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Detection of RTX Toxin Genes in Gram-Negative Bacteria with a Set of Specific Probes

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The family of RTX (RTX representing repeats in the structural toxin) toxins is composed of several protein toxins with a characteristic nonapeptide glycine-rich repeat motif. Most of its members were shown to have cytolytic activity. By comparing the genetic relationships of the RTX toxin genes we established a set of 10 gene probes to be used for screening as-yet-unknown RTX toxin genes in bacterial species. The probes include parts of *apxIA*, *apxIIA*, and *apxIIIA* from *Actinobacillus pleuropneumoniae*, *cyaA* from *Bordetella pertussis*, *frpA* from *Neisseria meningitidis*, *prtC* from *Erwinia chrysanthemi*, *hlyA* and *elyA* from *Escherichia coli*, *aaltA* from *Actinobacillus actinomycetemcomitans* and *lktA* from *Pasteurella haemolytica*. A panel of pathogenic and nonpathogenic gram-negative bacteria were investigated for the presence of RTX toxin genes. The probes detected all known genes for RTX toxins. Moreover, we found potential RTX toxin genes in several pathogenic bacterial species for which no such toxins are known yet. This indicates that RTX or RTX-like toxins are widely distributed among pathogenic gram-negative bacteria. The probes generated by PCR and the hybridization method were optimized to allow broad-range screening for RTX toxin genes in one step. This included the binding of unlabelled probes to a nylon filter and subsequent hybridization of the filter with labelled genomic DNA of the strain to be tested. The method constitutes a powerful tool for the assessment of the potential pathogenicity of poorly characterized strains intended to be used in biotechnological applications. Moreover, it is useful for the detection of already-known or new RTX toxin genes in bacteria of medical importance.

The exotoxins belonging to the family of pore-forming proteins named RTX toxins (RTX representing repeats in the structural toxin) (44) play an important role in the virulence of a variety of human and animal gram-negative bacterial pathogens. The RTX toxin family is still growing and currently consists of 16 proteins for which the genes have been completely sequenced (for a recent review, see reference 43). They include toxins of hemolytic *Escherichia coli* strains (13, 38), *Pasteurella haemolytica* (28) and *P. haemolytica*-like bacteria, (6), *Actinobacillus suis* (5), *Actinobacillus pleuropneumoniae* (7, 8, 23), *Actinobacillus actinomycetemcomitans* (24), *Bordetella pertussis* (18) and *Bordetella bronchiseptica* (1), *Neisseria meningitidis* (40, 41), *Erwinia chrysanthemi* (10), and *Serratia marcescens* (34). The known operons are similarly organized in a *CABD* pattern where *C* codes for the activation protein, *A* encodes the structural toxin, and *B* and *D* code for proteins involved in the secretion of the toxin. Not all RTX representatives have a secretion system of their own. ApxII, for example, takes advantage of the B and D proteins encoded by the *apxI* operon (9). An additional gene, which is *tolC* in the case of *E. coli* α-hemolysin, is needed for the secretion of the toxin but is not linked on the operon in the same cell (42). The *B. pertussis* cytolysin represents the only exception found so far, with the *tolC* analog being located on the same operon (26). As in the case of the hemolysin operon of *E. coli* (2), RTX toxin genes can be located either on large transmissible plasmids or on the chromosome, mainly on so-called "pathogenicity islands" (3, 31).

The common structural element of the RTX toxins is a domain of glycine-rich nonapeptide repeats with the consensus sequence L/I/F-X-G-G-X-G-N/D-D-X (43). The total number of repeats varies from 9 to 40 among the members of the RTX toxin family. These repeats bind calcium, which is needed for cytolytic activities exerted by most of the RTX toxins, and are therefore essential for toxin function, as deletion studies showed (4). The structural similarity of RTX toxins is to a certain extent also found on the nucleotide sequence level. Nevertheless, the entire family is heterogeneous enough to display little or no similarity in the nucleotide sequences of its most distant members. In this paper we report the design and testing of a limited number of probes to detect RTX toxins based on permutated sequence analysis and experimental evaluation as well as observations on the application of the probes to the screening of clinical isolates and bacterial strains used in biotechnology.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used as sources and positive controls for RTX toxins were hemolytic *E. coli* pHly152 (JF527), enterohemorrhagic *E. coli* CDC EDL933 (O157:H7; ATCC 43895), *A. pleuropneumoniae* reference strains (serotype 1, ATCC 27088; serotype 2, ATCC 27089; serotype 3, ATCC 27090; serotype 4, ATCC 33378; serotype 5a, ATCC 33377; serotype 5b, L20; serotype 6, ATCC 33590; serotype 7, WF83; serotype 8, 405; serotype 9, CVI 13261; serotype 10, 13039; serotype 11, 56153, and serotype 12, 8329 [15]), *P. haemolytica* type A1 (ATCC 14003), *E. chrysanthemi* B374 (I. Potrykus, Institute for Plant Sciences, ETH Zürich), *S. marcescens* JF1449, *B. pertussis* JF1418, *B. bronchiseptica* JF1398, *A. suis* ATCC 15558, *N. meningitidis* type B strains (HCUG 6114 and HCUG 5658) (R. Auckenthaler, National Center for Meningococci, Hôpital Cantonal Universitaire Geneva, Geneva, Switzerland), and *A. actinomycetemcomitans* serotype B (BE80 and BE86) (A. Mombelli, School of Dental Medicine, Bern, Switzerland). Field strains with no defined RTX toxin genes were isolated from clinical material of diseased humans and animals (see Table 3).

The *E. coli* K-12 derivatives HB101, XL1-Blue, C600, and 5K were used as negative controls (25).

DNA preparation. Lysates of the strains studied were done in 500 μl of lysis buffer containing 50 mM KCl, 20 mM dithiothreitol, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% (wt/vol) gelatin, 0.05 mg of proteinase K per ml, and 1.5

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TABLE 1. Currently known and sequenced genes of the RTX toxin family

Gene	Activity	Species	Reference	Accession no.
hlyA	Hemolysin	*E. coli*	13	M10133
elyA	Hemolysin	*E. coli*	38	X79839
lktA	Leukotoxin	*P. haemolytica*	28	M24197
pllktA	Leukotoxin	*P. haemolytica*-like sp.	6	L12148
cyaA	Cyclolysin	*B. pertussis*	17	Y00545
bbcyaA	Cyclolysin	*B. bronchiseptica*	1	Z37112
aaltA	Leukotoxin	*A. actinomycetemcomitans*	24	X16829
apxIA	Hemolysin	*A. pleuropneumoniae*	14	X68595
apxIIA	Hemolysin	*A. pleuropneumoniae*	8	M30602
apxIIIA	Leukotoxin	*A. pleuropneumoniae*	7	L12145
ashA	Hemolysin	*A. suis*	5	M90440
prtB	Protease	*E. chrysanthemi*	11	M60395
prtC	Protease	*E. chrysanthemi*	11	M60395
sprA	Protease	*S. marcescens*	34	X04127
frpA	Iron-reg.[a] protein	*N. meningitidis*	41	L06302
frpC	Iron-reg. protein	*N. meningitidis*	40	L06299

[a] Iron-reg., iron-regulating.

mM sodium dodecyl sulfate (SDS) at 37°C for 1 h. Proteinase K was inactivated for 10 min at 95°C.

