The \textit{lcrE} Gene Is Part of an Operon in the \textit{lcr} Region of \textit{Yersinia enterocolitica} O:3

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Received 15 November 1989/Accepted 17 March 1990

The low-calcium response (\textit{lcr}) region of the virulence plasmid of \textit{Yersinia pestis}, \textit{Yersinia pseudotuberculosis}, and \textit{Yersinia enterocolitica} has been associated with calcium-dependent growth of bacteria. Mutations in the previously identified \textit{lcr} locus within the \textit{lcr} region lack the repressor control of production of the \textit{lcr} specific proteins, \textit{Yersinia} outer membrane proteins (Yops) and V and W antigens. We sequenced a 3.3-kilobase-pair \textit{BamHI-Clal} fragment of the \textit{lcr} locus of \textit{pYVO3}, the virulence plasmid of \textit{Y. enterocolitica} O:3. The sequence of \textit{lcr} locus revealed six tightly packed open reading frames (ORFs), one of which was identified as the structural gene, \textit{lcrE}, of the 32.9-kilodalton outer membrane protein LcrE (formerly known as Yop4b or YopN). Detection of large (\textgtrsim 2.3-kilobase-pair) transcripts strongly supports the conclusion that the \textit{lcrE} gene and ORF1 to -5 function as an operon. Transcription of the \textit{lcrE}-containing operon and the adjacent \textit{lcrB} locus was found to be divergent, and the corresponding transcripts overlapped about 1,200 nucleotides. This extremely long overlap of the 5' ends of the transcripts produced from face-to-face promoters is a new finding; the longest overlap thus far found has been a few hundred nucleotides. Temperature was found to play the major role in regulation of transcription of the \textit{lcrE}-containing operon of \textit{pYVO3}, whereas Ca\textsuperscript{2+} concentration seemed to affect it only modestly.

There are three human pathogenic strains in the genus \textit{Yersinia}: \textit{Y. pestis}, \textit{Y. pseudotuberculosis}, and \textit{Y. enterocolitica}. \textit{Y. pestis} is the causative agent of a systemic disease, plague. Infection by \textit{Y. pseudotuberculosis} and \textit{Y. enterocolitica} is manifested as yersiniosis, which is characterized by fever, abdominal pain, and diarrhea. Acute mesenteric lymphadenitis and terminal ileitis are often described (9). Reactive arthritis and erythema nodosum sometimes occur as postinfection complications (1, 41, 43).

All pathogenic \textit{Yersinia} strains harbor a related plasmid of about 70 kb (kilobase pairs), designated \textit{pYV}, which is essential for virulence (17, 20, 47). This virulence plasmid carries genes for the low-calcium response (\textit{lcr} genes), i.e., for calcium-dependent growth of \textit{Yersinia} spp. At 37°C in the absence of calcium, a metabolic down-shift and cessation of growth occur (46). This phenomenon was described in \textit{Y. pestis} cultures in 1959 (27), but only in 1980 was it associated with plasmid \textit{pYV}. In plasmid \textit{pYV}, the \textit{lcr} genes are gathered in a conserved region of about 20 kb (14, 21, 33, 34). Low-calcium response is characterized by the synthesis of plasmid-encoded proteins, which include V and W antigens and outer membrane proteins (Yops) (6, 12, 35). Low-calcium response does not occur at 25°C; bacteria grow normally at room temperature even under Ca\textsuperscript{2+} restriction. Furthermore, Yops are secreted into the culture supernatant at 37°C under Ca\textsuperscript{2+}-deficient conditions (25). Secretion is blocked in the presence of 2.5 mM Ca\textsuperscript{2+}, but small amounts of Yops can still be found in the outer membrane (18).

