSH20 was integrated into plasmid pYV. For this purpose, we subcloned the hybrid *yopE'-CRA'-yopE"* gene as an *Xba*I-*Sal*I fragment in the suicide plasmid pKNG101, giving pKNG108. After transfer into strain *Y. enterocolitica* KNG1024 (pYV1024), we checked the homologous recombination between *yopE'-CRA'-yopE"* of pKNG108 and *yopE* of pYVe1024 by Southern blot and Northern (RNA) blot (Fig. 4A). The new plasmid was called pKNG1024. Immunoblot analysis showed that this plasmid governed secretion of the hybrid YopE'-CRA'-YopE" protein instead of YopE (Fig. 4B).

Stability of the integration of *CRA* in pKNG1024. Strain KNG1024(pKNG1024), the host strain for the *yopE'-CRA'-yopE"* hybrid gene, also carries the *luxAB* gene markers in its chromosome. In the presence of *n*-decanal, this strain emits light and can thus be easily detected and counted in tissues or feces of inoculated laboratory animals (18). To evaluate the in vivo stability of our genetic construct, we inoculated one group of five C57BL/6J mice by gastric intubation with 2.1×10^{10} live bacteria of strain KNG1024(pKNG1024). Four days after inoculation, mice were sacrificed and three Peyer's patches were excised from the ileal tissue. Bioluminescence assays and plating on MacConkey agar indicated that 9.7×10^3 to 1.3×10^5 bacteria were present per Peyer's patch. The stability of *CRA* in pYV1024 in these bacteria was monitored. Forty-six to forty-eight colonies per mouse were tested by replica plating and colony hybridization with a *CRA* probe. All of the 238 bacteria isolated from mice that received KNG1024(pKNG1024) still contained the *CRA* gene. Hence, the construct is stable in vivo. This finding is in contrast with results obtained with a pACYC184-derived plasmid expressing CRA. This plasmid was easily lost 3 days after inoculation. In five mice that received W22703 (pYVe227)(pMSH20), only 15, 23, 31, 48, and 98% of bacteria isolated from the Peyer's patches still contained pMSH20.

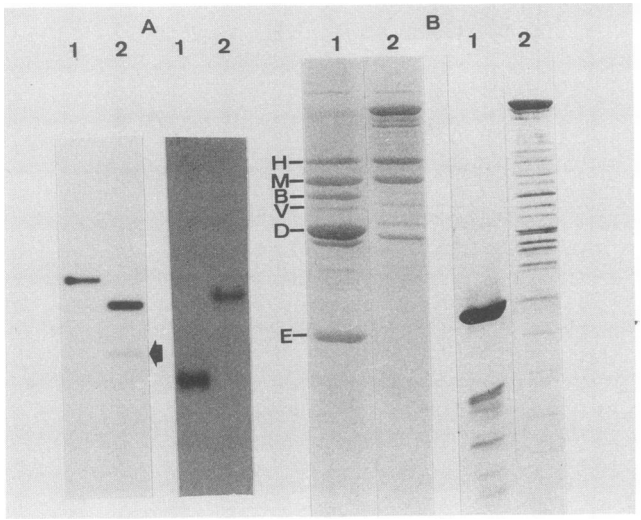

FIG. 4. Lanes: 1, *Y. enterocolitica* W1024(pYV1024); 2, *Y. enterocolitica* KNG1024(pKNG1024). (A) Southern (left) and Northern (right) blot analyses of *CRA* integrated into plasmid pYV. pYV1024 DNA was restricted with *Hin*dIII and *Asp*-718 and hybridized with the *Xba*I-*Sal*I fragment of pMSH20 containing *yopE* and *CRA* for the Southern blot and with pMS100 containing *yopE* for the Northern blot. As expected, a single band was detected in the parental plasmid (lane 1), and two fragments of 2.9 and 1.7 kb were in the recombinant plasmid pKNG1024 (lane 2). Northern blot analysis shows an mRNA with increased size in the recombinant strain (lane 2) compared with the wild type (lane 1). (B) Coomassie blue-stained SDS-polyacrylamide gel and immunoblot analysis of released proteins of bacteria incubated at 37°C in the absence of Ca^{2+}. The immunoblot was resolved with the anti-YopE monoclonal antibody 6G1 diluted 1/200. Yops are designated according to the conventional nomenclature.

Immunization of mice with a CRA-producing strain. We then examined whether gastric inoculation of mice with the recombinant *Y. enterocolitica* strain would elicit an immune response against CRA. We first monitored colonization of the intestinal lumen of mice by the strain producing YopE-CRA and by the parental strain by measuring the quantity of bacteria excreted in the feces. Two groups of 10 C57BL/6J mice were inoculated with either 1.4×10^{10} bacteria of strain KNG1024(pYV1024) or 2.1×10^{10} bacteria of strain KNG1024(pKNG1024). At days 6 and 11, feces were taken from five mice and tested by luminometry. At day 6, we detected 7.7×10^3 to 4.6×10^4 bacteria per mg of stools isolated from mice that received KNG1024(pKNG1024). In the same time, we detected 1.1×10^4 to 8.5×10^4 bacteria per mg of stools isolated from mice that received KNG1024(pYV1024). No bacteria could be detected by luminometry 11 days after the inoculation. At day 14, mice were inoculated again with 1.1×10^{10} or 1.6×10^{10} bacteria of strain KNG1024(pYV1024) or KNG1024(pKNG1024), respectively, and tested for the presence of bacteria in the intestinal lumen. Only a few bacteria (about 10^2/mg of feces) in a limited number of mice (one or two mice) were detected 4 days after the inoculation. There was no significant difference in the colonization capacity of the strain carrying pKNG1024 or the wild-type pYV1024 plasmid.