Genomic DNA was isolated by the guanidinium thiocyanate method of Pitcher et al. (36), treated with RNase, and purified by phenol-chloroform extraction.

Cloning procedures. Toxin gene fragments for cloning were generated by PCR with oligonucleotides containing restriction sites as primers. Plasmid or genomic DNA harboring the corresponding toxin genes was used as template.

Plasmids used were pJFF750 (*apxIA* [19]), pJFF810 (*apxIIA* [30]), pACT7 (*cyaA* [39]), pHLY152 (*hlyA* [35]), pCVD419 (partial *elyA* [38]), pUNCH216 (*frpA* [41]), and pRUW520 (*prtC* [11]).

Genomic DNA of *A. actinomycetemcomitans*, *P. haemolytica*, and *A. pleuropneumoniae* served as template for the amplification of fragments for the *aaltA*, *lktA*, and *apxIIIA* genes, respectively. PCR fragments were purified with the QIAquick PCR purification kit (QIAGEN, Basel, Switzerland). Plasmid pBluescriptII-SK$^-$ and purified PCR fragments were digested with the corresponding restriction enzymes, run on an agarose gel (Sigma, Buchs, Switzerland), purified by Jet-Sorb (Genomed, Research Triangle Park, N.C.), and ligated for 2 h at room temperature before transformation of *E. coli* K-12 strain DH5-α by the calcium chloride method (37). Isolated clones were sequenced in order to exclude cloning artifacts.

Sequencing and sequence analysis. Sequencing was done with the PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems/Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's recommendations by using vectors T3 and T7 matching primers flanking the cloned inserts in pBluescriptII-SK$^-$. The sequencing reactions were run on an Applied Biosystems DNA Sequenator AB373 automated sequencing device.

Sequence comparisons were done with the Genetics Computer Group (GCG) program package using FASTA (Wisconsin package; GCG, Madison, Wis.) or with the PCGene package (IntelliGenetics, University of Geneva, Switzerland). Phylogenetic relationship was established with PILEUP and by further analysis with the Mega 1.02 program: corrected distances were calculated with the Jukes-Cantor algorithm, and a tree was derived by the neighbor-joining method.

Probe preparation. To get pure, plasmid-contaminant-free probes, the cloned RTX gene fragments were excised with the appropriate restriction enzymes according to the suppliers' recommendations, purified twice over agarose gels with the Jet-Sorb kit (Genomed), and then used as template for PCR with internal primers. This yielded enough pure template to be used for the dot blot assay. PCR was performed with either a PE9600 or PE2400 automated thermocycler with MicroAmp tubes (Perkin-Elmer Cetus). The reaction was carried out in a 50-μl volume containing 5 μl of 10× buffer (supplied with *Taq*), 20 pmol of each primer, 1 mM deoxynucleoside triphosphate (dNTP), 2.5 U of *Taq* DNA polymerase (Boehringer GmbH, Mannheim, Germany) and 10 to 100 ng of DNA. Cycling conditions were as follows: 3 min at 94°C followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min. For the RTXCYAA and RTXPRTC probes the annealing temperature was 60°C.

Labelled PCR fragments were produced by adding alkali-labile digoxigenin-11 (DIG-11)–dUTP (Boehringer) at a final concentration of 20 pM to the PCR mixture. Total genomic DNA was labelled by random priming with the alkali-labile DIG-High Prime DNA labelling kit (Boehringer). The removal of DIG-labelled probes in order to rehybridize filters was achieved by alkaline wash according to the supplier's protocol (Boehringer).

Blotting and hybridization. Reverse dot blots were done with unlabelled specific PCR fragments applied on the filter, which was hybridized with labelled total genomic DNA of various strains. Approximately 200 or 20 ng of PCR product was solubilized in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), denatured by adding 50 μl of 1 M NaOH for 20 min at room temperature, and neutralized by adding 50 μl of 1 M HCl. The denatured DNA was then applied to the nylon membrane by using a Bio-dot apparatus (Bio-Rad Laboratories, Hercules, Calif.) according to the manufacturer's instructions, with 100 μl of DNA solution per well.

For dot blots used in the screening of different strains with a given labelled probe, 5 μl of lysate or 200 ng of genomic DNA was solubilized in 100 μl of TE buffer, denatured with 50 μl of 1 M NaOH for 20 min at room temperature, and neutralized by the addition of 50 μl of 1 M HCl. The sample was then applied to the nylon membrane by using a Bio-dot apparatus (Bio-Rad Laboratories) according to the manufacturer's instructions, with 100 ng of DNA per well.

Southern blotting was done by alkaline transfer of agarose gels onto positively charged nylon membranes (Boehringer) with an LKB 2016 VacuGene vacuum blotting pump (Pharmacia LKB Biotechnology AB, Bromma, Sweden). Gels were treated with 0.25 M NaOH prior to the blotting procedure. After blotting, filters were baked for 30 min at 80°C under vacuum. After at least 1 h of prehybridization, hybridization was carried out in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–1% blocking reagent (Boehringer)–0.1% *N*-lauroylsarcosine-Na-salt–0.02% SDS at 68°C overnight. Filters were washed under nonstringent conditions twice for 5 min each with 50 ml of 2× SSC–0.1% SDS per 100 cm^2 at room temperature, followed by medium-stringency washing twice for 15 min each with 50 ml of 0.2× SSC–0.1% SDS per 100 cm^2 at room temperature. The filters were then processed with phosphatase-labelled anti-DIG antibody according to the producer's protocol. Signals were produced with chemiluminescent substrate (CSPD or CDP-Star; Boehringer). Signal detection was done on X ray films with a GS-250 molecular imager (Bio-Rad).