The \textit{lcr} region has been previously studied by insertion mutation analysis. Within the region, several different loci have been identified, designated \textit{lcrA} to \textit{lcrF} and \textit{virA} to \textit{virF} (13, 21). The \textit{lcrA} locus was further divided into \textit{lcrE} and \textit{lcrD} loci, and the direction of transcription was shown to diverge between \textit{lcrE} and \textit{lcrB} loci (45). Within the \textit{lcrA} locus, Yother and Goguen characterized the so-called calcium-blind mutant (45). This mutant showed a constitutive \textit{lcr} phenotype [\textit{lcr(Con)}] and growth restriction at 37°C even in the presence of 2.5 mM calcium (45). A 3.8-kb DNA fragment of the \textit{lcrA} locus, which was designated by the authors as the \textit{lcrE} locus, was able to complement the calcium-blind \textit{lcr(Con)} mutation. In the \textit{lcrE} locus, the \textit{lcr(Con)} mutation was mapped within a 1-kb DNA fragment. Comparison of the results of Forsberg and co-workers (18) with those presented by Yother and Goguen (45) revealed that the 1-kb fragment overlaps the structural gene for Yop4b (also called YopN). These observations raised speculations about a link between Yop4b and Ca\textsuperscript{2+} regulation.

Bölin et al. showed that the restriction map of the structural gene for Yop4b is conserved in the pathogenic \textit{Yersinia} strains in \textit{BamHI} fragment 8 of \textit{Y. pestis} and \textit{Y. pseudotuberculosis} and in \textit{BamHI} fragment 6 of \textit{Y. enterocolitica} (5, 18). In sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), Yop4b is identified as a 34-kilodalton (kDa) peptide, which in \textit{Y. pestis} and \textit{Y. pseudotuberculosis} is hidden under the more abundant Yop4a of the same size, and requires two-dimensional gels for visualization (18). In \textit{Y. enterocolitica}, however, the size of Yop4a is larger, 36 kDa, which allows direct visualization of Yop4b from SDS-polyacrylamide gels (5).

Because of the apparent importance of Yop4b to Ca\textsuperscript{2+}-regulated functions, we cloned and sequenced the structural gene for Yop4b of \textit{Y. enterocolitica} O:3. On the basis of the results obtained in this study and those obtained from transcomplementation studies (A. Forsberg, A.-M. Viitanen, H. Wolf-Watz, and M. Skurnik, unpublished data), it has become evident that Yop4b (YopN) plays a major role in regulation of the low-calcium response. For that reason, the Yop4b (YopN) protein is hereafter designated LcrE. The corresponding gene is called \textit{lcrE}. We also show that \textit{lcrE} and five open reading frames (ORFs) constitute an operon.

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The sequence data together with Northern (RNA) blotting and primer extension analyses showed that the organization and regulation of the genes in this region are extremely complex.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used are described in Table 1.

**DNA methods.** Plasmid DNA isolations, restriction enzyme digestions, ligations, Northern blotting, Southern hybridizations, colony hybridizations, and electrophoresis of DNA fragments were performed as described previously (29). Transformations were done according to Hanahan (22). Either nitrocellulose filters (Millipore, Molsheim, France) or GeneScreen plus filters (Dupont, NEN Research Products, Boston, Mass.) were used as transfer membranes. Double-stranded DNA probes were labeled with an oligolabeling system (16). \([\alpha-32P]dATP\) was purchased from Amersham International (Amersham, United Kingdom), random hexanucleotide primers were supplied by Pharmacia Biotechnology International AB (Upsala, Sweden), and Klenow enzyme was supplied by Boehringer GmbH (Mannheim, Federal Republic of Germany). Oligonucleotide probes were radiolabeled by using a 5′ DNA terminus labeling system kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

