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A Novel Protein, LcrQ, Involved in the Low-Calcium Response of *Yersinia pseudotuberculosis* Shows Extensive Homology to YopH

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The plasmid-encoded *yop* genes of pathogenic yersiniae are regulated by the environmental stimuli calcium and temperature. A novel protein, LcrQ, which exhibits a key function in the negative calcium-controlled pathway, was identified. DNA sequence analysis revealed that LcrQ has a molecular mass of 12,412 daltons and its isoelectric point is 6.51. Overexpression of LcrQ in *trans* in wild-type *Yersinia pseudotuberculosis* YPIII(pIB102) changed the phenotype from calcium dependence to calcium independence and inhibited Yop expression. LcrQ is expressed from a monocistronic operon. *Trans* overexpression of LcrQ in *yopN* and *lcrH* mutants affected the phenotype of the *yopN* mutant (temperature sensitive to calcium independence) but not that of the *lcrH* mutant (temperature sensitive), suggesting that LcrQ acts between YopN and LcrH in the calcium-regulated pathway. An *lcrQ* mutant was found to be temperature sensitive for growth and showed derepressed Yop expression at 37°C in the presence of calcium in the growth medium. During these culture conditions, the *lcrQ* mutant secreted only LcrV and YopD into the culture supernatant. Removal of Ca^{2+} from the growth medium resulted in a Yop expression pattern of the mutant that was identical to that of the wild-type strain. The LcrQ protein was recovered from the culture supernatant. LcrQ shows 42% identity to the first 128 amino acids of the YopH virulence protein.

Virulent yersiniae possess a common virulence plasmid of about 70 kb (14, 15, 32, 43) which encodes a number of virulence determinants (3, 6, 10, 18, 31). Three of these, YopH, YopE, and YopM, have been shown by site-directed mutagenesis to be indispensable for virulence (5, 12, 24, 31). YopH and YopE are both involved in the ability of the pathogen to obstruct the primary host defense by inhibition of phagocytosis (35, 36). Moreover, YopH was recently shown to have a protein tyrosine phosphatase activity, suggesting that this protein acts by dephosphorylation of host structures (16). YopM, which shows high homology to GpIba, the platelet receptor of the von Willenbrand factor, prevents platelet aggregation (24, 25). These three virulence determinants belong to a class of plasmid-encoded proteins (Yops) (4) which are regulated by the external stimuli temperature and calcium concentration (5, 7, 8, 10, 12, 40). At 37°C, *yop* transcription is induced and the rate of transcription is regulated by the Ca^{2+} concentration of the growth medium (5, 9, 10, 12, 13, 40). In parallel to this transcriptional regulation, the Yops are secreted into the culture medium by a specific Ca^{2+}-regulated plasmid-encoded secretion system, i.e., at low Ca^{2+} concentrations the Yops can be recovered from the culture supernatant, while elevated levels of Ca^{2+} inhibit the secretory process (19, 28, 29, 36). One mutant has been isolated which shows derepressed *yop* transcription as well as derepressed Yop secretion at high Ca^{2+} concentrations (11). This mutant is defective in the ability to express surface-located protein YopN. These data suggest that YopN directly or indirectly senses the Ca^{2+} level and transmits this signal accordingly. YopN is likely to be close to the top of the regulatory hierarchy which, at the final step, involves a *yop* transcriptional repressor. LcrH, which is encoded by the polycistronic *lcrGVH-yopBD* operon (2, 31, 33), has recently been suggested to be this repressor (2, 34), since overproduction of LcrH in certain strains leads to repression of *yop* transcription (2). In response to an increase in temperature, synthesis of an AraC-homologous protein, LcrF (VirF), is induced (8). LcrF is an activator of *yop* transcription. Thus, the two stimuli temperature and calcium concentration affect two independent control systems of *yop* transcription (see the model in Fig. 8).

Wild-type strains of *Yersinia* spp. are defined as being calcium dependent (CD), since they are unable to form colonies at 37°C on plates lacking Ca^{2+} (14, 21). Plasmid-cured strains or certain plasmid mutants are calcium independent (CI), as they show the same plating efficiency with or without addition of Ca^{2+} to the plates. A third class of mutant strains are unable to grow at 37°C, irrespectively of the Ca^{2+} content of the medium (12, 31, 34, 42). Such plasmid mutants are defined as temperature sensitive (TS). Mutants defective in the positive control loop, as well as mutants showing constitutive non-Ca^{2+}-regulated high expression of the repressor are CI (7, 12, 42). TS mutants are affected in the ability to express the repressor (12). CI mutants are unable to express Yops at 37°C, while TS mutants show derepressed transcription of the *yop* genes even in the presence of Ca^{2+} (4, 5, 7, 8, 12, 13, 34).

We have identified a new plasmid-encoded protein, LcrQ, that is involved in the negative Ca^{2+}-controlled loop. LcrQ has a molecular mass of 12 kDa, and the corresponding structural gene is part of a monocistronic operon. LcrQ shows striking homology to the amino-terminal part of YopH.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Yersinia pseudotuberculosis* YPIII, carrying the different plasmids indicated in Table 1, was used. The *Escherichia coli* strains used were C600 (26), DH1 and DH5α (17), and MM383 (30). The liquid