RESULTS

Establishing the RTX toxin family. In order to investigate the relationships between the various toxins belonging to the RTX toxin family, sequence comparisons were carried out. Table 1 gives a list of the toxin genes, the toxin activities, the species the toxins were isolated from, and the accession numbers of the sequences that were used in this study. Since the structural and functional similarities of the glycine-rich repeats are determined within the toxin-coding part of the operon, the analysis focused on these regions. Hence, sequences for the genes encoding the structural toxin were phylogenetically analyzed by generating a tree with the Mega software and were also directly compared with FASTA of the GCG package. The phylogenetic tree is given in Fig. 1. From these data four major groups of RTX toxin genes can be distinguished. One contains *prtC* and *prtB* from *E. chrysanthemi*, which show 80% similarity, and *sprA* of *S. marcescens*, which is about 68% similar to the *prt* genes. A second group includes *frpA* and *frpC* of *N. meningitidis*, which have 90% similarity, and *cyaA* of *B. pertussis* and *bbcyaA* of *B. bronchiseptica*, which have only 54% similarity with the two *frp* genes but which share 98% identical nucleo-

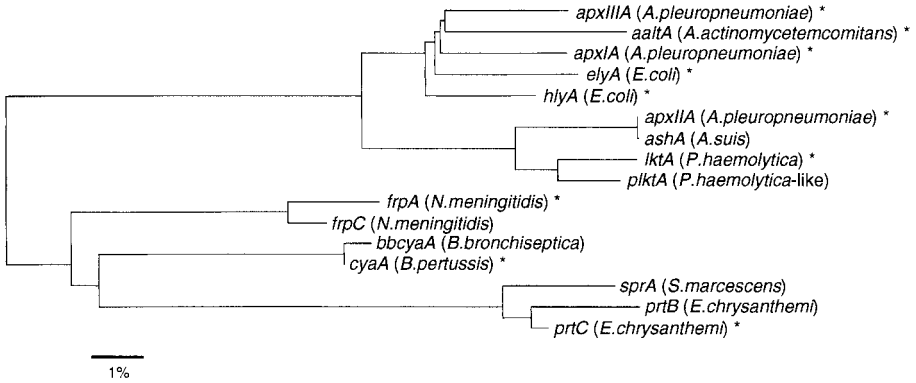

FIG. 1. Phylogenetic relation of the RTX toxin genes. The 1,500 bp at the 3′ ends of the genes, covering the glycine-rich repeats and being part of the probes (except for the RTXCYAA probe), were aligned with the PILEUP program of the GCG package followed by tree-building with the Mega 1.02 program. Distances were calculated by the Jukes-Cantor algorithm, and the tree was built with the "neighbor-joining" settings. Toxin genes used for the construction of probes are indicated by asterisks.

tides among themselves. The third group is defined by *apxIIA* of *A. pleuropneumoniae* and *ashA* of *A. suis* (which are virtually identical), *lktA* of *P. haemolytica* (67% similarity to *apxIIA*), and *plktA* of *P. haemolytica*-like strains (also 67% similarity to *apxIIA*). The latter shows 76% similarity to *lktA*. The largest and most heterogeneous group comprises the *E. coli* hemolysin genes *hlyA* and *elyA* (64% similarity), *apxIA* (60% homology to each of the hemolysin genes), and *apxIIIA*. The latter shows 60% similarity to *apxIA* and about 56% similarity to each of the *E. coli hly* genes. The *aaltA* gene of *A. actinomycetemcomitans*, which is also contained within this group, shares 60% identical nucleotides with the other members of the group.

Construction of probes. The phylogeny and similarity analyses led to the selection of 10 single probes for the RTX toxin genes that are indicated by asterisks in Fig. 1. They include parts of genes *prtC*, *frpA*, *cyaA*, *apxIA*, *apxIIA*, *apxIIIA*, *hlyA*, *elyA*, *lktA*, and *aaltA*. For safety reasons segments rather than entire genes were used for the construction of the probes in order to avoid the generation of strains harboring functional virulence genes. All fragments, except the *cyaA*-specific probe, cover the region coding for the characteristic glycine-rich repeat motif which shows the highest similarity among the various RTX toxin genes. The *cyaA*-specific probe contains the first half of the repeat region, which extends over more than 2 kb in this gene. Table 2 summarizes the genes and regions that were chosen to build the probes, the primers that were used to generate PCR fragments from the corresponding genes, and the different clones that were established. Restriction sites were included in primers in order to enable easy, and for some probes consecutive, cloning.

Plasmid pRTXAPXIA, which contains the probe based on the *apxIA* gene, was constructed by PCR with pJFF750 (19) as template and primers RTX1-L and RTX1-R. The resulting PCR product was digested with *Xba*I and ligated into the

TABLE 2. Plasmids containing RTX gene probes generated in this study

Plasmid[a]	Gene (segment)[b]	Primers		Length (bp)	Cloning site	PCR annealing temp (°C)
		Designation	Sequence[c] (5′ to 3′)			
pRTXAPXIA	*apxIA* (2102–2999)	RTX1-L	GT**TCTAGA**CATTCGAACTTGGGAACG	900	*Xba*I	52
		RTX1-R	CT**TCTAGA**CCGCCTGAGCCTACAGAT			
pRTXAPXIIA	*apxIIA* (1831–2530)	RTX2-L	CT**TCTAGA**ATCGCAAATTTAGGTGCT	700	*Xba*I-*Bam*HI	52
		RTX2-R	TT**GGATCC**ACCAATTCCCAATACGAA			
pRTXCYAA	*cyaA* (2709–3605)	RTX3B-L	GGC**GGATCC**AAGCACAGCATCAAC	895	*Bam*HI-*Eco*RI	60
		RTX3B-R	GCG**GAATTC**TCGATGTCATCGCTGAC			
pRTXFRPA	*frpA* (2250–2984)	RTX4-L	GA**GAATTC**GCAAAATGTAGGCTTTGG	735	*Eco*RI-*Kpn*I	52
		RTX4-R	AC**GGTACC**CTTCGCCGAAGACATAAG			
pRTXPRTC	*prtC* (893–1422)	RTX5-L	TG**GGTACC**CGGCGGCACCGACACCTT	530	*Kpn*I	60
		RTX5-R	TA**GGTACC**TGTGCCGCCTGACCGACA			
pRTXHLYA	*hlyA* (2098–2771)	RTX6II-L	TAT**GAATTC**ACTCATATCAATGG	675	*Eco*RI	52
		RTX6II-R	TCT**GAATTC**TGATTAGAGATATCACCTGACTC			
pRTXEHEC	*elyA* (2140–2922)	RTX7-L	GAT**GAATTC**AAAGGCGGTAA	790	*Eco*RI	52
		RTX7-R	TAA**GAATTC**ATCACCTGAATCGAAC			
pRTXALTA	*aaltA* (1870–2732)	RTX8-L	GTC**GGATCC**GGTTCAACAA	870	*Bam*HI	52
		RTX8-R	TAC**GGATCC**AATCCTGCCGATGAATT			
pRTXAPXIIIA	*apxIIIA* (2029–2576)	RTX9-L	GCG**GAATTC**TGTTACGCGCGAATTG	545	*Eco*RI	52
		RTX9-R	GCG**GAATTC**GCTACCATCGCCTCCT			
pRTXLKTA	*lktA* (1715–2537)	RTX10-L	GCG**GAATTC**AGATGGTGCAGCAAGTT	820	*Eco*RI	52
		RTX10-R	GCG**GAATTC**AAATCAGCCTCTCGGAAC			