**DNA cloning and sequencing.** BamHI restriction fragments of pYVO3 have been previously cloned into pBR322 (37). The internal Clal fragment of BamHI fragment 6, obtained from pYP6, was further cloned into pBR322 (Fig. 1). Two of the hybridization-positive clones (pYMS4073 and pYMS 4146; Fig. 1) were further analyzed by immunoblotting for the presence of an about 34-kDa protein. For sequencing, the Clal and BamHI fragments of pYMS4073 and pYP6 were cloned and subcloned into sequencing vectors M13mp18 and M13mp19. Single-stranded templates were subjected to DNA sequencing by the dyeoxy-chain termination method (38) with modified T7 DNA polymerase (Sequenase version 2.0 kit: U.S. Biochemical Corp., Cleveland, Ohio) and \([\alpha-32P]dATP\) (Amersham) as the label. Where needed, oligonucleotides (Table 2) deduced from the sequenced part of DNA were used as primers in sequencing reactions. DNA and amino acid sequences were handled and analyzed with computer programs Geneus (23, 24) and Wisconsin (15).

To obtain controlled production of LcrE, plasmid pl2.2 was constructed (Fig. 1). The strong isopropyl-\(\beta\)-d-thiogalactoside (IPTG)-inducible tac promoter was cloned from pICB1 (11) in a 400-base-pair (bp) EcoRI fragment into the EcoRI site of pYMS4146. Screening for tac-positive clones was done by colony hybridization, which showed clear differences in intensity between chromosomal background and plasmid-derived strong positivity. pl2.2 was maintained in Escherichia coli JM103 (30), the lac?-positive host, to avoid possible detrimental effects of overproduction of LcrE. Interestingly, JM103(pl2.2) was tetracycline resistant.

**RNA isolation and primer extension.** Strain 6471/76 was grown overnight at 22°C in brain heart infusion broth (BH; Difco Laboratories, Detroit, Mich.). For induction of Yops, bacteria from the overnight cultures were incubated at 37°C for 2 h in BHI supplemented with 20 mM sodium oxalate–20 mM MgCl2–0.2% glucose (BHIMOX). Alternatively, Pest medium, which is Higuchi minimal medium (28) supplemented with 1% tryptone (Difco), 0.5% yeast extract (Oxoid Ltd., Basingstoke, United Kingdom), and 0.2% glucose, was used. E. coli was cultured in Luria broth (LB). IPTG induction was performed as follows. An overnight culture of E. coli JM103(pl2.2) was diluted 1/20 in LB and grown to an A650 of 0.3. IPTG was added to a final concentration of 1 mM, and aeration was continued for 30 min at 37°C.

RNA was extracted by the hot phenol method and subjected to primer extension as described previously (31). Primers are listed in Table 2. Reverse transcriptase was purchased from Janssen Biochimica (Beerse, Belgium). Antibodies. Since monospecific or monoclonal anti-LcrE antibodies are not available, a rabbit antiserum specific for Yop4a/b (18), kindly donated by Åke Forsberg, University of Umeå, Umeå, Sweden, was used at a 1:100 dilution for immunoblotting. To differentiate LcrE from Yop4a, monoclonal antibody 4H11 specific for Yop4a (44), kindly donated by Christoph Schoerner, was used in parallel at a 1:500 dilution.

In the first phase of the study, antiserum specific for the released proteins (RP) (kindly provided by Outi Mäki-Ikola) was also used in immunoblotting. Anti-RP sera were prepared as follows. Rabbits were immunized with RPs isolated from Y. enterocolitica O:3 grown in Ca2+-deficient medium (26). A 400-μg sample of antigen was injected subcutane-
FIG. 1. Restriction maps of plasmids pYVO3, pYMS4073, pYMS4146, pYP6, and pl2.2 and the cloning strategy. Locations of the lcrE-containing operon and the lcrB and lcrD loci are defined according to reference 45 and the results of this study. See text for further details.