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TABLE 1. Plasmids used

Plasmid	Description	Reference or source
pIB1	Wild type	10
pIB102	Tn*5*::*yad* derived from pIB1	12
pIB103	Tn*5-132*::*yadA* derived from pIB102	12
pIB11	Kanamycin resistance fragment of Tn*5* inserted into the *Xho*I site of *lcrV* of pIB103	12
pIB13	Gene block (kanamycin) insertion mutant (*Nco*I) of *lcrH* of pIB103	2
pIB15	Gene block (kanamycin) insertion mutant (*Eco*RI) of *yopD* of pIB103	2
pIB22	Tn*5*::*lcrK* of pIB102 (Fig. 1)	12
pIB23	Gene block (kanamycin) mutant (*Bgl*II) of *lcrK* of pIB103 (Fig. 1)	36
pIB24	Gene block (kanamycin) mutant (*Cla*I) of *Bam*HI fragment 2 of pIB103 (Fig. 1)	36
pIB25	Insertion of suicide plasmid pNQ705 into *lcrQ* gene of pIB102	This study
pIB82	Gene block (kanamycin) replacing the 0.45-kb *Pst*I fragment of *yopN* in pIB103	36
pIB921	Gene block (kanamycin) inserted between *Bam*HI fragments 9 and 2 of pIB103	36
pBBH1	*Bam*HI-*Hin*dIII fragment from *Bam*HI fragment 2 of pIB1 cloned into pBluescript (Fig. 1)	This study
pBH57	*Bam*HI-*Hin*dIII fragment from *Bam*HI fragment 2 of pIB1 cloned into pACYC184 (Fig. 1)	36
pCF12	*Bam*HI-*Xba*I fragment from *Bam*HI fragment 2 cloned into pACYC184 with kanamycin resistance fragment in the *Cla*I site (Fig. 1)	This study
pPBC3	*Bam*HI-*Cla*I fragment from *Bam*HI fragment 2 cloned into pACYC184	This study
pPCM10	*Cla*I-*Ssp*I fragment containing *lcrQ* cloned into pACYC184 (Fig. 1)	This study
pCMS16	*Cla*I-*Ssp*I fragment containing *lcrQ* cloned into pBluescript (Fig. 1)	This study
pCKMS16	Gene block (kanamycin) inserted into the *Eco*RV site of *lcrQ* in pCMS16	This study
pNQ705	*Xho*I-*Acc*I fragment (chloramphenicol resistance) of pACYC184 inserted into *Pst*I-*Sal*I-digested pGP704, derivative of suicide plasmid pJM703.1 (Pir dependent)	29a, 31a
pNQ109	97-bp internal PCR fragment of *lcrQ* gene, positions 301 to 397 (Fig. 2), inserted into *Kpn*I-*Eco*RV sites of pNQ705	This study

growth medium for *Yersinia* strains consisted of brain heart infusion broth (BHI) supplemented with either 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] and 20 mM MgCl (BHI minus Ca^{2+}) or 2.5 mM $CaCl_2$ (BHI plus Ca^{2+}). The solid medium was blood agar base containing 20 mM Na oxalate, 20 mM $MgCl_2$, and 0.2% glucose (MOX plates; Ca^{2+} free) or blood agar base supplemented with only 2.5 mM Ca^{2+}. *E. coli* strains were grown in Luria broth or on Luria agar.

DNA methods. Preparation of plasmid DNA, restriction enzyme digests, ligation, and transformation of *E. coli* were performed essentially as described by Maniatis et al. (26). Transformation of *Y. pseudotuberculosis* was performed as described previously (12).

DNA sequencing. The 4.5-kb *Bam*HI-*Hin*dIII fragment from *Bam*HI fragment 2 of pIB1 (see Fig. 1) was cloned into the pBluescript SK(−) vector. The plasmid obtained, pBBH1, was digested with either *Apa*I-*Hin*dIII or *Sac*I-*Bam*HI, and a deletion library was created by using exonuclease III-mung bean nuclease as described in references 20, 22, and 40a.

Various DNA fragments obtained from restriction endonuclease digests were subcloned into M13 vectors mp18 and mp19, and single-stranded DNA templates were prepared and sequenced in accordance with reference 1a.

The sequencing procedure for double- and single-stranded DNAs was carried out in accordance with the dideoxy-chain termination method of Sanger et al. (38) with [α-^{35}S]dATP (Amersham Corp., Little Chalfant, United Kingdom) as the label.

The enzymes used in the sequencing reactions were T7 DNA polymerase (Pharmacia), Sequenase Version 2.0 (United States Biochemical Corp., Cleveland, Ohio), *Taq* DNA polymerase (New England BioLabs), and TAQuence *Taq* DNA polymerase (United States Biochemical Corp.).

In some cases, 7-deaza-dGTP was substituted for dGTP in the labeling and termination mixtures to avoid compressions due to strong secondary structures. Primers of specific oligonucleotides were purchased from Scandinavian Gene Synthesis AB (Köping, Sweden) and Symbicom AB (Umeå, Sweden).

DNA and protein sequences were analyzed by the GCG (Genetics Computer Group [University of Wisconsin, Madison] sequence analysis software package) and PC/GENE (IntelliGenetics, Inc./Genofit, S.A.) computer programs.

Analysis of *yop* expression. *Yersinia* strains were grown at 26°C to an optical density of 0.1 (550 nm). The cultures were then shifted to 37°C and grown for an additional 3 h before being harvested. The secreted proteins were precipitated with 10% trichloroacetic acid as described before (10), analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 12% polyacrylamide gels (23), and visualized by Coomassie blue R staining.

Samples of whole bacteria were also analyzed by immunoblotting using rabbit total-*yop* antiserum as described earlier (10).

Minicell analysis. Minicells were prepared from *E. coli* P678-54 (1) carrying hybrid plasmids. The minicells were labeled with [^{35}S]methionine (Amersham) and subjected to SDS-PAGE on 15% polyacrylamide gels as described previously (10).

Definition of phenotypes. The strains tested were diluted in physiological NaCl. The serial dilutions were spread on agar plates with or without Ca^{2+} (plate composition is described above under bacterial strains and growth conditions). The plates were incubated at 26 or 37°C for about 40 h. Strains unable to grow at 37°C without addition of Ca^{2+} are defined as CD, which is the wild-type phenotype of *Yersinia* spp. CI mutants are able to grow at 37°C irrespectively of the Ca^{2+} concentration. Strains unable to grow at 37°C are defined as TS.