[a] The designation of the gene probe is the same as that for the plasmid without the prefix "p."
[b] The segment is given in the nucleotide coordinates of the corresponding toxin gene sequence.
[c] Bases in boldface indicate restriction enzyme cleavage sites.

corresponding site of pBluescript. For gene probe preparation the 900-bp insert was cut with *Xba*I and a second PCR amplification was done on the purified insert with the same primers. Plasmid pRTXAPXIIA, which is based on the *apxIIA* gene, was constructed with pJFF810 (30) as a template and the primers RTX2-L and RTX2-R for PCR amplification. The PCR product was double digested with *Bam*HI-*Xba*I and subsequently ligated into pBluescript digested with the same enzymes. The 700-bp gene probe was amplified with the purified insert of pRTXAPXIIA, which had been excised with *Bam*HI-*Xba*I, as template and the primers RTX2-L and RTX2-R in a PCR. Plasmid pRTXAPXIIIA was constructed with genomic DNA of *A. pleuropneumoniae* serotype 2 as a template and the primer pair RTX9-L and RTX9-R in a PCR amplification. The resulting fragment was restricted with *Eco*RI and cloned accordingly into pBluescript. The gene probe was produced by PCR with the purified *Eco*RI fragment from pRTXAPXIIIA as a template and the primer pair RTX9-L and RTX9-R, resulting in a 545-bp probe.

Plasmid pRTXCYAA, based on the *cyaA* gene of *B. pertussis*, was constructed with pACT7 (39) as template and the primers RTX3B-L and RTX3B-R. The resulting 895-bp fragment was then simultaneously digested with *Bam*HI and *Eco*RI and ligated into the matching sites of pBluescript. Afterwards, the gene probe was amplified with the same primers and the purified insert cut out of pRTXCYAA with *Bam*HI-*Eco*RI as template.

Plasmid pRTXFRPA, containing part of *frpA*, was constructed by PCR amplification with pUNCH216 (41) as a template and primers RTX4-L and RTX4-R. The PCR product was digested with *Eco*RI-*Kpn*I and subsequently ligated into the equivalent sites of pBluescript. For gene probe production, the 735-bp insert was excised with *Eco*RI and *Kpn*I, and a second PCR was performed on the purified fragment with the same primer pair.

pRTXPRTC is the plasmid specific for the *prtC* gene found in *E. chrysanthemi* and was constructed with pRUW520 (11) as a template and primers RTX5-L and RTX5-R in a PCR amplification. The 530-bp product was digested with *Kpn*I and cloned into the matching site of pBluescript. The gene probe was then synthesized by PCR with the purified *Kpn*I fragment from pRTXPRTC as a template and primers RTX5-L and RTX5-R.

Plasmids harboring the probes for the hemolysin gene clusters found in uropathogenic *E. coli* and for the enterohemolysin genes of enterohemorrhagic *E. coli* strains were constructed as follows. pRTXHLYA was assembled by using pHLY152 (35) as a template and the primers RTX6II-L and RTX6II-R in a PCR amplification. The resulting *hlyA*-specific fragment was digested with *Eco*RI and cloned into the corresponding site of vector pBluescript. The 680-bp fragment was afterwards excised with *Eco*RI, purified, and applied as a template in a second PCR with the same primer pair. Plasmid pRTXEHEC was created with pCVD419 (38) as a template and primers RTX7-L and RTX7-R in a PCR amplification. The product was subsequently digested with *Eco*RI and cloned into the same site of pBluescript. Preparation of this *elyA* (enterohemorrhagic *E. coli hlyA*)-specific 780-bp gene probe was done with the purified insert excised with *Eco*RI as a template and the primer pair RTX7-L and RTX7-R in a PCR amplification.

A plasmid specific for *aaltA* was produced with genomic DNA of *A. actinomycetemcomitans* as a template and primers RTX8-L and RTX8-R in a PCR amplification. Digestion of the 870-bp PCR product with *Bam*HI and its cloning into the corresponding site in pBluescript resulted in plasmid pRTX ALTA. The PCR probe was generated with the purified *Bam*HI insert of pRTXALTA as a template and the primers RTX8-L and RTX8-R.

Plasmid pRTXLKTA was generated by PCR amplification of a characteristic part of *lktA* with genomic DNA of *P. haemolytica* JF1253 as template and primers RTX10-L and RTX10-R. The resulting 820-bp fragment was then cloned into the *Eco*RI site of pBluescript. The *lktA*-specific gene probe was amplified with the purified *Eco*RI insert of pRTXLKTA as a template and primer pair RTX10-L and RTX10-R.

Screening with individual probes. The 10 single probes listed in Table 2 were tested for redundancy by using them as templates for sequential hybridization with all the single probes. Figure 2 shows the extent of cross-hybridization between a few probes.

To evaluate the probes, we analyzed three groups of selected bacteria as shown in Table 3: (i) bacteria known to contain the RTX toxins used for probe construction as a control, (ii) bacteria known or supposed to contain other RTX toxins, and (iii) bacteria with no known RTX toxins. As a negative control we used *E. coli* K-12 derivatives known to be devoid of virulence genes (32, 33).

We hybridized DNA from different bacterial strains with the RTX probes listed in Table 1 by the dot blot technique. The results of this screening are summarized in Table 3. In order to standardize the signal intensities, which might vary due to different amounts of DNA applied to the filters, the first probe was removed and the filters were rehybridized with a probe specific for the 16S rRNA gene. Blots with a strong deviation signal for the 16S rRNA were repeated with adjusted amounts of target DNA.

When a simple method was used to label the probes, which involved direct labelling with DIG during PCR with the universal T7 and T3 primers matching the flanking segments of the vector's cloning sites, high levels of background signals were observed. These signals were detected as relatively strong hybridization signals of *E. coli* K-12 strains with virtually all probes. Such signals were absent with highly purified DNA probes, as described in Materials and Methods.

