Journal of Bacteriology, Apr. 1999, p. 2501–2506 Vol. 181, No. 8
0021-9193/99/$04.00+0
Copyright © 1999, American Society for Microbiology. All Rights Reserved.

Cloning and Characterization of Two Bistructural S-Layer-RTX Proteins from *Campylobacter rectus*

MARTIN BRAUN, PETER KUHNERT, JACQUES NICOLET, ANDRÉ P. BURNENS, and JOACHIM FREY*

Institute for Veterinary Bacteriology, University of Berne, CH-3012 Berne, Switzerland

Received 22 September 1998/Accepted 8 February 1999

Campylobacter rectus **is an important periodontal pathogen in humans. A surface-layer (S-layer) protein and a cytotoxic activity have been characterized and are thought to be its major virulence factors. The cytotoxic activity was suggested to be due to a pore-forming protein toxin belonging to the RTX (repeats in the structural toxins) family. In the present work, two closely related genes, *csxA* and *csxB* (for *C. rectus* S-layer and RTX protein) were cloned from *C. rectus* and characterized. The Csx proteins appear to be bifunctional and possess two structurally different domains. The N-terminal part shows similarity with S-layer protein, especially SapA and SapB of *C. fetus* and Crs of *C. rectus*. The C-terminal part comprising most of CsxA and CsxB is a domain with 48 and 59 glycine-rich canonical nonapeptide repeats, respectively, arranged in three blocks. Purified recombinant Csx peptides bind Ca^{2+}. These are characteristic traits of RTX toxin proteins. The S-layer and RTX domains of Csx are separated by a proline-rich stretch of 48 amino acids. All *C. rectus* isolates studied contained copies of either the *csxA* or *csxB* gene or both; *csx* genes were absent from all other *Campylobacter* and *Helicobacter* species examined. Serum of a patient with acute gingivitis showed a strong reaction to recombinant Csx protein on immunoblots.**

Campylobacter rectus, formerly *Wolinella recta* (39), is a gram-negative, anaerobic, motile bacterium which is associated with several forms of human periodontal diseases (9, 34, 45). Little is known about the molecular mechanisms of pathogenicity and host cell interactions; current investigations have concentrated on characterization of a surface-layer (S-layer) protein, Crs, which is assumed to be involved in resistance of *C. rectus* to phagocytic uptake and to bactericidal activity of serum (31, 41). Recently two groups have simultaneously cloned and sequenced the gene encoding the S-layer protein and named the gene *crs* (41) and *slp* respectively (29); in this study, the designation *crs* will be used. Studies revealed that depending on the strain, the Crs protein varies slightly in size (from 150 to 166 kDa) and amino acid sequence (30, 41). S-layer proteins form regularly arranged structures on the outer surface of various bacteria. They are assumed to play a role in virulence of several pathogens by rendering the bacteria resistant to complement killing and providing structures for adherence to host cells (4). Cultures of *C. rectus* strains that had lost most of S-layer structures by continuous passage many times on growth medium showed reduced virulence in a mouse abscess model compared to low-passage cultures (10, 20). The primary task of S-layer proteins seems to be to provide the bacterium with a stable shape and surface so as to resist the harsh conditions encountered during the process of infection. S-layer proteins have also been identified in *Campylobacter fetus* subsp. *fetus* but not other *Campylobacter* or *Helicobacter* species (Table 1).

Cytotoxic activity of gram-negative bacteria such as proposed for *C. rectus* (16), however, is often due to pore-forming protein toxins belonging to the family of RTX (repeats in the structural toxin) proteins. RTX toxins, which include the α-hemolysin HlyA of *Escherichia coli* (11), the leukotoxins LktA of *Pasteurella haemolytica* (26) and AaltA of *Actinobacillus actinomycetemcomitans* (21), the cytotoxins ApxIA, ApxIIA, and ApxIIIA of *Actinobacillus pleuropneumoniae* (14), and the adenylate cyclase CyaA of *Bordetella pertussis* (17), are major virulence factors of these pathogens (42, 43). The RTX proteins are characterized by a domain located generally in the C-terminal part of the protein consisting of a variable number of highly conserved glycine-rich nonapeptide repeats with the consensus sequence (L/I/F)XGGXG(N/D)DX (42). This domain has a high Ca^{2+}-binding capacity and is involved in binding of the toxin to the target cell (3, 27). *C. rectus* was suggested to contain genes encoding RTX proteins as shown by Kuhnert et al. (22). Furthermore, Gillespie et al. (16) described a cytotoxic fraction of *C. rectus* which reacted serologically with antiserum directed against leukotoxin of *A. actinomycetemcomitans*. Surprisingly, the N-terminal amino acid sequence of the main protein of this fraction is identical to that of the Crs protein described by Wang et al. (41). However, neither native nor recombinant Crs showed cytotoxicity against HL-60 cells or human peripheral leukocytes (29). The present study was therefore undertaken to analyze the presence of two new genes in *C. rectus* coding for bistructural S-layer-RTX proteins named CsxA and CsxB (*C. rectus* S-layer-RTX protein).

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmid cloning vectors. *C. rectus* strains (Table 1) were grown at 37°C in an atmosphere of 10% CO_2 and 1% O_2 in H_2 either on blood agar plates (Trypticase soy agar supplemented with 0.1% $CaCl_2$ and 5% sheep blood) supplemented with sodium formate (Merck, Darmstadt, Germany) and disodium fumarate (Fluka, Buchs, Switzerland) (each at 0.6 g/liter) or in mff broth described by Gillespie and Holt (15) (PPLO broth without CV [20 g/liter] [Difco Laboratories, Detroit, Mich.] supplemented with 25 mM sodium formate, 50 mM sodium fumarate, and 5 mg of hemin [Sigma-Aldrich, St. Louis, Mo.]). The other *Campylobacter* spp. and the *Helicobacter* spp. were routinely cultured at 37°C on blood agar plates under microaerophilic conditions (7% CO_2, 7% H_2, 80% N_2, 6% O_2).