Transcomplementation assays. Various DNA fragments obtained from the 4.5-kb *Bam*HI-*Hin*dIII region (see Fig. 1) were subcloned into pACYC184 or the pBluescript SK(−) vector. The different hybrid plasmids (Table 1) were introduced into different *Yersinia* strains, and their phenotypes were determined as described above.

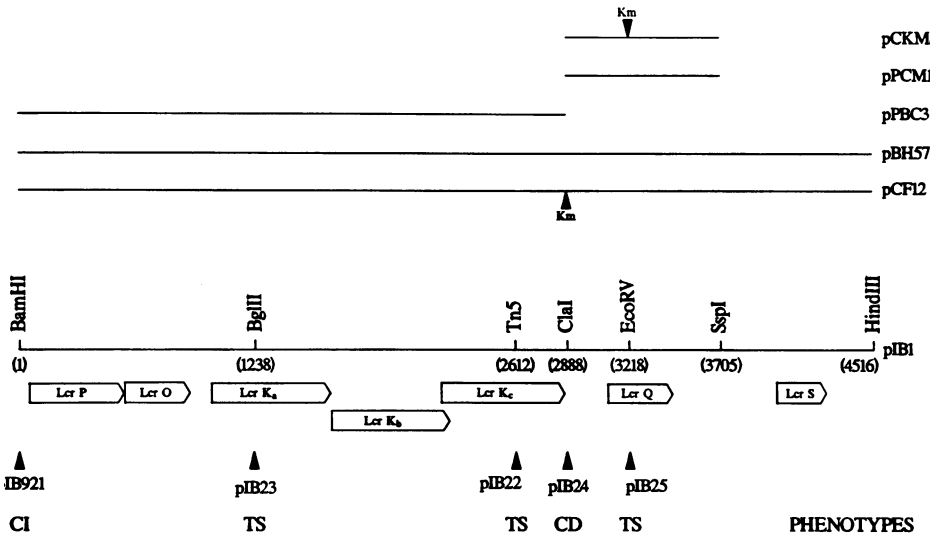

FIG. 1. Restriction endonuclease map of the *lcrQ*-containing DNA region of pIB1. The nucleotides are labelled starting with +1 at the *Bam*HI endonuclease restriction site of *Bam*HI fragment 2 in pIB1 of *Y. pseudotuberculosis*. Arrowheads indicate the positions of different gene block (kanamycin resistance) insertions. Below the arrowheads are the names of the mutant plasmids and the phenotypes of strain YPIII carrying these plasmids. Plasmids used in transcomplementation tests are shown above.

N-terminal amino acid sequencing. The LcrQ protein was purified from the culture supernatant as described earlier (10). Amino acid sequencing was performed in a Beckman automatic amino acid sequencer.

Construction of YPIII(pIB25). The *lcrQ*::pNQ109 mutant was obtained by the following procedure. pNQ705 is a derivative of pGP704 into which the *Xho*II-*Acc*I chloramphenicol resistance-encoding fragment obtained from pACYC184 has been inserted (32a). This fragment replaced the *Pst*I-*Sal*I fragment of pGP704, and thus pNQ705 generates resistance to chloramphenicol but does not confer ampicillin resistance. A polymerase chain reaction (PCR) fragment of the *lcrQ* gene was cloned into the unique *Kpn*I and *Eco*RV restriction endonuclease sites of the polylinker of pNQ705, generating pNQ109. The PCR fragment generated had an engineered terminal *Kpn*I site, and the second *Eco*RV site was the internal *Eco*RV site of the *lcrQ* gene (see Fig. 1 and 2). pNQ109 contained an internal 97-bp DNA sequence of the *lcrQ* gene (positions 301 to 397; see Fig. 2). Since plasmid pNQ109 is dependent upon the Pir protein for replication, this plasmid cannot replicate in *Y. pseudotuberculosis*; therefore, to maintain pNQ109 in this strain it must integrate into the genome of the host cell. This integration most frequently occurs by a single homologous recombination event, thereby generating a mutant of the target gene if an integral part of the gene is used. Strain YPIII(pIB102) (wild type; kanamycin resistant) was mated with *E. coli* S17-1 (*pir*) containing pNQ109. Offspring were selected on Luria agar plates containing chloramphenicol and kanamycin. One colony which had pNQ109 integrated into the *lcrQ* gene of pIB102 was obtained. This strain was designated YPIII (pIB25) (*lcrQ*::pNQ109). Successful integration of pNQ109 was confirmed by *Bam*HI restriction enzyme analysis and PCR analysis. One PCR primer was derived from the *lcrQ* upstream region between positions 130 and 154 (see Fig. 2), i.e., far outside of the presumed duplicated region of pIB25. The reversed primer was derived from an internal pNQ705 DNA sequence (ACATGTGGAATTGTGAGCGGATAACA ATTTgtg[**gaattc** *Eco*RI site of the polylinker]).

Nucleotide sequence accession number. The sequence depicted in Fig. 1 has been submitted to GenBank under accession no. M83986.

RESULTS

We had earlier shown that the *lcrK* mutant YPIII(pIB22) of *Y. pseudotuberculosis* was TS for growth at 37°C and that it showed derepressed transcription of *yop* genes at 37°C, irrespectively of the Ca^{2+} concentration of the culture medium (12). In addition, it was unable to secrete Yops into the culture medium (36). Therefore, this mutant was suggested to be defective in Yop export (36). To investigate the *lcrK* mutant in more detail, we first mapped the site of transposon Tn*5* insertion into the plasmid between the *Bgl*II and *Cla*I restriction endonuclease sites of *Bam*HI fragment 2 of pIB1 (Fig. 1). A number of new mutants in this region were constructed by insertion of a DNA fragment that encodes resistance to kanamycin into unique restriction endonuclease sites within this region (Fig. 1). These mutants were tested for response to Ca^{2+}. Mutant YPIII(pIB921) was found to be CI (Fig. 1), while mutant YPIII(pIB24) was CD, showing the same phenotype as the corresponding wild-type strain, YPIII(pIB102) (Fig. 1). Mutant strains YPIII(pIB22) and YPIII(pIB23) were both TS (Fig. 1). These results indicated that *lcrK* is located between the *Bam*HI and *Cla*I restriction endonuclease sites, as shown in Fig. 1.