In order to evaluate the significance of positive dot blot hybridization signals, a few medically and biotechnologically relevant strains which gave a positive signal with probe RTX CYAA were further analyzed by Southern blotting (Fig. 3). Genomic DNA was digested with *Hin*dIII, run on an agarose gel, and transferred to a nylon membrane. The filter was then hybridized with probe RTXCYAA. The strains of *Campylobacter rectus*, *Aeromonas* spp., *Yersinia enterocolitica*, *Pseudomonas putida*, and *Xanthomonas campestris* showed discrete hybridization bands (Fig. 3), thus strengthening the assumption that RTX-like toxin genes were present. Only faint and multiple bands were observed with the *Salmonella* strains, and no hybridization signal was obtained with *Shigella* spp. or *Pseudomonas aeruginosa* (Fig. 3).

Analysis of potential RTX toxin genes in specific strains. In order to get a comprehensive picture of potential RTX toxin genes in a given strain, we developed a method which enables a rational broad-range screening for this group of genes. For this purpose we bound plasmid-free PCR amplification products of the 10 unlabelled RTX probes onto nylon membranes and hybridized these membranes with labelled total genomic DNA. Figure 4 shows the results of such an experiment using *Pasteurella aerogenes* JF1319 and *E. coli* K-12 strain XL1-Blue. *P. aerogenes* strongly hybridized with the RTXAPXIIIA probe and also slightly with RTXAPXIA and RTXALTA. In the case of *E. coli* K-12 a hybridization signal was obtained solely with the control probe but not with any of the RTX toxin gene probes.

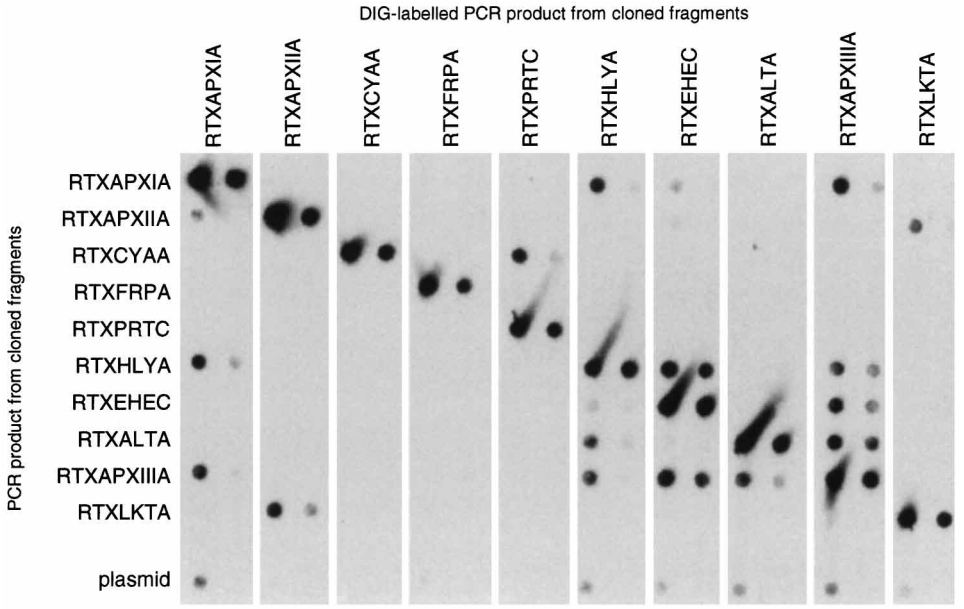

FIG. 2. Cross-hybridization of the RTX probes. Dot blot filters with approximately 100 and 10 ng of PCR product were prepared. PCR fragments were produced with internal primers by using twice-purified inserts of the corresponding RTX probes as templates. Probes were labelled by the addition of DIG-dUTP to the PCR mixture. Ten identical filters were produced and hybridized individually with all the single probes.

DISCUSSION

We report the establishment and evaluation of a method for the detection of RTX toxin genes and their use in the screening of gram-negative bacteria. The DNA probes used in this method are based on the *A* genes of RTX toxin operons coding for the structural toxins. The *B* and *D* genes involved in secretion of RTX toxins were not considered in this study since they belong to a family of ATP-dependent transporter genes (22, 29). They would therefore detect genes which are not related to RTX toxins. Moreover, certain RTX toxin operons are devoid of secretion genes and would remain undetected with probes derived from *B* and *D* genes.

Of the 16 known structural RTX toxin genes, the 10 most divergent genes were chosen for the development of the probes by a comparative phylogenetic analysis. The aim was to construct a set of probes which is able to detect the broadest possible range of RTX and RTX-like toxin genes. The weak cross-hybridization seen among these probes (Fig. 2) supports this approach. Initial attempts to combine the individual probes by cloning them onto a single plasmid and using the interlinked probes as a general probe for RTX toxin genes failed. The DNA fragment containing the combined RTX probes resulted in high background due to unspecific hybridization, as shown with *E. coli* K-12 derivatives. In addition the combined probe showed a strongly decreased sensitivity for certain RTX genes which were included in the probe. Mixing the labelled single probes revealed the same problems as those of the combined probes, i.e., high background and loss of sensitivity for certain RTX toxin genes. We conclude that the nonspecific hybridization leading to a high background was caused by minor amounts of plasmid sequences in the probes resulting from linear amplification of plasmid sequences. These hybridize strongly with *E. coli* and other strains as seen in Fig. 4. The loss of sensitivity for certain RTX toxin genes is supposed to be due to competition of hybridization among different probes which do contain to some extent repeated sequences. It is therefore essential that highly purified probes be used individually for the hybridizations, which must be done under low-stringency conditions.

Table 3 summarizes the results of the screening of a large number of gram-negative bacteria with the 10 RTX probes. All strains containing the RTX genes from which the probes were derived reacted with the corresponding gene probe (Table 3). They also reacted to some extent with other probes, as expected from the cross-hybridization experiments. All strains known to contain RTX toxin genes which were not used for the construction of the probes gave clear hybridization signals with at least the probe of the most closely related RTX toxin gene (Table 3). Thus, e.g., both strains of *S. marcescens*, known to possess the *sprA* gene, hybridized with the RTXPRTC probe (Table 3), which is derived from the related *prtC* gene of *E. chrysanthemi* (Fig. 1).