E. coli K-12 strain XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r)]) (6) and BL21(DE3) [*E. coli* B F′ *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3)] (37) were used for cloning and gene expression, respectively. Plasmid pBluescriptIISK$^-$ (Stratagene, La Jolla, Calif.) was used as the cloning vector. The T7 promoter-based expression vector pETHIS-1 (36a), which is derived from cloning vector pET14b (Novagen, Madison, Wis.)

* Corresponding author. Mailing address: Institute for Veterinary Bacteriology, Länggasstrasse 122, CH-3012 Berne, Switzerland. Phone: 41 31 631 24 84. Fax: 41 31 631 26 34. E-mail: jfrey@vbi.unibe.ch.

source: https://doi.org/10.7892/boris.39165 | downloaded: 19.4.2024

TABLE 1. *Campylobacter* and *Helicobacter* strains used

Species	Strain[a]	*csx* gene(s)[b]	Gene encoding S-layer protein (reference)
C. rectus	CCUG11640	*csxA*	*crs*[c]
	CCUG11643	*csxA*	*crs*[c]
	CCUG27948	*csxA*	*crs*[c]
	CCUG20446B[T]	*csxA* and *csxB*	*crs*[c], *crs* (41), *slp* (29)
	CCUG11642	*csxA*	*crs*[c]
	CCUG11645	*csxB*	*crs*[c]
C. curvus	ATCC 35224[T]	Neg.	NR[d]
C. concisus	ATCC 33237[T]	Neg.	NR
C. fetus subsp. *fetus*	ATCC 27374[T]	Neg.	*sapA* (5) and *sapB* (8)
C. jejuni	NCTC11351[T]	Neg.	NR
C. upsaliensis	LMG8850[T]	Neg.	NR
C. coli	LMG6440[T]	Neg.	NR
C. sputorum biovar *sputorum*	LMG7795[T]	Neg.	NR
H. pylori	ATCC 43504[T]	Neg.	None (7)
H. cinaedi	ATCC 35683[T]	Neg.	NR
H. pullorum	NCTC12824[T]	Neg.	NR

[a] CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures and Pathogenic Fungi, London, England; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium.

[b] Determined by Southern blotting and partial sequencing. The gene with higher sequence similarity within the determined part (see text) is given. Neg., negative (no hybridization signal).

[c] Determined by PCR with primers BCRSLP-L and BCRSLP-R (Table 2) and subsequent partial sequencing of the PCR product with primer BCRSLP-L.

[d] NR, no reference found.

and allows addition of polyhistidine tails at both N- and C-terminal ends of cloned proteins, was used for expression of cloned genes. *E. coli* strains were grown at 37°C in Luria-Bertani broth supplemented when necessary with ampicillin (50 μg/ml) for selection and maintenance of recombinant plasmids. For blue-white selection with pBluescriptIISK$^-$, 125 μM X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) were added.

Gene cloning and DNA sequencing. DNA was extracted by the method of Pitcher et al. (33). Plasmid libraries were constructed by cloning restricted total DNA of the *C. rectus* type strain into pBluescriptIISK$^-$, using conventional techniques (2). Recombinant plasmids were screened by a colony blot assay (2) using digoxigenin (DIG)-labelled DNA probes detected either with CDP-Star (Boehringer Mannheim, Mannheim, Germany) or with NBT (4-nitroblue tetrazolium chloride)-BCIP (5-bromo-4-chloro-3-indolylphosphate) (Boehringer Mannheim) according to the manufacturer's protocol.

Subclones were generated either with a double-stranded nested deletion kit (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) or by cutting with restriction enzymes and religating with T4 DNA ligase (Boehringer Mannheim). Sequencing reactions were performed with a Taq Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems/Perkin-Elmer Cetus, Norwalk, Conn.) with either T3 and T7 matching primers flanking the inserts or custom-synthesized internal primers such that all sequences ultimately were read from both directions. Reaction products were analyzed on an ABI Prism 310 genetic analyzer (Applied Biosystems/Perkin-Elmer Cetus).

For direct DNA sequencing from genomic DNA, extraction of DNA was performed with the QIAamp tissue kit (Qiagen GmbH, Hilden, Germany). Seven micrograms of DNA was preincubated with 2 μg of RNase at 37°C for 10 min followed by addition of 16 μl of sequencing premix (ABI Prism Bigdye ABI Primer Cycle Sequencing Ready Reaction kit; Perkin-Elmer), 21 pmol of primer, and an appropriate amount of water to make up total volume of 40 μl. After 3 min at 95°C, 99 cycles of 30 s at 95°C, 20 s at 55°C, and 4 min at 65°C with primer CRS1HisKin (Table 2) and 99 cycles of 30 s at 95°C and 5 min at 65°C with primer CRS1HisKin2 were performed. Reaction products were analyzed on an ABI Prism 310 genetic analyzer.

Sequence data analyses. Sequence alignment and editing were done with the software Sequencher (Gene Codes Corporation, Ann Arbor, Mich.). Sequence comparisons were done as described by Altschul et al. (1), alignments were done with the Wisconsin Package (Genetics Computer Group, Inc. [GCG], Madison, Wis.), and PEST scores were calculated by the method of Rechsteiner and Rogers (36). RNA secondary structures were calculated by using the PCGENE software (Oxford Molecular Ltd., Oxford, England) or the GCG package. The theoretical isoelectric pHs (pIs) and molecular masses of proteins were calculated with the GCG software.