TABLE 2. Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence</th>
<th>Sequence positions</th>
</tr>
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<tr>
<td>AMV-2</td>
<td>5'-ggg act tgt ctc ctc gcg gc-3'</td>
<td>2315-2334</td>
</tr>
<tr>
<td>AMV-3</td>
<td>5'-ggg ctc aat atg ggg gcc agg-3'</td>
<td>2175-2159</td>
</tr>
<tr>
<td>AMV-6</td>
<td>5'-cgg cac aga ttc ttc cgg gcc-3'</td>
<td>766-751</td>
</tr>
<tr>
<td>AMV-11</td>
<td>5'-cac tgt agt gcc acg tcc tc-3'</td>
<td>361-380</td>
</tr>
<tr>
<td>AMV-12</td>
<td>5'-cac tgt tgg cca tga tgc acg gc-3'</td>
<td>227-208</td>
</tr>
</tbody>
</table>

RESULTS

Cloning of the lcrE-containing operon. According to previous work (18), the lcrE gene should be located in the internal 2.7-kb Clal fragment of BamHI fragment 6 of pYV6471/76. Therefore, the 2.7-kb Clal fragment of pYP6 was cloned into pBR322. The advantage of Clal site cloning is the possibility for the cloned fragment to utilize promoter P1 of pBR322, in this case for transcription of lcrE, provided that the fragment is in the correct orientation. A number of hybridization-
positive clones were analyzed by immunoblotting, using antibodies specific for released proteins. Plasmid pYMS4073 contained the gene apparently in the correct orientation, since a faint band of about 34 kDa could be detected in Western blotting (data not shown). pYMS4146 (also hybridization positive) did not express the 34-kDa protein. Detailed restriction maps of pYMS4073 and pYMS4146 were constructed and compared with that of pYP6 (Fig. 1). The maps showed that a 2.7-kb ClaI fragment was in opposite orientations in plasmids pYMS4073 and pYMS4146. To ascertain the presence of lcrE in the clones, the tac promoter was cloned into the EcoRI site of pYMS4146 (Fig. 1). The resultant plasmid, pI2.2, showed IPTG-inducible expression of LcrE at both the transcriptional (see Fig. 6) and translational (Fig. 2) levels. Weaker expression of LcrE was also detected from pYMS4073, whereas pYP6 and pYMS 4146 did not express it (Fig. 2).

Nucleotide sequence of the lcrE-containing operon. The 3.3-kb BamHI-ClaI fragment of pYVO3 was sequenced (Fig. 3). Altogether, eight ORFs became evident. In the position where the lcrE gene was previously localized, we found an 879-bp ORF with the anticipated orientation. This ORF starts with initiation codon ATG and ends with stop codon TGA. A putative ribosome-binding site, GGAG, is apparent upstream of the initiation codon. On the basis of the Western blotting analysis, we conclude that this ORF codes for LcrE protein.

Downstream of lcrE, the lcrE-containing operon is filled with five partially overlapping ORFs (ORF1 to -5) (Fig. 3). ORF5 extends beyond the distal ClaI site, and our sequence only indicates that ORF5 is longer than 476 bp. The organization of the genes, their possible initiation and stop codons, and their Shine-Dalgarno sequences are shown in Fig. 4. No apparent ribosome-binding site for ORF4 was found. The initiation codon of ORF2 and ORF3 was GUG. Upstream of lcrE, two ORFs were evident in reverse orientation to lcrE and ORF1 to -5 (Fig. 3 and 4). The sequenced DNA contains only part of ORF7, which starts in the middle of ORF6 and extends beyond the BamHI site. The presence of divergent ORFs suggests that the border between the lcrE-containing operon and the lcrB locus might be between ORF6 and lcrE.

The upstream sequence of lcrE had no evident homology with the unique A+T-rich sequence located upstream of yopE (19), yopH (7), and virF (13); however, the DNA sequence upstream of lcrE is similarly A+T rich. The previously noted occurrence of dam methylase in Yersinia species (40) may explain the presence of one undigested ClaI site in the sequence at position 1740.