Of the 139 strains of various species for which no RTX toxin genes are described (Table 3), 40 strains gave hybridization signals with the probes used. For a few of these species RTX-like proteins have been postulated. Thus, *P. aeruginosa*, which reacted with probes RTXCYAA and RTXPRTC, has been described as containing an RTX-like protease (AprA) which seems to use a secretion pathway similar to the PrtB protease of *E. chrysanthemi* (12). The hybridization signal with RTX CYAA which was observed in 8 of 12 strains of *Yersinia* spp. might indicate the presence of the *yopB* gene, which is supposed to encode an RTX-like virulence protein (20). Moreover, RTX determinants are postulated to be in *Haemophilus* spp. (27). They might have led to hybridization with the RTX FRPA probes in 7 of the 13 strains tested. An RTX-like toxin has also been proposed for *C. rectus* (16), which gave a positive signal with the RTXCYAA probe both on dot blots and Southern blots. However, *C. rectus* did not hybridize with the RTX ALTA probe for the leukotoxin AaltA of *A. actinomycetemcomitans*, although a cytotoxin similar to AaltA was hypothesized (16).

For the other species which gave positive hybridization signals, no RTX-like proteins have ever been proposed. The

TABLE 3. Hybridization signals with bacterial species by using RTX probes

Species or strain	No. of strains positive/no. analyzed	Probe yielding major hybridization signal[d]	RTX toxin gene(s)[e]
A. actinomycetemcomitans[a]	1/1	ALTA	*aaltA*
A. pleuropneumoniae (serotype 10)[a]	1/1	APXIA	*apxIA*
A. pleuropneumoniae (serotypes 7 and 12)[a]	2/2	APXIIA	*apxIIA*
A. pleuropneumoniae (serotypes 1, 5, 9, and 11)[a]	6/6	APXIA, APXIIA	*apxIA*, *apxIIA*
A. pleuropneumoniae (serotypes 2, 3, 4, 6, and 8)[a]	9/9	APXIIA, APXIIA	*apxIIA*, *apxIIIA*
B. pertussis[a]	4/4	CYAA	*cyaA*
E. chrysanthemi[a]	1/2	PRTC	*prtC*, *prtB*
E. coli hemolytic (non-EH[f]) sp.[a]	31/31	HLYA	*hlyA*
E. coli hemolytic (EH) sp.[a]	3/3	EHEC	*elyA*
N. meningitidis[a]	2/2	FRPA	*frpA*, *frpC*
P. haemolytica[a]	9/10	LKTA	*lktA*
Actinobacillus lignieresii[b]	1/2	APXIA	*apxI*-like gene
Actinobacillus rossii[b]	1/1	APXIIA	*apxII*-like gene
A. suis[b]	1/1	APXIIA	*ashA*
B. bronchiseptica[b]	9/9	CYAA	*bbcyaA*
P. haemolytica-like sp.[b]	2/2	LKTA	*plktA*
Proteus vulgaris[b]	1/1	HLYA	*hlyA*-like gene
S. marcescens[b]	2/2	PRTC	*sprA*
Actinobacillus spp.[c]	0/8	—	Unknown
Aeromonas spp.[c]	2/2	CYAA	Unknown
Agrobacterium tumefaciens[c]	0/1	—	Unknown
Branhamella ovis[c]	0/2	—	Unknown
C. rectus[c]	1/1	CYAA	Unknown
Campylobacter spp.[c]	0/5	—	Unknown
Citrobacter freundii[c]	0/1	—	Unknown
Enterobacter cloacae[c]	0/1	—	Unknown
E. coli K-12[c]	0/4	—	Negative control
E. coli B[c]	0/2	—	Unknown
E. coli B (TOPP)[c]	0/1	—	Unknown
Flavobacterium indologenes[c]	0/1	—	Unknown
Haemophilus spp.[c]	7/13	FRPA	Unknown
Hafnia alvei[c]	0/1	—	Unknown
Helicobacter spp.[c]	0/2	—	Unknown
Klebsiella spp.[c]	0/3	—	Unknown
Legionella pneumophila[c]	0/1	—	Unknown
Morganella morganii[c]	0/1	—	Unknown
P. aerogenes[c]	1/3	APXIIIA	Unknown
Pasteurella spp.[c]	0/45	—	Unknown
Paracoccus denitrificans[c]	1/1	FRPA	Unknown
P. aeruginosa[c]	3/3	CYAA	Unknown
P. putida[c]	1/1	CYAA	Unknown
Proteus mirabilis[c]	0/2	—	Unknown
Salmonella spp.[c]	10/12	CYAA	Unknown
Shigella spp.[c]	4/5	CYAA	Unknown
Vibrio cholerae[c]	0/3	—	Unknown
Xanthomonas spp.[c]	2/2	CYAA	Unknown
Yersinia spp.[c]	8/12	CYAA	Unknown

[a] Bacteria possessing RTX genes contained in probes.
[b] Bacteria possessing potential RTX genes not contained in probes.
[c] Species containing no known RTX genes.
[d] Probes are indicated without the prefix RTX. Where several probes hybridized, only the most intense is given. —, no signal.
[e] RTX toxin gene(s) present in the species which is most likely reacting with the probe.
[f] EH, enterohemorrhagic.

presence of such genes, however, seems to be highly probable for many of them. In certain cases, including *Aeromonas* spp., *X. campestris*, and *P. putida*, the results of the dot blots were confirmed by Southern blots which showed clearly defined bands hybridizing with the corresponding probes. For *P. aerogenes*, which hybridized strongly with the RTXAPXIIIA probe, the presence of a complete RTX toxin operon has even been demonstrated by gene cloning and DNA sequence analysis (21). For a few strains, including *Salmonella* spp., *Shigella* spp., and *P. aeruginosa*, the hybridization results observed on dot blots gave no distinct bands by Southern blot analysis and might therefore represent nonspecific hybridization with probe RTXCYAA.

Of particular interest for the screening of potential RTX toxin genes in given strains is the reverse dot blot method. We tested the possibility of binding unlabelled RTX probes on nylon membranes and hybridizing them with total, labelled genomic DNA of a certain strain. This is demonstrated in Fig. 4 with *P. aerogenes*, which in the screening experiments was found to hybridize with the RTXAPXIIIA probe. This was