To determine whether *lcrK* mutants YPIII(pIB22) and YPIII(pIB23) could be complemented in *trans*, a number of different hybrid plasmids, all based on the pACYC184 replicon and carrying different parts of the appropriate DNA region (Fig. 1), were introduced into these strains and into control strains. The resulting constructs were tested for calcium response (Table 2). pBH57 (*Bam*HI-*Hin*dIII) was the only plasmid that conferred the wild-type phenotype on TS mutants YPIII(pIB22) and YPIII(pIB23) (Fig. 1). Although the TS loci had been mapped to the 2.9-kb *Bam*HI-*Cla*I fragment, pPBC3 carrying this DNA fragment did not complement the mutations in pIB22 and pIB23 (Fig. 1).

TABLE 2. Transcomplementation of *lcrK*

Strain (genotype) and phenotype	Phenotype[a] conferred by:			
	pPCM10	pPBC3	pBH57	pCF12
YPIII(pIB102) (wild type) CD	CI	NT[b]	CD	NT
YPIII(pIB22) (*lcrK*) TS	CI	TS	CD	CD
YPIII(pIB23) (*lcrK*) TS	CI	TS	CD	CD

[a] All of the plasmids used are based on the pACYC184 replicon (Table 1).
[b] NT, not tested.

These results indicated that the 1.6-kb *Cla*I-*Hin*dIII DNA region (Fig. 1) contained information that affected the phenotype of the TS mutants. Therefore, pPCM10 (*Cla*I-*Ssp*I [Fig. 1]) carrying a part of this region of DNA was used in *trans* complementation tests of the two TS mutants. Both strains became CI (Table 2). The best explanation for this finding was that pPCM10 overexpressed a protein that exerts a negative control function on the low-calcium response. If so, pPCM10 should also change the wild-type phenotype from CD to CI. This idea was tested and confirmed (Table 2).

DNA sequence analysis and expression of *lcrQ*. The 4.5-kb *Bam*HI-*Hin*dIII fragment was sequenced, and seven open reading frames (LcrP, LcrO, LcrKa, LcrKb, LcrKc, LcrQ, and LcrS) were identified (Fig. 1). During the preparation of this report, the corresponding DNA sequence of *Yersinia enterocolitica* was presented. The researchers named the corresponding gene products Ysc (Yop secretion) (28a). When the two sequences from *Y. pseudotuberculosis* and *Y. enterocolitica* were compared, it was found that they showed a high degree of homology (98.2% identity). Thus, the putative gene products of the operon were conserved, showing homologies of 95 to 99%. No major differences were observed. Therefore, we show only a part of this DNA sequence (see Fig. 2). The calculated molecular masses of the putative proteins were as follows: LcrP (YscH), 18.2 kDa; LcrO (YscI), 12.7 kDa; LcrKa (YscJ), 27.0 kDa; LcrKb (YscK), 24.0 kDa; LcrKc (YscL), 24.9 kDa; and LcrQ (YscM), 12.4 kDa.

As shown above, the 820-bp *Cla*I-*Ssp*I DNA fragment, which encodes only the LcrQ protein (Fig. 2), exhibited a pronounced effect on the phenotype of the wild type (CD to CI) when LcrQ was expressed in *trans* from a multicopy plasmid (Table 2). This argues that *lcrQ* constitutes a monocistronic operon, although we were unable to find a good consensus promoter region of *lcrQ* (Fig. 2). In support of this assumption was also the observation that strain YPIII (pIB24), which carries a kanamycin gene block insertion between *lcrK* and *lcrQ*, showed the wild-type phenotype (Fig. 1). Moreover, we put *lcrQ* (the *Cla*I-*Ssp*I fragment) into several different plasmid vectors in opposite orientations, and in all of the cases studied it was found that *lcrQ* has an endogenous promoter activity (data not shown).

Analysis of the expression of pPCS16 (*lcrQ*), using the *E. coli* minicell system, supported the conclusions drawn from DNA sequence analysis (Fig. 3). Only one polypeptide showing a molecular mass of 12 kDa could be connected to the *Cla*I-*Ssp*I fragment. In addition, these experiments showed that the level of expression of *lcrQ* was high in *E. coli* and exceeded the level of expression of the beta-lactamase encoded by the vector.

When grown in rich medium lacking Ca^{2+}, yersiniae secrete considerable amounts of Yop proteins into the culture medium. These proteins can easily be recovered by trichloroacetic acid precipitation. In previous studies, we had noticed that four additional polypeptides having molecular masses below 18 kDa precipitated with the Yop proteins. We anticipated that one of these could be LcrQ. Therefore, these four polypeptides were purified and their N-terminal ends were determined. It was found that one of the proteins, which showed a molecular mass of about 12