**Primer extension analysis and promoters.** Computer analysis indicated the presence of divergent promoters (P2, P4, P5, and P6; Fig. 4) between ORF6 and lcrE. To verify whether these promoters were functional, the transcriptional start points of the lcrE-containing operon and the lcrB locus were determined. RNA for primer extension analysis was isolated from Y. enterocolitica O:3 strain 6471/76 grown at 37°C in BHIMOX. It became soon evident that there was unexpected complexity at the transcriptional level. Primer AMV11 (Fig. 4; Table 2) was used when the start point of transcription was analyzed for lcrB. A strong band extending about 658 nucleotides from the primer was detected (Fig. 5). The transcription start point and the putative promoter, P3 (Fig. 4), would be within the lcrE gene. The best candidate for the start point was the T residue at position 1019 (Fig. 3). A putative promoter motif was identified upstream (Fig. 3). The −10 box TTATAGT and the −35 box TAAGTT resemble only weakly the consensus promoter sequence of E. coli.

Promoter extensions with AMV6 and AMV12 as primers were performed to find the transcriptional start point of lcrE. With AMV6, the reverse transcriptase stops extended all through the gel, i.e., beyond the BamHI site in Fig. 4. Similarly, primer extension with AMV12 extended beyond the BamHI site. One faint stop was detected apparently 134 bp downstream of the BamHI site (Fig. 5), but the main stop seemed to extend still further, and the exact location and structure of promoter P1 (Fig. 4) remain to be elucidated.

Promoters P1 and P3 revealed by the primer extension analysis are divergent face-to-face promoters (Fig. 4). The 5′ termini for the transcripts seem to overlap more than 1,200 bp. Computer program analysis identified four other possible promoter motifs in the A+T-rich region just upstream of lcrE: P2, P4, P5, and P6 (Fig. 3 and 4). Toward the lcrD locus, three possible promoter motifs were found (P7, P8, and P9). The fact that pl2.2 was tetracycline resistant indicates either the functional existence of one of these promoters or, alternatively, that a few transcripts originating from the tac promoter, even in the absence of IPTG, extend to the tet gene.

**Terminator sequences.** Possible hairpin loops of DNA sequence with calculated AG values of less than −13 kcal (ca. −54 kJ/mol) (42) are shown in Fig. 4. Possible terminators were found downstream of each ORF. Many stable hairpins were seen downstream of ORF6 and ORF1. We used also another terminator search program (10), which compares the sequence with known terminator sequences and then looks for hairpins from those hits. These terminator-like structures are indicated in Fig. 4.

**Northern analysis.** Total RNA isolated from Y. enterocolitica...
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**FIG. 3.** Nucleotide sequence of the 3.3-kb BamHI-Clal fragment of the **lcrE**-containing operon of *Y. enterocolitica* 6471/76 (GenBank accession no. M32097). The nucleotides are numbered starting from the BamHI site. Some restriction sites are underlined. The start and end of each ORF are boxed. The transcription start of the lcrB locus is marked with an asterisk, and the putative promoter motif of P3 is shown upstream. The hypothetical promoters P2 to P9 are also indicated. Amino acid sequences are shown for all ORFs; because of the opposite directions of ORF6 and ORF7, both strands of the sequence are shown for the first 480 bp.

*a. enterocolitica* 6471/76 grown at 22 or 37°C in media both with and without calcium was analyzed by Northern blotting, using double-stranded probes. Probes are illustrated in Fig. 4, and Northern hybridizations are shown in Fig. 6. From bacteria grown at 37°C in BHIMOX, the internal 450-bp *PstI* fragment of the **lcrE** gene recognized transcripts of about 1.1, 1.4, 1.5, 1.8, 2.3, and 2.8, and >3 kb in size (Fig. 6A). The shorter transcripts were predominant, and the >3-kb band was hardly visible. The 420-bp EcoRV-Clal probe hybridized to the two largest messengers (2.8 and >3.0 kb) and weakly to a 1.5-kb transcript (Fig. 6B). The whole 2.7-kb Clal fragment detected the same bands as the *PstI* probe, but the >3-kb transcript was more evident (Fig. 6D). The 600-bp *BamHI-Clal* fragment hybridized with <700-1.1-, 1.5-, 1.8-, and 2.8-kb transcripts (Fig. 6C). Induction of the **lcrE** gene was seen only after the temperature shift. Supplementation of the culture medium with 2.5 mM Ca<sup>2+</sup> decreased transcription of the **lcrE** gene about threefold (Fig. 6E). Calcium concentration had no effect on transcription of the **lcrE** gene in bacteria grown at 22°C, and transcripts were detectable only after prolonged exposure (data not shown).