We then studied the antibody response to CRA in these mice. Plasma samples were collected 12 days after the first inoculation and 11 days after the second inoculation. Analysis by anti-CRA ELISA (Fig. 5) showed that all the mice

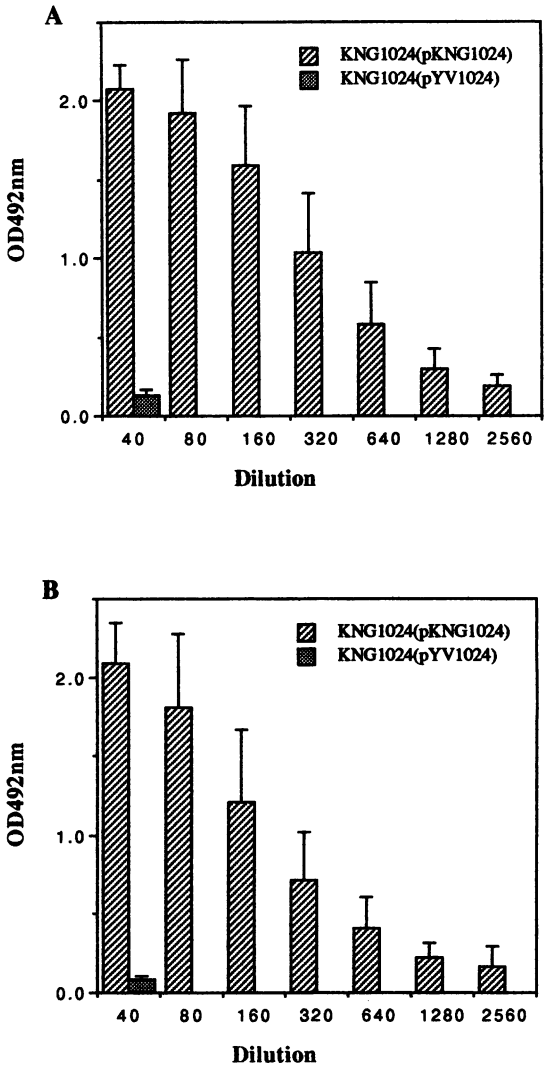

FIG. 5. Anti-CRA ELISA of plasma samples from mice inoculated with strain KNG1024(pYV1024) or KNG1024(pKNG1024). Plasma samples were tested 12 days after the first inoculation (A) and 11 days after the second inoculation (B). Each column shows the mean optical density (OD) and standard deviation.

that received KNG1024(pKNG1024) developed significant levels of antibodies against CRA. No increase in this response was observed after the second inoculation.

DISCUSSION

We showed previously that inoculation of mice with a *Y. enterocolitica* strain producing intracellular CT-B elicits a serum and mucosal antibody response against CT-B (33). However, when CT-B is produced as a hybrid YopH–CT-B protein, no antibody response is detected (33). This result probably reflects the inability of the chimeric protein to assemble into the characteristic B pentamer of CT-B. Thus, this model did not enable assessment of the value of Yops as in vivo-secreted carriers of foreign epitopes.

As a new model, we used the cytoplasmic CRA protein of the agent of Chagas' disease. By fusion of the *CRA* gene to *yopE*, we obtained secretion of a hybrid protein by *Y. enterocolitica*. To our knowledge, this is the first time that a

eukaryotic protein has been found to be produced and secreted by *Y. enterocolitica*. This bacterium, like *Bacillus subtilis*, could thus be used to secrete biotechnologically important proteins. Indeed, *Y. enterocolitica* grows easily and has a specific and powerful secretion system. These two properties are relevant to the use of this bacterium as an in vitro producer of recombinant proteins. Its pathogenic capacity is now quite well understood and could easily be disarmed. The chromosome gene encoding the enterotoxin Yst (9) should be removed, and the virulence could be decreased by removing one or the other *yop* gene or eventually *yadA* (for a recent review, see reference 8).

In this study, the CRA protein was produced as a hybrid Yop-CRA protein. When live recombinant bacteria were given by gastric intubation to mice, a good antibody response was obtained, confirming that *Y. enterocolitica* can be used to deliver heterologous proteins to the immune system. In this work, we studied only the serum antibody response. However, the production of hybrid Yop-CRA proteins could be used to analyze the serum, secretory, and cell-mediated immune responses elicited by hybrid proteins secreted during infection by recombinant *Y. enterocolitica*.

To stabilize the production of CRA, we integrated the fusion gene in plasmid pYV itself. Integration into pYV by a double-homologous recombination allowed maximal stability of the construction. Indeed, all bacteria isolated from the intestinal tissue 4 days after inoculation still harbored the *CRA* gene.

Production of the hybrid YopE′-CRA′-YopE″ in *Y. enterocolitica* resulted in a decrease in Yop production. Since the number of copies of *CRA* is the same as that of the other *yop* genes, the decrease in Yop production could not result from the titration of VirF or some other transcriptional regulator. One could speculate that it results from titration of the secretion factors by the hybrid protein.

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