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1   GAAGCACTTTCTCGGGCAATATCTACCACTTTAGGACAAATGAAAGTTACAGAATAGGAAT   61
    GluAlaLeuSerArgAlaIleSerThrThrLeuGlyGlnMetLysValThrGlu***
      ClaI                                              lcrKc
62  AACTATCGATATATGTTTAGTGTTATCTATTATAAGATTGAGTTATCTACCTAAATTGGAT  122
123 TTTTCATCCTCGTTTTATGAGAATGATTCCCAAGAATAATTTTTTATTGTGATTTTCTGTT  183
184 TAAAAGCCGATTAAAAAAATAAATCGTCTACGACAGTAGTTTAGCAAAAATAAATAACTTA  244
245 GAATATCGTAGAGATAATTATAGCGACAGGAGACTCGATGAAAATCAATACTCTTCAATCG  305
                              rbs      MetLysIleAsnThrLeuGlnSer
                                       lcrQ
306 TTAATAAATCAACAAATTACCCAAGTGGGACACGGGGGGCAGGCCGGTCGTCTCACTGAAA  366
    LeuIleAsnGlnGlnIleThrGlnValGlyHisGlyGlyGlnAlaGlyArgLeuThrGluT
                                 EcoRV
367 CTAACCCACTCACAGAGAATAGTCATCAGATATCTACCGCCGAAAAAGCCTTTGCCAATGA  427
    hrAsnProLeuThrGluAsnSerHisGlnIleSerThrAlaGluLysAlaPheAlaAsnGl
                                                          Ser
428 GGTGCTGGAACATGTGAAAAATACGGCTCTCAGTCGTCACGATATTGCCTGCTTATTACCA  488
    uValLeuGluHisValLysAsnThrAlaLeuSerArgHisAspIleAlaCysLeuLeuPro
489 CGCGTTTCTAATTTGGAACTAAAGCAGGGCAAGGCAGGGGAAGTGATAGTGACCGGCTTGC  549
    ArgValSerAsnLeuGluLeuLysGlnGlyLysAlaGlyGluValIleValThrGlyLeuA
550 GTACTGAACAACTCTCGCTTAGCGATGCTAAATTATTGCTAGAAGCCGCCATGCGCCAGGA  610
    rgThrGluGlnLeuSerLeuSerAspAlaLysLeuLeuLeuGluAlaAlaMetArgGlnAs
611 TACGGCGGCTGACGGCTGAGATAATATATATCTACTGTATATTGAGGCGATAATATCCCCC  671
    pThrAlaAlaAspGly***
672 AGGTTGATTTACGTAACCATTTTTCAAGGAGTCATGTATCAATTCTTTCCCCTGAGCCAAT  732
733 TTAGAATAATAATACACCTCCTTCGGTGATCCCCTGAAGTGGGGGTATTTATCAGTAGAGT  793
794 CTGCTCCTCATATAAATTGAGAGAATTAGGATGAAAGATCACATTGTAGCGACTGCCGGGT  854
                              SspI
855 TATGGTTATTTTGATGTCATCCCAGTCAATATT      887
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FIG. 2. DNA sequence of *lcrQ*. The nucleotide sequence of *lcrQ* and the surrounding DNA is shown. The amino acid sequence of LcrQ is shown below. The asterisks indicate the stop codon of *lcrQ*. The putative Shine-Dalgarno (39) region is indicated (rbs).

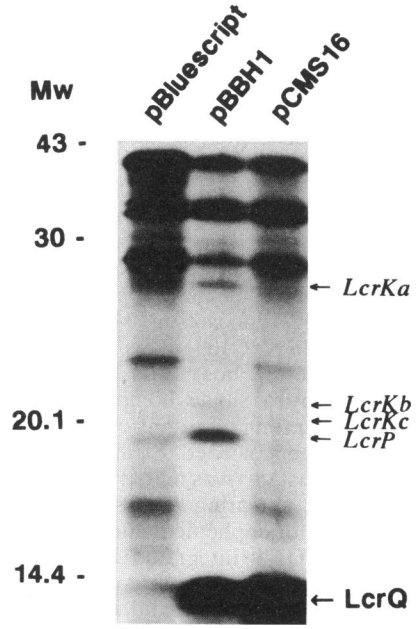

FIG. 3. Expression of *lcrQ* in *E. coli* minicells. Minicells were prepared from *E. coli* P678-54 harboring plasmids pBBH1 (*Bam*HI-*Hin*dIII) and pCMS16 (*Cla*I-*Ssp*I) and the cloning vector pBluescript. The positions of different gene products are indicated. The three products of *lcrK* are indicated by LcrKabc. Lanes: 1, P678-54(pBluescript); 2, P678-54(pBBH1); 3, P678-54(pCMS16). Mw, molecular weight (10^3).

TABLE 3. Extragenic suppression of *yopN* mutant YPIII(pIB82) and phenotype change in the wild-type strain due to *trans* overexpression of *lcrQ*

Plasmid in strain YPIII	Relevant genotype	Phenotype	Phenotype conferred by transcomplementing plasmid[a]:	
			pCMS16 (*lcrQ*$^+$)	pCKMS16 (*lcrQ*)
pIB102	Wild type	CD	CI	CD
pIB11	*lcrV1*	TS	TS	NT[b]
pIB13	*lcrH1*	TS	TS	NT
pIB15	*yopD*	TS	TS	NT
pIB82	*yopN*	TS	CI	TS
pIB25	*lcrQ*	TS	CI	TS

[a] pBluescript vector.
[b] NT, not tested.

kDa, had an N-terminal amino acid sequence that was identical to that of LcrQ, as deduced from DNA sequence analysis (Fig. 2).

LcrQ is involved in the low-calcium response. As shown above, expression of LcrQ in *trans* in wild-type strain YPIII(pIB102) or in *lcrK* mutants YPIII(pIB22) and YPIII-(pIB23) changed the phenotype of these strains to CI (Table 2). To examine at which level in the low-calcium response LcrQ acts, we introduced plasmid pPCM10 into different mutants of *Y. pseudotuberculosis*. We had earlier shown that overproduction of LcrH in *trans* resulted in a changed phenotype of certain mutant strains (2). Mutants carrying gene block insertions in the *yopN* gene or in the *lcrGVH-yopBD* operon are phenotypically TS (2, 12, 31, 34, 41, 42). When these strains were tested with respect to overexpression of LcrQ in *trans*, we observed that *yopN* mutant YPIII(pIB82) changed its phenotype from TS to CI, while the TS *lcrGVH-yopBD* operon mutants were unaffected by the increased levels of LcrQ (Table 3). To prove that *lcrQ* was essential, we also used plasmid pCKMS16, which has a gene block insertion in the unique *Eco*RV site of *lcrQ* (Fig. 1). When this plasmid was used in experiments similar to those described above, no effect on the wild-type phenotype or on the phenotypes of the different mutants was seen (Table 3).