Southern blot analysis. DNA fragments used as probes were purified twice over an agarose gel, using a JETsorb gel extraction kit (Genomed GmbH, Bad Oeynhausen, Germany) and DIG labelled overnight with a High Prime DIG labelling kit (Boehringer Mannheim). Southern blotting was done by alkaline transfer onto positively charged nylon membranes (Boehringer Mannheim) with an LKB 2016 VacuGene vacuum blotting pump (Pharmacia LKB). Gels were depurinated for 10 min in 0.25 M HCl, and subsequent transfer was performed with 0.4 M NaOH. 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 M sodium citrate)–1% blocking reagent (Boehringer Mannheim)–0.1% *N*-lauroylsarcosine sodium salt–0.02% sodium dodecyl sulfate (SDS) at 68°C overnight. Filters were washed under nonstringent conditions twice for 5 min each with 50 ml of 0.2× SSC–0.1% SDS per 100 cm^2 at room temperature (RT), 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 RT. The filters were then processed with phosphatase-labelled anti-DIG antibody according to the producer's protocol. Signals were produced with chemiluminescent substrate (CDP-Star; Boehringer Mannheim) or with the chromogene substrate NBT-BCIP. Luminescence was detected on X-ray films.

Expression of His-tailed fusion proteins and mouse immunization. Sequences of the primers used to amplify segments of the *csxB* gene are given in Table 2. Amplification was done on a DNA thermal cycler (GeneAmp 9600; Perkin-Elmer Cetus), using an Expand long template PCR kit (Boehringer Mannheim) an annealing temperature of 55°C, 2-min expansion time, and genomic DNA of *C. rectus* CCUG20446B[T] (type strain) as the template. The PCR products were purified by using a QIAquick PCR purification kit (Qiagen). Plasmid pJFFCsxBN-His (Fig. 1), encoding the polyhistidine-tailed N-terminal part of CsxB (CsxBN-His; amino acids [aa] 1 to 723) was obtained by cloning the PCR product amplified with primers CRSAP1N-L and CRSAP1N-R into the *Bam*HI-*Spe*I restriction sites of pETHIS-1. Plasmid pJFFCsxBC-His (Fig. 1), encoding the polyhistidine-tailed C-terminal part of CsxB (CsxBC-His; aa 670 to 1238), was obtained with the same procedure, using primers CRSAP1C-L and CRSAP1C-R. The clones were sequenced for confirmation of the gene fusions with the vector and transformed into BL21(DE3) cells. One of each positive

TABLE 2. Oligonucleotide primers

Name	Sequence[a]	Position[b]	Annealing temp (°C)
CRSAP1C-L	ggactagtgATCACCTCGCATAAAGAT	2560–2577	55
CRSAP1C-R	cgcggatccCATACCTGCAAGACCGAC	4293–4276	55
CRSAP1N-L[c]	ggactagtgATGTCCCTAACCCAGTCC	583–600	55
CRSAP1N-R	cgcggatccCAAGCGCGGTTTTGATTGTT	2775–2757	55
BCR1B391[c]	AGCGTATCGTTTAGCTCGCC	3914–3895	55
CRS1HisKin	TTTTGTATGACGAGCGCAGCTG	5368–5389	55
CRS1HisKin2	AGATGGTTTTAGAGGCGGTTTTTATG	5551–5576	65
BCRSLP-L[d]	GGCGCCTCTCAAGATTTGTT		57
BCRSLP-R[e]	CCGCCGTCGATATTTGTTCT		57

[a] Lowercase letters indicate nucleotides added to create restriction enzyme recognition sites (underlined) for cloning.

[b] Based on nucleotide sequence AF035192 (*csxB*).

[c] Common primers for *csxA* and *csxB*.

[d] Based on nucleotide sequence AF010143 (*crs*) positions 552 to 575.

[e] Based on nucleotide sequence AF010143 (*crs*) positions 1681 to 1662.

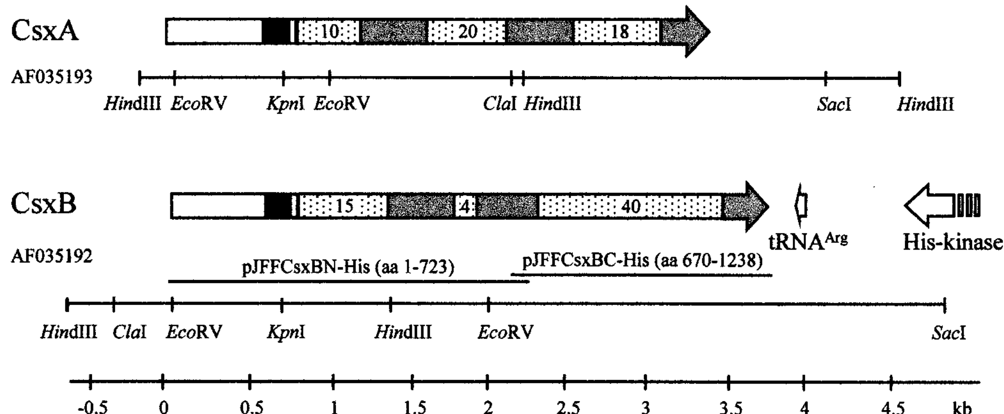

FIG. 1. Physical and genetic map of the gene clones containing the genes *csxA* and *csxB*. Boxes with arrowheads indicate ORFs. Black boxes indicate the Pro-rich region. Dotted boxes represent the regions with the Gly-rich nonapeptide repeats; numerals indicate the number of repeats. Grey boxes represent the regions found interspaced and at the ends of the nonapeptide repeat regions. White boxes at the N terminus represent the S-layer-homologous region. The cloned segments of *csxB* in pJFFCsxBN-His and pJFFCsxBC-His used for His-tailed protein expression are indicated. Note that the N-terminal part (white) and the Pro-rich region (black) are identical for CsxA and CsxB.