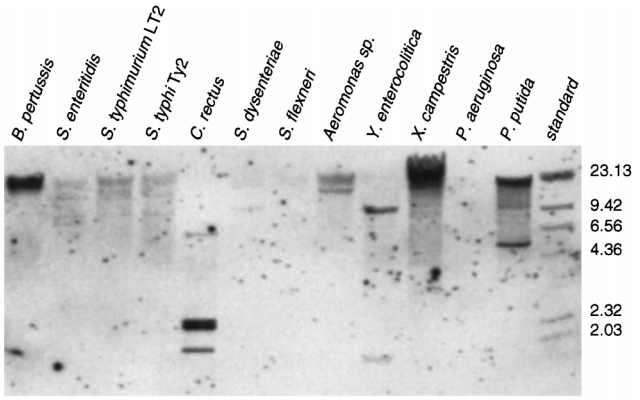

FIG. 3. Southern blot hybridization of genomic DNA of selected medically and biotechnologically relevant bacterial strains with probe RTXCYAA. One hundred nanograms of genomic DNA was digested with *Hin*dIII, run on agarose gels, and transferred to nylon membranes. Hybridization was done with RTX CYAA as the probe. Strains are as follows: *B. pertussis* JF1419 (positive control), *Salmonella enteritidis* NZ46-94, *Salmonella typhimurium* LT2 (ATCC 23564), *Salmonella typhi* Ty2 (JF246), *C. rectus* ATCC 33238^T, *Shigella dysenteriae* NZ1403-94, *Shigella flexneri* NZ936-94, *Aeromonas* sp. strain E367-93, *Y. enterocolitica* BG2 (NZ1775-92), *X. campestris* JF261, *P. aeruginosa* JF1608, and *P. putida* JF8. The standard is lambda DNA digested with *Hin*dIII.

confirmed by the reverse dot blot procedure. Moreover, there was also a signal detectable with the RTXAPXIA and RTX ALTA probes, showing the redundancy in the probe set already observed in Fig. 2. As a control we used *E. coli* K-12, which showed no reaction with any of the RTX probes. This reverse dot blot has the advantage of providing at the same time information about the nature of the RTX toxin gene that is detected.

In summary, we describe a set of probes that detects all known RTX toxin genes and that was able to identify several new RTX toxin genes. The probes have been applied in a screening of gram-negative bacteria, thereby showing that RTX toxin genes are probably widely distributed in this group. The DNA probes were also utilized in a reverse dot blot method which allows the analysis of a given strain with all probes in a single step. We present this method to test bacterial strains to be used in biotechnological processes for the presence of potential RTX toxin genes; it should be a valuable tool in biosafety assessment. Moreover, this method allows the identification of specific virulence factors of bacterial strains isolated from clinical specimens in medical research and diagnostics.

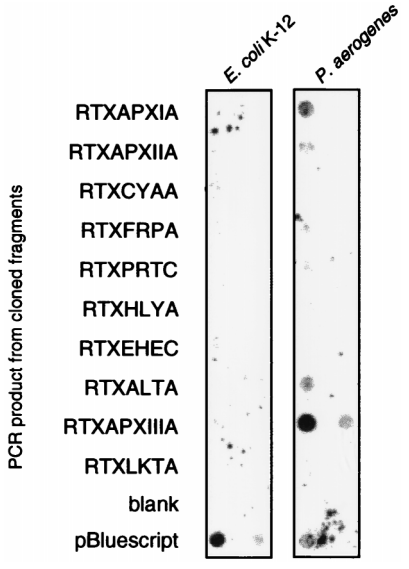

FIG. 4. Dot blot hybridization of RTX probes with DIG-labelled total genomic DNA. Dot blot filters with approximately 100 and 10 ng of DNA were prepared. PCR fragments were produced by using twice-purified inserts of the corresponding RTX probes as templates with internal primers. Plasmid pBluescriptII-SK$^-$ was used as a positive control for *E. coli* K-12. Left panel, hybridization with DIG-labelled genomic DNA of *E. coli* K-12 strain XL1-Blue; right panel, hybridization with DIG-labelled genomic DNA of *P. aerogenes* JF1319.

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REFERENCES

1. **Betsou, F., O. Sismeiro, A. Danchin, and N. Guiso.** 1995. Cloning and sequence of the *Bordetella bronchiseptica* adenylate cyclase-hemolysin-encoding gene: comparison with the *Bordetella pertussis* gene. Gene **162:**165–166.
2. **Blum, G., V. Falbo, A. Caprioli, and J. Hacker.** 1995. Gene clusters encoding the cytotoxic necrotizing factor type 1, Prs-fimbriae and α-hemolysin from the pathogenicity island II of the uropathogenic *Escherichia coli* strain J96. FEMS Microbiol. Lett. **126:**189–196.
3. **Blum, G., M. Ott, A. Lischewski, A. Ritter, H. Imrich, H. Tschäpe, and J. Hacker.** 1994. Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. Infect. Immun. **62:**606–614.
4. **Boehm, D. F., R. A. Welch, and I. S. Snyder.** 1990. Domains of *Escherichia coli* hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes. Infect. Immun. **58:**1959–1964.
5. **Burrows, L. L., and R. Y. C. Lo.** 1992. Molecular characterization of an RTX toxin determinant from *Actinobacillus suis*. Infect. Immun. **60:**2166–2173.
6. **Chang, Y.-F., D.-P. Ma, J. Shi, and M. M. Chengappa.** 1993. Molecular characterization of a leukotoxin gene from a *Pasteurella haemolytica*-like organism, encoding a new member of the RTX toxin family. Infect. Immun. **61:**2089–2095.
7. **Chang, Y.-F., J. Shi, D.-P. Ma, S. J. Shin, and D. H. Lein.** 1993. Molecular analysis of the *Actinobacillus pleuropneumoniae* RTX toxin-III gene cluster. DNA Cell Biol. **12:**351–362.
8. **Chang, Y. F., R. Young, and D. K. Struck.** 1989. Cloning and characterization of a hemolysin gene from *Actinobacillus* (*Haemophilus*) *pleuropneumoniae*. DNA **8:**635–647.
9. **Chang, Y.-F., R. Young, and D. K. Struck.** 1991. The *Actinobacillus pleuropneumoniae* hemolysin determinant: unlinked *appCA* and *appBD* loci flanked by pseudogenes. J. Bacteriol. **173:**5151–5158.
10. **Delepelaire, P., and C. Wandersman.** 1989. Protease secretion by *Erwinia chrysanthemi*: protease B and C are synthesized and secreted as zymogens without a signal peptide. J. Biol. Chem. **264:**9083–9089.
11. **Delepelaire, P., and C. Wandersman.** 1990. Protein secretion in gram-negative bacteria: the extracellular metalloprotease B from *Erwinia chrysanthemi* contains a C-terminal secretion signal analogous to that of *Escherichia coli* alpha-hemolysin. J. Biol. Chem. **265:**17118–17125.
12. **Duong, F., A. Lazdunski, B. Cami, and M. Murgier.** 1992. Sequence of a cluster of genes controlling synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretory pathways. Gene **121:**47–54.
13. **Felmlee, T., S. Pellett, and R. A. Welch.** 1985. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. J. Bacteriol. **163:**94–105.
14. **Frey, J., A. Haldimann, J. Nicolet, A. Boffini, and P. Prentki.** 1994. Sequence analysis and transcription of the *apxI* operon (hemolysin I) from *Actinobacillus pleuropneumoniae*. Gene **142:**97–102.
15. **Frey, J., and J. Nicolet.** 1990. Hemolysin patterns of *Actinobacillus pleuropneumoniae*. J. Clin. Microbiol. **28:**232–236.
16. **Gillespie, M. J., J. Smutko, G. G. Haraszthy, and J. J. Zambon.** 1993. Isolation and partial characterization of the *Campylobacter rectus* cytotoxin. Microb. Pathog. **14:**203–215.
17. **Glaser, P., D. Ladant, O. Sezer, F. Pichot, A. Ullmann, and A. Danchin.** 1988. The calmodulin-sensitive adenylate cyclase of *Bordetella pertussis*: cloning and expression in *Escherichia coli*. Mol. Microbiol. **2:**19–30.