Yop expression was also determined in the different constructs described above. As expected, when the secreted Yops from strains YPIII(pIB102) and YPIII(pIB102, pPCM10) (wild type overexpressing LcrQ in *trans*) were analyzed, we found that the presence of plasmid pPCM10 suppressed Yop expression in the absence of calcium in the growth medium (Fig. 4). Similarly, overexpression of LcrQ in the *yopN* mutant YPIII(pIB82) abolished Yop expression (Fig. 4). In contrast, the presence of pPCM10 in *lcrH* mutant strain YPIII(pIB13) had no affect on Yop expression.

An *lcrQ* mutant is affected in the low-calcium response. The results presented above strongly argue for the idea that LcrQ has a key role in the low-calcium response of virulent yersiniae. To examine the role of LcrQ in more detail, we constructed a knockout mutant of *lcrQ*, YPIII(pIB25). This was done by allowing suicide plasmid pNQ705, which carried an internal PCR fragment of *lcrQ* (positions 301 to 397; Fig. 2), to integrate into *lcrQ* by a single crossover event. As expected, *lcrQ* mutant YPIII(pIB25) was unable to form colonies on agar plates at 37°C, irrespectively of whether Ca^{2+} was added to the plates or not. Thus, the *lcrQ* mutant showed a TS phenotype (Table 3). Introduction of a hybrid plasmid which carried the *lcrQ* gene into YPIII(pIB25) resulted in a strain that showed the CI phenotype (Table 3).

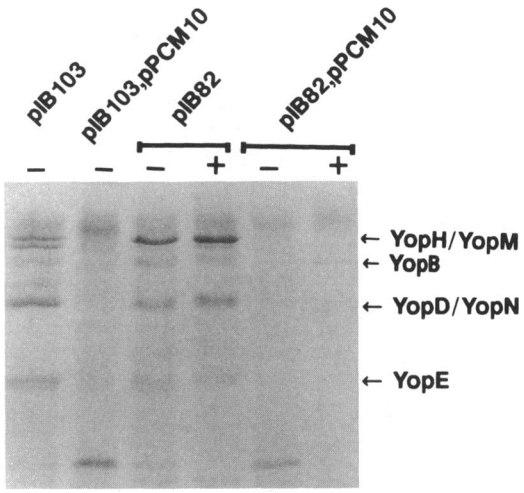

FIG. 4. Suppression of Yop expression by *trans* overexpression of LcrQ in the wild-type and *yopN* mutant strains. Strains YPIII (pIB102, pCM10) (wild type) and YPIII(pIB82, pCM10) (*yopN* mutant) were grown for 3 h at 37°C in Ca^{2+}-containing (+) and Ca^{2+}-depleted (–) media. The secreted Yop proteins were precipitated and subjected to SDS-PAGE. The positions of the different gene products are indicated.

The procedure used to generate the *lcrQ* mutant is now commonly used by us. We noticed in this case that the frequency to obtain the *lcrQ* mutant was unusually low. In fact, we were able to isolate only one single colony. Moreover, mutant YPIII(pIB25) was extremely unstable and reverted at high frequency to the CI phenotype by second-site suppression mutations. This could explain why Michiels et al. were unable to isolate a corresponding *Y. enterocolitica* mutant (28a). When the ability to express Yop proteins

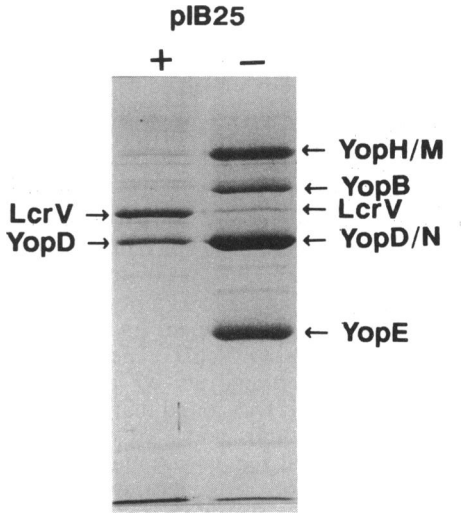

FIG. 5. Secretion of Yop proteins and LcrV from *lcrQ* mutant YPIII(pIB25). *lcrQ* mutant YPIII(pIB25) was grown at 37°C in BHI medium containing (+) or lacking (–) Ca^{2+}. The cells were separated from the culture supernatant by centrifugation, and the proteins of the supernatant were precipitated with trichloroacetic acid, subjected to SDS-PAGE, and stained with Coomassie blue.

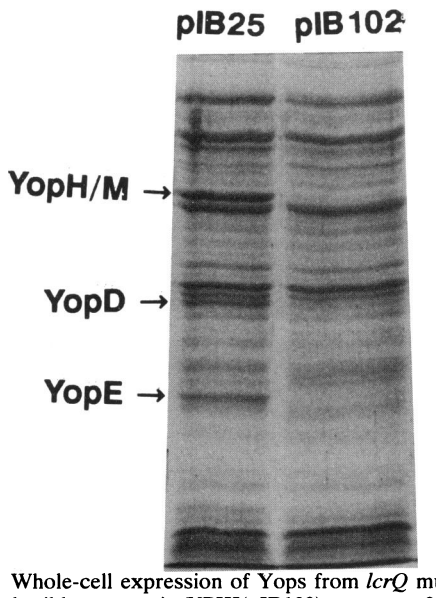

FIG. 6. Whole-cell expression of Yops from *lcrQ* mutant YPIII (pIB25) and wild-type strain YPIII(pIB102) grown at 37°C in BHI media supplemented with 2.5 mM Ca^{2+}. Whole cells were isolated by centrifugation, lysed in SDS sample buffer, and then subjected to SDS-PAGE. The gel was stained with Coomassie blue.

was tested, it was found that the *lcrQ* mutant showed a novel Yop expression pattern (Fig. 5). At 37°C in the absence of Ca^{2+}, the pattern of Yop expression was indistinguishable from that of the wild type (Fig. 5). In contrast to the wild type, the *lcrQ* mutant secreted high amounts of LcrV and YopD into the culture supernatant in the presence of Ca^{2+} (Fig. 5). Both LcrV and YopD were identified by Western blot (immunoblot) analysis using monospecific anti-LcrV and anti-YopD rabbit sera.