clone was inoculated in 50 ml of LB-ampicillin at 37°C to an optical density at 650 nm of 0.3 and induced with 10 mM IPTG for another 3 h. The cells were sedimented by centrifugation at 4,000 rpm, resuspended in 5 ml of PN buffer (50 mM NaH_2PO_4, 300 mM NaCl; pH 8), sonicated with a microtip for 4 min at 50% and 1-s interval in a Branson Sonifier 250 (Branson Ultrasonics, Danbury, Conn.), and then centrifuged at 15,000 rpm for 20 min. The supernatant consisting of the cytosolic fraction was kept, and the pelleted cell debris was resuspended in 5 ml of PN buffer (cell debris). Analysis of the sonicated fractions on SDS–10% acrylamide gels showed the induced protein to be in the cytosolic fraction. The cytosolic fraction was loaded onto a prewashed 1.25-ml-bed-volume Ni-nitrilotriacetic acid-agarose column (Qiagen) and washed once more with 10 ml of buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl [pH 8]). Elution of the protein was performed with a 40-ml buffer B-to-buffer F (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl [pH 3]) gradient with a flow rate of 0.25 ml/min and collection of fractions of 1 ml with a HiLoad system (Pharmacia LKB). The fractions were analyzed on SDS–10% acrylamide gels. Those containing the purified fusion protein were pooled and dialyzed overnight against PN buffer.

The purified and dialyzed recombinant protein CsxBN-His was mixed 1:1 with complete Freund's adjuvant (Difco Laboratories) and 100 μl of the emulsion containing 0.5 μg of recombinant protein and then injected into a female mouse. The mouse was booster immunized with the same amount of protein emulsified with Freund's incomplete adjuvant 20 days later. On day 33 after the first immunization, the mice were bled, and serum was collected and stored at −20°C.

Acid extraction of *C. rectus* S-layer protein. Cells from 48-h cultures of *C. rectus* CCUG20446B^T of three blood agar plates were suspended in 10 ml of 0.9% NaCl and centrifuged for 6 min at 20,000 rpm (SS-34 rotor, 4°C). The cell pellet was resuspended in 10 ml of 0.1 M glycine and 500 μl of 1 M HCl were added, bringing the pH to 2.2. After being shaken on ice for 30 min, the suspension was centrifuged for 1 h at 25,000 rpm (Ti-50 rotor, 4°C); the supernatant was neutralized by addition of 1 ml of 1 M Tris and incubated on ice for another 30 min. The supernatant was then centrifuged under the same conditions as before. The final supernatant containing the S-layer protein was stored at −20°C until further use.

Analyses of proteins in the supernatant of *C. rectus* cells grown in broth. A 20-h culture of *C. rectus* type strain at an optical density at 600 nm of 0.25 grown in 100 ml of mff broth was centrifuged for 20 min at 4,000 rpm and 4°C; 2 ml of the supernatant was added to 18 ml of 4°C ethanol and centrifuged for another 30 min at 20,000 rpm. The ethanol precipitant was resuspended in 100 μl of SDS loading buffer, and 20 μl thereof was analyzed in parallel with 3 μl of the cell pellet resuspended in 20 μl of SDS loading buffer on an SDS–10% polyacrylamide gel.

SDS-PAGE and immunoblot analyses. Proteins were separated by SDS–10% polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (23) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, Calif.). Western blots were used either for the Ca^{2+} binding assay or for immunoblotting. For immunoblotting, Western blots were blocked with 1% milk buffer for 30 min and then incubated with the antiserum (1:1,000) in milk buffer overnight at 4°C. After a thorough wash with water, the appropriate phosphatase-labelled conjugate (Ac to human [catalog no. 15-10-06] or goat anti-mouse immunoglobulins G plus M [heavy plus light chain] [catalog no. 15-18-09]; Kirkegaard & Perry, Gaithersburg, Md.) diluted 1:2,000 in milk buffer was added, and the reaction was visualized 90 min later by incubation with BCIP-NBT as the substrate.

Ca^{2+} binding assay. The binding of Ca^{2+} to proteins was detected by ^{45}Ca autoradiography. Western blots were soaked and washed in calcium binding buffer (60 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole hydrochloride [pH 7.2]) for 10 min followed by 10 min of incubation in binding buffer supplemented with 1.0 μCi of $^{45}CaCl_2$ per ml with a specific activity of 0.02 mCi/μg (Amersham, Little Chalfont, Buckinghamshire, England). The membranes were then rinsed with deionized water for 5 min, dried at room temperature, and exposed to autoradiography.

Nucleotide sequence accession numbers. The sequences derived from clones were submitted to EMBL, and the assigned accession numbers are AF035193 for *csxA* and AF035192 for *csxB*.

RESULTS

Cloning and sequence analyses of two RTX genes, *csxA* and *csxB*. Analysis of different *Campylobacter* spp. with the broad-range RTX gene probes developed by Kuhnert et al. (22) revealed a signal for a potential RTX gene for *C. rectus* with probe RTXCYAA, derived from the *Bordetella pertussis cyaA* toxin gene. This probe was then used to screen a gene library of *C. rectus* CCUG20446B^T made by cloning *Hin*dIII-digested genomic DNA fragments in cloning vector pBluescript SK$^-$. Two clones containing two different partial RTX genes were found. To obtain the full genes, two additional gene libraries made from *Kpn*I/*Sac*I-digested DNA and from *Cla*I/*Hin*dIII-digested DNA were made and screened with gene probes derived from the initial clones. This strategy allowed us to obtain composite clones of 4,649 and 5,473 bp containing two highly similar genes which were designated *csxA* and *csxB* (Fig. 1). Nucleotide sequence analysis done on both strands of the two cloned genes revealed 83% identity (BESTFIT parameters, gap weight = 50 and length weight = 1) with 22 gaps.