**Amino acid sequences.** The amino acid sequence of the **LcrE** polypeptide was deduced from the DNA sequence. **LcrE** contains 293 amino acids with only one cysteine residue. The presence of only one cysteine in the polypeptide chain indicates that **LcrE** has the potential to form disulfide bridges to other proteins or may exist naturally as a dimer. No signal peptide was detected when NH<sub>2</sub>-terminal amino acids of the predicted protein were compared with essential features for signal sequences (36). The calculated molecular mass for **LcrE** is 32,924 kDa, which is only a little lower than that predicted from SDS-PAGE. **LcrE** is an acidic protein; the calculated isoelectric point is 4.92, which also agrees with the previous results (18). The data for amino acid sequences of the other ORFs are shown in Fig. 4. Comparison of the amino acid sequences with the known
sequences of different Ca\(^{2+}\)-binding proteins revealed no apparent homologies.

**DISCUSSION**

We sequenced a 3.3-kb BamHI-ClaI fragment of pYV03. This sequence comprises most of the lcrE-containing operon, the region between the divergent lcrB locus and lcrE-containing operon, and the beginning of the lcrB locus. The hypothetical organization of the lcrE-containing operon based on our present knowledge is presented in Fig. 4. Eight ORFs were detected. The lcrE gene and ORF1 to -5 belong to the lcrE-containing operon, and ORF6 and -7 belong to the lcrB locus.

There has been indirect evidence that the lcrE locus of *Y. pestis* (45) is identical with the structural gene for LcrE (formerly called Yop4b or YopN) of *Y. pseudotuberculosis* and *Y. enterocolitica* (18). Here we show for the first time directly that the gene for LcrE (Yop4b[YopN]) is in the lcrE locus. Definite proof was obtained by Western blotting analysis, where expression of LcrE was detected from the lcrE gene, which was cloned downstream of the IPTG-inducible tac promoter. That the calculated size of LcrE is 32,924 Da and that the calculated pl value was the same as that obtained for Yop4b (18) supported this conclusion.

Downstream of lcrE, five ORFs were detected. Detection of long (>2.3-kb) transcripts with both the PsI and EcorV-ClaI probes strongly supports the conclusion that the region containing lcrE and ORF1 to -5 functions as an operon. Whether the observed smaller messengers are primary transcripts or products of posttranscriptional processing is not clear. The presence of relatively stable hairpins (for example, in ORF2) might indicate that some of the transcripts terminate there and others extend beyond the ClaI site (Fig. 4). We could not identify any ribosome-binding site for ORF4. Whether ORF4 or the other ORFs of the lcrE-containing operon code for proteins remains to be elucidated.

With primer extension analysis, the start point for lcrE transcription was not mapped within BamHI fragment 6 of pYV03 but instead was mapped >800 bp upstream of the lcrE gene. This finding is supported also by the transcomplementation experiments, which were performed with several calcium-independent mutants of the lcrE-containing operon of *Y. pseudotuberculosis* YPIII(pIpB1) (A. Forsberg, Ph.D. thesis, University of Umeå, Umeå, Sweden, 1989; Forsberg et al., unpublished data). Mutations within the lcrE gene, i.e., insertion in the BglII site or deletion of the internal PsI fragment, could not be transcomplemented to
calcium dependence with plasmid pAF8, which contained the whole BamHI fragment 8 of pIB1 (equivalent of BamHI fragment 6 of pYVO3). Transcomplementation was achieved with plasmid pAF82, which in addition to BamHI fragment 8 carries about 2 kb of the adjacent BamHI fragment 6 of pIB1. Two facts allow extrapolation of the transcomplementation results for Y. pseudotuberculosis to Y. enterocolitica. First, the restriction maps of the lcrE-containing operon and lcrB loci of Y. pseudotuberculosis and Y. enterocolitica are identical; second, the sequence of the lcrE gene of Y. pseudotuberculosis is 98% homologous to that of the Y. enterocolitica lcrE gene (Forsberg et al., unpublished data).