Usually the TS phenotype is coupled to a general derepression of Yop expression of bacteria grown in the presence of Ca^{2+} at 37°C (12). To investigate whether only expression of LcrV and YopD was derepressed under these circumstances, whole cells were subjected to SDS-PAGE analysis (Fig. 6). It was found that *lcrQ* mutant YPIII(pIB25), in contrast to wild-type strain YPIII(pIB102), was able to express Yops in large amounts after incubation in a Ca^{2+}-containing medium at 37°C (Fig. 6). The Yop proteins indicated in Fig. 6 were also identified by Western blot analysis using specific anti-Yop rabbit serum (data not shown). Thus, these experiments confirm the earlier conclusions that LcrQ has a key role as a negative element in the low-calcium response of yersiniae.

LcrQ is homologous to YopH. The LcrQ protein was subjected to computer analysis by using the GCG and PC/GENE programs. It was found that LcrQ had a molecular mass of 12,412 daltons and a pI of 6.51. No obvious features, such as signal sequences, membrane-spanning regions, or DNA-regulatory sequences, were found. However, when the amino acid sequence of LcrQ was used to search for proteins showing homologous sequences, one yersinia protein, YopH, which showed significant homology to LcrQ was found (Fig. 7). On the amino acid level, LcrQ and residues 1 to 128 of YopH exhibited 42% identity, indicating a close relationship between these two proteins.

```
LcrQ   1 ..MKINTLQ...S.LINQQITQ.VG..HGGQAGRLTET..NPLTENSHQI 39
           :.:..|:   | |:.|: .: .|  :|. |:. .|| ..|| .| .
YopH   1 MNLSLSDLHRQVSRLVQQESGDCTGKLRGNVAAN.KETTFQGLTIAS.GA 48

LcrQ  40 STAEKAFANEVLEHVKNTALSRHDIACLLP.....RVSNLELKQ.GKAGE 83
         ...||.||..||.|| |..|...|.| ||.     .:.|.:|:  |.:..
YopH  49 RESEKVFAQTVLSHVANVVLTQEDTAKLLQSTVKHNLNNYDLRSVGNGNS 98

LcrQ  84 VIVTGLRTEQLSLSDAKLLLEAAMRQDTAADG..................  115
         |:| :||.:|:.| |||:|||||:||:.:| |
YopH  99 VLV.SLRSDQMTLQDAKVLLEAALRQESGARGHVSSHSHSALHAPGTPVR 147
```

FIG. 7. Sequence homology between LcrQ and YopH of *Y. pseudotuberculosis*. Identical residues are connected by bars. Double dots between residues indicate amino acids of the same hydrophilic or hydrophobic grouping.

DISCUSSION

Expression of Yop proteins in yersiniae is regulated by two independent control systems: one positive loop and one negative loop (2, 12, 41). The negative loop is Ca^{2+} controlled, and as we show here LcrQ acts as a negative element in this regulatory pathway. When LcrQ was expressed in *trans* from a multicopy plasmid vector, the phenotype of wild-type strain YPIII(pIB102) was changed from CD to CI and Yop expression was suppressed. In contrast, *lcrQ* mutant YPIII(pIB25) showed a TS growth phenotype and the mutant had the ability to express Yops even in the presence of Ca^{2+} at 37°C. Thus, LcrQ does not affect the regulation of the LcrF activator; rather, LcrQ has a key function in the regulation of Yop expression in response to the extracellular concentration of calcium.

Interestingly, *lcrQ* mutant YPIII(pIB25) showed a novel phenotype with respect to the Yop secretion pattern. The mutant was TS and was derepressed for Yop expression in the presence of Ca^{2+}. However, only LcrV and YopD were secreted in large quantities into the culture supernatant under these conditions. LcrV was recovered in much higher amounts than normally found when the bacteria were incubated to allow maximal Yop expression (Fig. 5). This may indicate that LcrQ specifically regulates expression-secretion of the *lcrGVH-yopBD* operon, since both LcrV and YopD are expressed from this operon (2). Since all Yops are expressed but only LcrV and YopD are secreted, this also suggests that LcrV and YopD may be secreted via a mechanism different from that of the other Yops.

We succeeded in identifying *lcrQ* by the fact that pBH57, which carries *lcrK* and *lcrQ* (Fig. 1), had the ability to transcomplement *lcrK* mutants to the wild-type phenotype with respect to the response to calcium, as well as the ability to secrete Yops (36), while pPBC3, which carries only *lcrK* (Fig. 1), was unable to do so. From the results presented here, it is not evident why pPBC3 is unable to transcomplement the *lcrK* mutants, since this plasmid contains the essential genetic information and since a mutant having a gene block insertion in the *Cla*I site (pIB24; Fig. 1) still showed the wild-type phenotype. This *Cla*I site is very close to the stop codon of *lcrKc*, the last gene of the operon (Fig. 3). Therefore, it is possible that the transcript of this operon is affected in pPBC3, resulting in a low level of expression. Another possibility is that *lcrK* regulates *lcrQ* expression by a *cis*-acting mechanism. This idea is attractive from the point of view that *lcrK* mutants which are known to be defective in Yop export are also TS for growth at 37°C (28a, 36). It is not obvious why export-defective mutants are TS. Therefore, the TS phenotype of *lcrK* mutants could be explained if *lcrQ* expression were concomitantly lowered in these strains.