Analysis of the sequenced DNA showed a low G+C content of 42.2%, which is below the average G+C content for *C. rectus* of 45.4% (39). The *csxA* open reading frame (ORF) is located from positions 136 to 3507, with an ATG initiation codon and TGA stop codon. It encodes a protein with 1,123 amino acid residues and a calculated molecular mass of 118 kDa. The ORF for *csxB* is 3,717 bp long, starting with ATG at position 583 and ending with TAA at position 4299. It encodes a protein with 1,238 amino acid residues and a calculated molecular mass of 130.8 kDa. For both ORFs, a putative ribosomal binding site (RBS), AGGAG, was found 5 bp upstream of the ATG. Upstream of the RBS of *csxA*, a putative promoter −10 box (TATAAT) but no canonical −35 box was

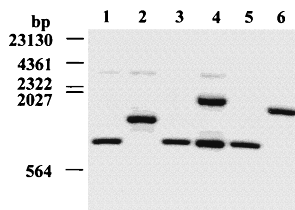

FIG. 2. Southern hybridization analysis of total genomic DNA from *C. rectus* with a *csxA* probe. Genomic *C. rectus* DNA was cut with *Eco*RV and probed with the DIG-labelled 263-bp *Eco*RV-*Kpn*I fragment from *csxA*. Samples of 1 μg of total genomic DNA of strains CCUG11640 (lane 1), CCUG11643 (lane 2), CCUG27948 (lane 3), CCUG20446B^T (lane 4), CCUG11642 (lane 5), and CCUG11645 (lane 6) were separated by gel electrophoresis and transferred to a positively charged nitrocellulose membrane. Positions of DIG-labelled lambda *Hin*dIII fragments and their sizes are indicated on the left.

found. The *csxA* gene is flanked by two hairpins, one of them 21 bp upstream of the ATG consisting of a 13-bp stem and a 5-bp loop with a ΔG of -24.4 kcal/mol and the other positioned 17 bp downstream of the stop codon with a 11-bp stem, 6-bp loop, and ΔG of -21.8 kcal/mol. These structures could serve as rho-independent transcription termination signals. The *csxB* gene is preceded by a characteristic promoter with -35 (TAAACT) and -10 (TATAAT) sequences identical to those found upstream of *crs* (41). Between the promoter and RBS of *csxB*, there are two 11-bp direct repeats (AAATATA TTAT). The *csxB* gene is followed by a 19-bp palindrome (TTTTTAATTTAATTAAAAA) 19 bp downstream of the stop codon.

Analyses of sequences downstream of *csxB*. The 19-bp palindrome downstream of *csxB* is followed by a sequence with high similarity to the *Helicobacter pylori* tRNA^{Arg}(GCG) gene sequence (GenBank/EMBL accession no. AE000551; bp 73 to 149) in the opposite direction to *csxB* (Fig. 1). Folding analysis by the program of Zuker et al. (46) gave an energy of -19.3 kcal/mol (13). The typical prokaryotic 3′ CCA terminus (12, 40) and the signatures A_{20}, C_{35}, G_{36}, and A_{74} (38) further confirm this sequence to be a tRNA^{Arg} gene with GCG as the anticodon.

The tRNA^{Arg} gene is preceded by a sequence with high similarity to histidine protein kinase (His-kinase) genes (TIGR database no. HP1364; 29% identity, 45% similarity) (Fig. 1). The conserved residues for the N, D/F, and G boxes (32) among members of the His-kinase superfamily are also present within this sequence. To determine regions of this gene which are not present on the plasmid clones, genomic sequencing was performed with the primers CRS1HisKin and CRS1HisKin2. This allowed the determination of a further 300 bp of the putative His-kinase with the characteristic H-box consensus (FIRDTTHEINTPLSVILM) for His-kinases.

Specificity of *csx* genes for *C. rectus*. Southern blot analysis of *Eco*RV-digested genomic DNA of different *Campylobacter* and *Helicobacter* species (Table 1) with a probe common for *csxA* and *csxB* made from the 263-bp *Kpn*I-*Eco*RV fragment of *csxA* (Fig. 1) revealed two bands representing *csxA* and *csxB* for *C. rectus* CCUG20446B^T and only one band for the other *C. rectus* strains analyzed (Fig. 2). Other *Campylobacter* and *Helicobacter* species (Table 1) showed no hybridization signals. Further determination of the *csx* genes in *C. rectus* strains which showed only one gene on Southern blots was performed by PCR amplification with primers CRSAP1N-L and BCR1B931, which are common for both *csx* genes, and subsequent partial sequencing of the PCR product with primer BCR1B931, which differentiated *csxA* from *csxB*. These analyses revealed that five of the six strains, i.e., CCUG20446B^T, CCUG11640, CCUG11642, CCUG11643, and CCUG27948, harbored a copy of gene *csxA*. In these strains, *csxA* showed some variability which result in 3 to 6% different amino acid positions of CsxA compared to that of the type strain. One strain analyzed, CCUG11645, did not harbor *csxA* but contained only a copy of *csxB*.

To determine whether the *C. rectus* strains analyzed in this study for the presence of *csxA* and *csxB* coharbor the *crs* gene encoding the S-layer protein, we have developed a *crs*-specific PCR using primers BCRSLP-L and BCRSLP-R (Table 2). PCR analysis revealed the presence of the *crs* gene in all six *C. rectus* strains analyzed as shown by the amplification of the specific 1,130-bp fragment and subsequent partial DNA sequence determination of the PCR products using oligonucleotide primer BCRSLP-L.

Structural analysis of the CsxA and CsxB sequences. The amino acid sequences for CsxA and CsxB were deduced from the genes, using the universal genetic code. As expected from the high nucleotide similarity, CsxA and CsxB showed a very similar amino acid sequence. Both proteins have high contents of Gly (16.6 and 15.3%) and Asp (15.1 and 14.0%) residues and a calculated pI of 4.1.

Both (CsxA and CsxB) show two main domains with different structural features (Fig. 1). The first 100 aa, which are identical between CsxA and CsxB, show structures typical for S-layer proteins and exhibit high similarity with the S-layer protein Crs of *C. rectus* (41) (58% identical and 78% similar amino acid residues), SapA of *C. fetus* (5) (36% identity and 74% similarity), and SapB of *C. fetus* (8) (34% identity and 76% similarity).