It is possible that transcription of the lcrE-containing operon starts from two separate promoters, P1 and P2 or P4, and stops at two separate terminators. The hypothesis of two promoters is supported by the facts that extension stops were seen in the primer extension analysis around promoters P2 and P4 (data not shown) and that a 1.4-kb messenger was exclusively detected with the PsrI probe, not with the BamHI-ClaI probe. It is tempting to speculate that transcription of this messenger could start from the hypothetical promoter P2 or P4 and terminate after ORF1. At a minimum, four transcripts of different lengths are produced. More thorough analysis of the transcripts is needed to clarify which promoters and terminators are indeed functional.

In Northern blottings, the shorter transcripts are predominant. Temperature seems to play the major role in regulation of transcription of the lcrE-containing operon of pYVO3, whereas Ca^{2+} concentration has only a moderate effect at 37°C. At room temperature, calcium has no effect on transcription. Experiments with mini-Mu(Kan lac) and Mu d1(Ap lac) insertions in the genes in this region did not reveal any effect of Ca^{2+} on transcription even at 37°C (14, 21). It is possible that in these experiments, the large insertion fusions in the genes disturbed the normal regulatory circuits of the region.

Transcriptional direction was previously shown to diverge at the region between the lcrE-containing operon and the lcrB locus by using mini-Mu(Kan lac) and Mu d1(Ap lac) insertions (14, 21). We could verify this by primer extension analysis. Surprisingly, primer extension analysis revealed that the start point of transcription of the lcrB locus lies inside the lcrE gene. The face-to-face promoters for the lcrE-containing operon and the lcrB locus produce transcripts that overlap about 1,200 nucleotides. This explains why pAF82 (see above) was not able to transcomplement the insertion in the ClaI site (at position 578) (Forsberg, Ph.D. thesis, 1989; Forsberg et al., unpublished data), since that insertion affects both the lcrB locus and the lcrE-containing operon (Fig. 4). The extremely long overlap of the
5' ends of the transcripts is a new finding; the longest overlap found thus far has been a few hundred nucleotides (4). The phenomenon probably plays important role in regulation of the region, since no ORFs were detected from the 5' ends of the transcripts. The presence of ORF6 and ORF7 in the beginning of lcrB is in concordance with the results of Forsberg, who has localized genes coding for 13- and 50-kDa proteins upstream of lcrE (Forsberg, Ph.D. thesis, 1989).

According to Yother and Goguen, the lcrE locus of _Y. pestis_ maps, in their kilobase scale, from kb 49 to 45.5 (45). Position 45.5 kb corresponds the site about 300 bp downstream of the _ClaI_ site in ORF5 (Fig. 4). The results of Yother and Goguen also predict that the size of the _lcrD_ locus should be about 1 kb. We detected a faint 1.5-kb transcript with the _ClaI-EcoRV_ probe, suggesting that transcription of the _lcrD_ locus in _Y. enterocolitica_ starts from one of the hypothetical promoters P7 to P9. pAF8 (see above) was not able to transcomplement a mutation in the _ClaI_ site equivalent to that at position 3361 in _Fig. 4_ (Forsberg, Ph.D. thesis, 1989; Forsberg et al., unpublished data), which possibly relates to the effect of this mutant to the transcription of the _lcrD_ locus.