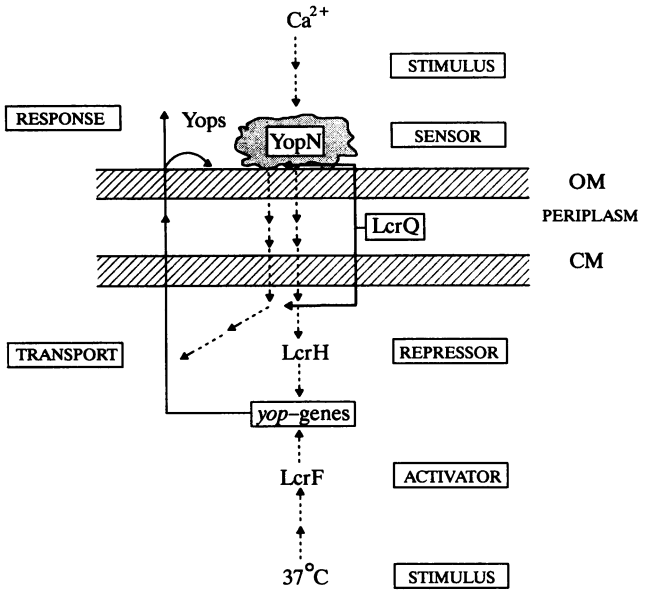

FIG. 8. Model of the low-calcium response of yersiniae. Surface-located protein YopN has been shown by Forsberg et al. to be involved in the low-calcium response (11). LcrH is also involved in the low-calcium response, and it has been shown by Bergman et al. that LcrH acts at the end of this pathway (2). LcrF is the *Y. pseudotuberculosis* counterpart of VirF of *Y. enterocolitica*, which is the AraC-like temperature-regulated activator identified by Cornelis et al. (7). In this study, we show that LcrQ is involved in the low-calcium response and that LcrQ may act close to the calcium signal. Note, however, that the spatial localization of LcrQ has not been determined. OM, outer membrane; CM, cytoplasmic membrane.

Other possibilities cannot be excluded, and more work is needed to solve this question. What we can conclude, however, is that LcrQ must act after LcrK in the Ca^{2+}-controlled negative regulatory loop.

LcrQ is the first protein to be described that changes the phenotype of the wild-type strain after overexpression in *trans*. We have recently presented results which show that TS mutants of the *lcrGVH-yopBD* operon become CI after overexpression of LcrH from the *tac* promoter (2). However, in this case, the wild type was unaffected by the increased levels of LcrH. A *yopN* mutant is also temperature sensitive for growth at 37°C (11, 42). YopN is a surface-located protein, and the *yopN* mutant not only shows derepressed expression of Yops at 37°C in the presence of Ca^{2+}, but it also shows derepressed Yop export (11). On the basis of these results, we suggest a model of Ca^{2+} regulation of yersiniae in which YopN is high in the regulatory hierarchy and YopN may even be the Ca^{2+} sensor per se (Fig. 8) (11). On the other hand, LcrH is at the end of this regulatory pathway and has been suggested to be the repressor which interacts with the *yop* operator sequences (2, 34). Therefore, it was interesting to find that expression of LcrQ in *trans* in the *yopN* mutant resulted in a phenotype change from TS to CI which also could be seen as a general down regulation of Yop expression while *lcrH* mutant YPIII-(pIB13) maintained its TS phenotype. This would place LcrQ between YopN and LcrH in the regulatory cascade (Fig. 8).

Since the two pathways, Ca^{2+}-regulated transcriptional control and Yop secretion, have regulatory signals in common, we may ask whether LcrQ is involved in both pathways or not. The results obtained from studies using the *lcrQ* mutant suggest that LcrQ is involved in regulation of Yop expression, since the mutant has the ability to secrete Yops in a Ca^{2+}-dependent manner, like the wild type (with the exception of LcrV and YopD). This suggests that LcrQ has no control function in Yop export but a negative role in regulation of *yop* transcription. Interestingly, the LcrQ protein was recovered from the culture supernatant, which may suggest that LcrQ acts close to the Ca^{2+} signal. We cannot conclude from the results presented here that LcrQ acts outside the cell. It is possible that only a fraction of LcrQ having no obvious function can be recovered from the culture supernatant and that the active form of LcrQ is still within the cell. We cannot resist speculating, however, about whether LcrQ is exported to the culture supernatant as a way to lower the intracellular concentration of LcrQ to allow full expression of the *yop* genes.

LcrQ shows extensive sequence homology to amino acids 1 to 128 of YopH. YopH is a plasmid-encoded virulence determinant of yersiniae (5, 27). The identity between the two proteins was as high as 42%, suggesting a gene duplication event during evolution. Thus, YopH may be divided into three different domains. The first domain involves the first 48 amino acids of the N-terminal end of the protein, which has been shown to be essential for the specific plasmid-encoded Yop secretion system (28, 29). The second domain, ranging from amino acids 48 to 128, is the LcrQ domain, which may contain the autoregulatory signal, as suggested by Cornelis and coworkers (9). This model suggests that Yop expression is regulated by a feedback mechanism. When the Yops are not secreted, an intracellular increase in Yop concentration occurs which leads to down regulation of *yop* transcription, presumably by interaction of one Yop protein with a DNA region. We have been able to exclude the possibility that LcrQ and YopH form a complex which is the actual regulatory signal, since a mutant having all of *yopH* deleted responded to the signal promoted by LcrQ (data not shown). The last domain exhibits a protein tyrosine phosphatase activity. It is likely that this activity is important for the ability of the pathogen to counteract the primary immune defense by inhibition of phagocytosis (5, 16, 36).

In conclusion, we have described here a novel protein, LcrQ, which is involved in regulation of Yop expression in relation to the extracellular concentration of calcium. The fact that the protein is expressed from a monocistronic operon will facilitate our studies concerning the role of LcrQ in regulation. Questions that now are being asked are as follows. Is LcrQ a DNA-binding protein? Does LcrQ interact with a specific promoter structure? Does LcrQ interact with other proteins? Is LcrQ secretion an active part of the regulatory system?

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