The S-layer domain of each of CsxA and CsxB is followed by a proline-rich segment from aa 222 to 268 containing 23 Pro residues out of 47 aa. This region also shows a very high PEST score of +25, with a molar fraction of the amino acids P, E, D, S, and T of 0.68 and a hydrophobicity index of 24. The remaining part of each protein contains the typical RTX structures. CsxA contains three blocks of 10, 20, and 18 glycine-rich nonapeptide repeats interspaced and ended by a segment of 140 aa which is present twice between the blocks and partially at the C-terminal end of CsxA (Fig. 1). A very similar modular structure is observed in the slightly larger protein CsxB. The 310 N-terminal aa including the S-layer structure and the proline-rich segment are identical in CsxA. In the RTX part, CsxB contains three blocks of 15, 4, and 40 glycine-rich repeats which are interspaced and flanked C terminally by segments homologous to those found in CsxA, the segments showing 38 to 79% identity (45 to 82% similarity) within and between the Csx proteins. More detailed investigations in the Gly-rich nonapeptide repeat sequences of CsxA and CsxB reveal, respectively, 13 and 22 of the total of 48 and 59 repeats to fit the consensus (L/I/F)XGGXG(N/D)D.

Ca^{2+} binding. Autoradiography of the $^{45}Ca^{2+}$ blots indicates that recombinant CsxBN-His, representing the N-terminal part of CsxB, which contains two blocks of 15 and 4 glycine-rich nonapeptide repeats, and CsxBC-His, representing the C-terminal part of CsxB, which contains one block of 40 glycine-rich nonapeptide repeats both (CsxBN-His and CsxBC-His), strongly bound Ca^{2+} (Fig. 3B). Analysis of the proteins obtained by acid extraction, which is supposed to extract preferentially proteins with S-layer characteristics, revealed a distinct band in the 120-kDa range on $^{45}Ca^{2+}$ blots (Fig. 3B). This band is also seen in total cell protein but is strongly enriched in the acid glycine extract.

Antigenic properties of Csx. Mouse serum directed against the recombinant peptide CsxBN-His, representing the N-ter-

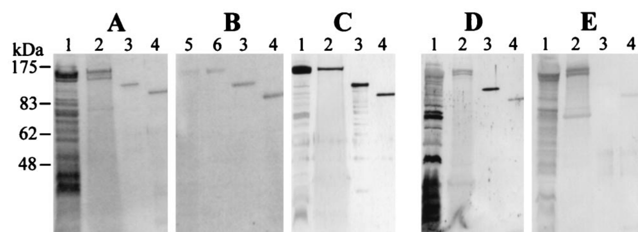

FIG. 3. Ca^{2+} binding and antigenicity of *csx*. SDS-PAGE (Coomassie blue-stained gel) (A), $^{45}Ca^{2+}$ binding (B), and immunoblot (C to E) analysis of *C. rectus* CCUG20446B^T grown in broth. Total cells (lanes 1), culture supernatant (lanes 2), Ni-nitrilotriacetic acid-purified recombinant CsxBN-His (lanes 3), and CsxBC-His (lanes 4) were used. Total cells of *C. rectus* grown on blood agar plates (lane 5) and acid extraction thereof (lane 6) fractions are represented on the $^{45}Ca^{2+}$ binding blot (B). The following sera were reacted on the immunoblots: polyclonal monospecific mouse serum raised against the N terminus of CsxB (anti-CsxBN-His) (C), serum from a human patient with acute gingivitis from whom *C. rectus* was isolated (D), and serum from a human with no known *C. rectus* infection (E).

minal part of CsxB, strongly reacted with proteins in the 120- to 130-kDa size range of total cells and of culture supernatants of all *C. rectus* strains analyzed (Table 1) except strain CCUG11645, lacking CsxA as shown above. The 120- to 130-kDa protein reacting with anti-CsxBN-His was also found in the acid-extracted protein fraction of *C. rectus* CCUG20446B^T. The serum also reacted with the recombinant CsxBN-His and CsxBC-His due to domains shared between these peptides (Fig. 1). An immunoblot with serum from a patient suffering from acute gingivitis and from whom *C. rectus* had been isolated showed a very strong reaction to recombinant CsxBN-His and a clear but weaker reaction against CsxBC-His (Fig. 3D). This serum also reacted against several other antigens of total cells of *C. rectus* CCUG20446B^T; the reaction in the 100- to 130-kDa range of the culture supernatant was weak. Serum from a patient with no history of gingivitis and who never had dental problems showed very weak reactions with recombinant CsxBN-His and CsxBC-His peptides (Fig. 3E). Such weak reactions were also observed in nine sera from randomly chosen blood donors.

DISCUSSION

By applying RTX-specific broad-range gene probes (22), we were able to detect and characterize two new RTX genes in *C. rectus*. The strong relatedness found within the amino acid sequences of the two proteins, CsxA and CsxB, encoded by these genes indicate that they have common function, whereas the variability found upstream of the genes suggests that they are regulated differently. The proteins show a two-domain structure. The N-terminal S-layer domain and the C-terminal part with RTX domains are separated by a sequence with a high PEST score, giving the protein a two-domain structure. High PEST scores indicate possible proteolytic signals (36) and imply a potential cleavage of the two domains.

The N-terminal parts of CsxA and CsxB show significant similarity to S-layer proteins. In addition, they have the low cysteine content, low pI, and high molecular weight which are typical for S-layer proteins. The N-terminal parts of CsxA and CsxB may therefore be involved in binding of lipopolysaccharide as was demonstrated for S-layer proteins from *C. fetus* (44). CsxA and CsxB are both distinguished by their large number of glycine-rich nonapeptide repeats which are arranged in three blocks. We attribute the potential to bind Ca^{2+} by these proteins to these RTX structures. RTX structures are involved together with Ca^{2+} binding in receptor recognition on the target cell and have been shown for certain toxins to determine the host cell specificity (24, 25). Thus, the RTX domains of Csx are suggested to be involved in target binding. No potential function could be attributed to the domain which is located between and C terminally to these RTX sequences. Thus far, we cannot suggest any possible toxic function for CsxA or CsxB. It remains, however, to be clarified whether the cytotoxic activity as measured by Gillespie et al. (16) might be due to CsxA or CsxB protein rather than to the S-layer protein Crs (29).