Transcription of the _lcrB_ locus also seems to start from, in addition to P3, a separate promoter, since at least one transcript, smaller than 700 bp, was detected with the _BamHI-ClaI_ probe but not with the _PstI_ probe. This transcript could originate from promoter P5 or P6 and contain only the ORF6 gene.

Within the _lcrA_ locus, the _lcrE_ locus was defined to be the area that is involved in the Ca\(^{2+}\)-blind _[lcr(Con)]_ phenomenon (45). In the Ca\(^{2+}\)-blind construction, the point mutation _lcrE<sup>l</sup>_, was localized in _HindIII_ fragment J between the _BamHI_ and _HindIII_ sites, which means that it is situated either within the structural gene for LcrE or just upstream of it (18). At present, all genetic studies suggest that LcrE protein plays an important role in regulation of the low-calcium response of _Yersinia_ spp. The fact that it is found excreted into the culture medium or bound to the outer

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**FIG. 5.** Primer extension analysis of _Y. enterocolitica_ 6471/76 grown at 37°C in BHIMOX. (A) Analysis in which the DNA sequence of M13mp18 was used as a size standard (lanes a, c, g, and t). Other lanes: 1, primer AMV6; 2, primer AMV11 (see panel B); 3, primer AMV12. A faint band of 354 nucleotides is indicated with an arrow in lane 3. A strong cDNA band detected from 6471/76 RNA with AMV11 as a primer is marked with an arrow in lane 2. (B) Long run of primer extension analysis with AMV11 as a primer.

**FIG. 6.** Northern hybridization analysis of transcription of the _lcrE_-containing operon. A 3-μg sample is isolated RNA was run in each lane. Probes used were the _PstI_ fragment (A), the _EcoRV-ClaI_ fragment (B), the _BamHI-ClaI_ fragment (C), and the 2.7-kb _ClaI_ fragment (D). Lanes: 1, _Y. enterocolitica_ 6471/76 grown at room temperature in BHI; 2, _Y. enterocolitica_ 6471/76 grown at 37°C in BHIMOX. (E) Examination of the effect of calcium on transcription of the _lcrE_-containing operon, examined with the _PstI_ probe. Lanes: 1, JM103(pl2.2); 2, _Y. enterocolitica_ 6471/76 grown at 37°C in Pest medium (see Materials and Methods) supplemented with 2.5 mM Ca\(^{2+}\); 3, _Y. enterocolitica_ 6471/76 grown at 37°C in Pest medium; 4, _Y. enterocolitica_ 6471/76 grown at 37°C in BHI supplemented with 2.5 mM Ca\(^{2+}\); 5, _Y. enterocolitica_ 6471/76 grown at 37°C in BHIMOX. (F) Transcription of _lcrE_ from pl2.2. Lanes: 1, JM103(pl2.2); 2, JM103(pl2.2) induced with IPTG; 3, _Y. enterocolitica_ 6471/76 grown at 37°C in BHIMOX; 4, _Y. enterocolitica_ 6471/76 grown at room temperature in BHI. The positions of 23S (2,904 bases) and 16S (1,542 bases) rRNA bands are marked.
membrane makes it difficult to understand how it carries out its regulatory function. Also, speculations of its direct involvement in the sensing of environmental Ca\textsuperscript{2+} concentration were not supported by its amino acid sequence. No evident similarities were found between LcrE, or any of the other deduced polypeptides of the lcrE-containing operon, and calmodulin or parvalbumin or even with their Ca\textsuperscript{2+}-binding domains. There is the possibility that one of ORF1 to -5 plays the key role in the low-calcium response. Detailed analysis of function of LcrE in the low-calcium response will also clarify the regulation of the lcrE-containing operon.

ACKNOWLEDGMENTS

We thank Hans Wolf-Watz, University of Umeå, Umeå, Sweden, for critical review of the manuscript. This study was supported by the Sigrid Juselius Foundation, Emil Aaltonen Foundation, and Research and Science Foundation of Farmos.

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