18. **Glaser, P., H. Sakamoto, J. Bellalou, A. Ullmann, and A. Danchin.** 1988. Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-hemolysin bifunctional protein of *Bordetella pertussis*. EMBO J. **7:**3997–4004.
19. **Gygi, D., J. Nicolet, C. Hughes, and J. Frey.** 1992. Functional analysis of the Ca^{2+}-regulated hemolysin I operon of *Actinobacillus pleuropneumoniae* serotype 1. Infect. Immun. **60:**3059–3064.
20. **Hakansson, S., T. Bergman, J.-C. Vanooteghem, G. Cornelis, and H. Wolf-Watz.** 1993. YopB and YopD constitute a novel class of *Yersinia* Yop proteins. Infect. Immun. **61:**71–80.
21. **Heyberger-Meyer, B., P. Kuhnert, J. Nicolet, and J. Frey.** Unpublished data.
22. **Higgins, C. F., M. P. Gallagher, M. L. Mimmack, and S. R. Pearce.** 1988. A family of closely related ATP-binding subunits from prokaryotic and eukaryotic cells. Bioessays **8:**111–116.
23. **Jansen, R., J. Briaire, E. M. Kamp, A. L. J. Gielkens, and M. A. Smits.** 1993. Structural analysis of the *Actinobacillus pleuropneumoniae*-RTX-toxin I (ApxI) operon. Infect. Immun. **61:**3688–3695.
24. **Kraig, E., T. Dailey, and D. Kolodrubetz.** 1990. Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*: homology to the alpha-hemolysin/leukotoxin gene family. Infect. Immun. **58:**920–929.
25. **Kuhnert, P., J. Nicolet, and J. Frey.** 1995. Rapid and accurate identification of *Escherichia coli* K-12 strains. Appl. Environ. Microbiol. **61:**4135–4139.
26. **Laoide, B. M., and A. Ullmann.** 1990. Virulence dependent and independent regulation of the *Bordetella pertussis cya* operon. EMBO J. **9:**999–1005.
27. **Lo, R. Y.** 1990. Molecular characterization of cytotoxins produced by *Haemophilus*, *Actinobacillus*, *Pasteurella*. Can. J. Vet. Res. **54**(Suppl.)**:**S33–S35.
28. **Lo, R. Y. C., C. A. Strathdee, and P. E. Shewen.** 1987. Nucleotide sequence of the leukotoxin genes of *Pasteurella haemolytica* A1. Infect. Immun. **55:**1987–1996.
29. **Lobo, A. L., and R. A. Welch.** 1994. Identification and assay of RTX family of cytolysins. Methods Enzymol. **235:**667–678.
30. **Maier, E., N. Reinhard, R. Benz, and J. Frey.** 1996. Channel-forming activity and channel size of the RTX toxins ApxI, ApxII, and ApxIII of *Actinobacillus pleuropneumoniae*. Infect. Immun. **64:**4415–4423.
31. **McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper.** 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc. Natl. Acad. Sci. USA **92:**1664–1668.
32. **Mühldorfer, I., G. Blum, A. Donohue-Rolfe, H. Heier, T. Ölschläger, H. Tschäpe, U. Wallner, and J. Hacker.** 1996. Characterization of *Escherichia coli* strains isolated from environmental water habitats and from stool samples of healthy volunteers. Res. Microbiol. **147:**625–635.
33. **Mühldorfer, I., and J. Hacker.** 1994. Genetic aspects of *Escherichia coli* virulence. Microb. Pathog. **16:**171–181.
34. **Nakahama, K., K. Yoshimura, R. Marumoto, M. Kikuchi, I. S. Lee, T. Hase, and H. Matsubara.** 1986. Cloning and sequencing of *Serratia marcescens* protease gene. Nucleic Acids Res. **14:**5843–5855.
35. **Noegel, A., U. Rdest, and W. Goebel.** 1981. Determination of the functions of hemolytic plasmid pHly152 of *Escherichia coli*. J. Bacteriol. **145:**233–247.
36. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction method of bacterial genomic DNA with guanidinium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. **Schmidt, H., L. Beutin, and H. Karch.** 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. Infect. Immun. **63:**1055–1061.
39. **Sebo, P., P. Glaser, H. Sakamoto, and A. Ullmann.** 1991. High-level synthesis of active adenylate cyclase toxin of *Bordetella pertussis* in a reconstructed *Escherichia coli* system. Gene **104:**19–24.
40. **Thompson, S. A., L. L. Wang, and P. F. Sparling.** 1993. Cloning and nucleotide sequence of *frpC*, a second gene from *Neisseria meningitidis* protein similar to RTX cytotoxins. Mol. Microbiol. **9:**85–96.
41. **Thompson, S. A., L. L. Wang, A. West, and P. F. Sparling.** 1993. *Neisseria meningitidis* produces iron-regulated proteins related to the RTX family of exoproteins. J. Bacteriol. **175:**811–818.
42. **Wandersman, C., and P. Delepelaire.** 1990. TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. Proc. Natl. Acad. Sci. USA **87:**4776–4780.
43. **Welch, R. A., M. E. Bauer, A. D. Kent, J. A. Leeds, M. Moayeri, L. B. Regassa, and D. L. Swenson.** 1995. Battling against host phagocytes: the wherefore of the RTX family of toxins. Infect. Agents Dis. **4:**254–272.
44. **Welch, R. A., C. Forestier, A. Lobo, S. Pellett, W. Thomas, and G. Rowe.** 1992. The synthesis and function of the *Escherichia coli* hemolysins and related RTX exotoxins. FEMS Microbiol. Immunol. **105:**29–36.