The genes *csxA* and *csxB* seem to be located on monocistronic operons, as indicated by the flanking putative transcription termination signals. The presence of a $tRNA^{Arg}$(GCG) gene, which represents a low-abundance Arg codon in *Helicobacter* and *Campylobacter* species, immediately downstream of *csxB* is noteworthy in view of the frequent association of tRNA genes with pathogenicity islands (18). For *Corynebacterium diphtheriae*, a $tRNA^{Arg}$ gene was reported to be the chromosomal integration site for toxinogenic bacteriophages (35).

Immediately next to the $tRNA^{Arg}$ gene we found a gene encoding a His-kinase. His-kinases commonly function as transmitter domains within the sensors of two-component signal transduction systems. The two-component system is the major mechanism of signal transduction in bacteria (19) and is often involved in the regulation of expression of virulence genes in pathogenic bacteria by sensing environmental signals (28).

Southern blot analysis, PCR amplifications, and partial DNA sequence analysis of the PCR amplification products revealed that the *csxA* gene was present in five of six *C. rectus* strains tested. The *csxB* gene was present in only two strains, the type strain CCUG20446B^T (together with *csxA*) and strain CCUG11645. Interestingly, the latter strain did not react on immunoblots with anti-CsxBN-His, whereas all other strains did. This result might be interpreted as signifying that anti-CsxBN-His recognizes protein CsxA in all strains where it would be expressed from gene *csxA*, while *csxB* would not be expressed under the given culture conditions, thus leading to an apparent Csx$^-$ phenotype in strain CCUG11645. It also should be noted that the very N-terminal parts of CsxA and CsxB show similarity to the analogous part of the S-layer protein Crs (41), whose gene is present in all *C. rectus* strains analyzed (Table 1) and therefore could generate to some extent serological cross-reactions.

The strong immunological reaction of a serum from a patient with a confirmed *C. rectus* infection with purified recombinant CsxBN-His peptide suggests the N-terminal half of Csx to be highly immunogenic upon infection of *C. rectus*, while the C-terminal part seems to be less antigenic. While in this patient tissue invasion by *C. rectus* apparently led to a strong immune response, the weak reaction to *C. rectus* proteins observed in normal sera suggests that exposure to this agent is limited in the general population.

In summary, we have shown that a putative RTX homologue detected in *C. rectus* by screening with broad-range RTX gene probes (22) represents a new class of putative bifunctional proteins containing both RTX and S-layer domains. This study also confirms the value of using probes for the general detection of toxin families.

ACKNOWLEDGMENTS

We thank Jürg Meyer, Department of Preventive Dentistry, University of Basel, Basel, Switzerland, for the serum of a patient with acute gingivitis, Yvonne Schlatter for technical support, and Isabelle Brodard for culturing *Campylobacter* strains.

This work was supported by the Priority Program Biotechnology of the Swiss National Science Foundation (grant 5002-045027).

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
3. **Bauer, M. E., and R. A. Welch.** 1996. Association of RTX toxins with erythrocytes. Infect. Immun. **64:**4665–4672.
4. **Beveridge, T. J., P. H. Pouwels, M. Sara, A. Kotiranta, K. Lounatmaa, K. Kari, E. Kerosuo, M. Haapasalo, E. M. Egelseer, I. Schocher, U. B. Sleytr, L. Morelli, M. L. Callegari, J. F. Nomellini, W. H. Bingle, J. Smit, E. Leibovitz, M. Lemaire, I. Miras, S. Salamitou, P. Beguin, H. Ohayon, P. Gounon, M. Matuschek, K. Sahm, H. Bahl, R. GrogonoThomas, J. Dworkin, M. J. Blaser, R. M. Woodland, D. G. Newell, M. Kessel, and S. F. Koval.** 1997. Functions of S-layers. FEMS Microbiol. Rev. **20:**99–149.
5. **Blaser, M. J., and E. C. Gotschlich.** 1990. Surface array protein of *Campylobacter fetus*. Cloning and gene structure. J. Biol. Chem. **265:**14529–14535. (Erratum, **265:**19372.)
6. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high frequency efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–378.
7. **Dunn, B. E., G. I. Perez-Perez, and M. J. Blaser.** 1989. Two-dimensional gel electrophoresis and immunoblotting of *Campylobacter pylori* proteins. Infect. Immun. **57:**1825–1833.
8. **Dworkin, J., and M. J. Blaser.** 1996. Generation of *Campylobacter fetus* S-layer protein diversity utilizes a single promoter on an invertible DNA segment. Mol. Microbiol. **19:**1241–1253.
9. **Dzink, J. L., S. S. Socransky, and A. D. Haffajee.** 1988. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J. Clin. Periodontol. **15:**316–323.
10. **Ebersole, J. L., L. Kesavalu, S. L. Schneider, R. L. Machen, and S. C. Holt.** 1995. Comparative virulence of periodontopathogens in a mouse abscess model. Oral Dis. **1:**115–128.
11. **Felmlee, T., S. Pellett, E. Y. Lee, and R. A. Welch.** 1985. *Escherichia coli* hemolysin is released extracellularly without cleavage of a signal peptide. J. Bacteriol. **163:**88–93.
12. **Fournier, M. J., and H. Ozeki.** 1985. Structure and organization of the transfer ribonucleic acid genes of *Escherichia coli* K-12. Microbiol. Rev. **49:**379–397.
13. **Freier, S. M., R. Kierzek, J. A. Jaeger, N. Sugimoto, M. H. Caruthers, T. Neilson, and D. H. Turner.** 1986. Improved free-energy parameters for predictions of RNA duplex stability. Proc. Natl. Acad. Sci. USA **83:**9373–9377.
14. **Frey, J.** 1995. Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. Trends Microbiol. **3:**257–261.
15. **Gillespie, J., and S. C. Holt.** 1987. Growth studies of *Wolinella recta*, a gram-negative periodontopathogen. Oral Microbiol. Immunol. **2:**105–111.
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. **Hacker, J., G. Blum-Oehler, I. Muhldorfer, and H. Tschape.** 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. **23:**1089–1097.
19. **Hoch, J. A., and T. J. Silhavy (ed.).** 1995. Two-component signal transduction. American Society for Microbiology, Washington, D.C.
20. **Kesavalu, L., S. C. Holt, R. R. Crawley, R. Borinski, and J. L. Ebersole.** 1991. Virulence of *Wolinella recta* in a murine abscess model. Infect. Immun. **59:**2806–2817.
21. **Kolodrubetz, D., T. Dailey, J. Ebersole, and E. Kraig.** 1989. Cloning and expression of the leukotoxin gene from *Actinobacillus actinomycetemcomitans*. Infect. Immun. **57:**1465–1469.
22. **Kuhnert, P., B. Heyberger-Meyer, A. P. Burnens, J. Nicolet, and J. Frey.** 1997. Detection of RTX toxin genes in gram-negative bacteria with a set of specific probes. Appl. Environ. Microbiol. **63:**2258–2265.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
24. **Lally, E. T., E. E. Golub, and I. R. Kieba.** 1994. Identification and immunological characterization of the domain of *Actinobacillus actinomycetemcomitans* leukotoxin that determines its specificity for human target cells. J. Biol. Chem. **269:**31289–31295.
25. **Lally, E. T., I. R. Kieba, A. Sato, C. L. Green, J. Rosenbloom, J. Korostoff, J. F. Wang, B. J. Shenker, S. Ortlepp, M. K. Robinson, and P. C. Billings.** 1997. RTX toxins recognize a beta 2 integrin on the surface of human target cells. J. Biol. Chem. **272:**30463–30469.
26. **Lo, R. Y., C. A. Strathdee, and P. E. Shewen.** 1987. Nucleotide sequence of the leukotoxin genes of *Pasteurella haemolytica* A1. Infect. Immun. **55:**1987–1996.
27. **Ludwig, A., T. Jarchau, R. Benz, and W. Goebel.** 1988. The repeat domain of *Escherichia coli* haemolysin (HlyA) is responsible for its Ca^{2+}-dependent binding to erythrocytes. Mol. Gen. Genet. **214:**553–561.
28. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1–7.
29. **Miyamoto, M., H. Maeda, M. Kitanaka, S. Kokeguchi, S. Takashiba, and Y. Murayama.** 1998. The S-layer protein from *Campylobacter rectus*: sequence determination and function of the recombinant protein. FEMS Microbiol. Lett. **166:**275–281.
30. **Nitta, H., S. C. Holt, and J. L. Ebersole.** 1997. Purification and characterization of *Campylobacter rectus* surface layer proteins. Infect. Immun. **65:** 478–483.
31. **Okuda, K., T. Kigure, S. Yamada, T. Kaneko, K. Ishihara, T. Miura, T. Kato, and I. Takazoe.** 1997. Role for the S-layer of *Campylobacter rectus* ATCC33238 in complement mediated killing and phagocytic killing by leukocytes from guinea pig and human peripheral blood. Oral Dis. **3:**113–120.
32. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26:**71–112.
33. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
34. **Rams, T. E., D. Feik, and J. Slots.** 1993. *Campylobacter rectus* in human periodontitis. Oral Microbiol. Immunol. **8:**230–235.
35. **Ratti, G., A. Covacci, and R. Rappuoli.** 1997. A $tRNA_2^{Arg}$ gene of *Corynebacterium diphtheriae* is the chromosomal integration site for toxinogenic bacteriophages. Mol. Microbiol. **25:**1179–1181. (Letter.)
36. **Rechsteiner, M., and S. W. Rogers.** 1996. PEST sequences and regulation by proteolysis. Trends Biochem. Sci. **21:**267–271.

36a. **Segers, R., and J. Frey.** Unpublished data.

37. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
38. **Tamura, K., H. Himeno, H. Asahara, T. Hasegawa, and M. Shimizu.** 1992. In vitro study of *E. coli* tRNA(Arg) and tRNA(Lys) identity elements. Nucleic Acids Res. **20:**2335–2339.
39. **Vandamme, P., E. Falsen, R. Rossau, B. Hoste, P. Segers, R. Tytgat, and J. De Ley.** 1991. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. Int. J. Syst. Bacteriol. **41:**88–103.
40. **Vold, B. S.** 1985. Structure and organization of genes for transfer ribonucleic acid in *Bacillus subtilis*. Microbiol. Rev. **49:**71–80.
41. **Wang, B., E. Kraig, and D. Kolodrubetz.** 1998. A new member of the S-layer protein family: characterization of the *crs* gene from *Campylobacter rectus*. Infect. Immun. **66:**1521–1526.
42. **Welch, R. A.** 1991. Pore-forming cytolysins of gram-negative bacteria. Mol. Microbiol. **5:**521–528.
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. **Yang, L. Y., Z. H. Pei, S. Fujimoto, and M. J. Blaser.** 1992. Reattachment of surface array proteins to *Campylobacter fetus* cells. J. Bacteriol. **174:**1258–1267.
45. **Zambon, J. J., H. Reynolds, J. G. Fisher, M. Shlossman, R. Dunford, and R. Genco.** 1988. Microbiological and immunological studies of adult periodontitis in patients with noninsulin-dependent diabetes mellitus. J. Periodontol. **59:**23–31.
46. **Zuker, M.** 1989. Computer prediction of RNA structure. Methods Enzymol. **180:**262–288.